

Research Article

Early isolated V-lesion may not truly represent rejection of the kidney allograft

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Intimal arteritis is known to be a negative prognostic factor for kidney allograft survival. Isolated v-lesion (IV) is defined as intimal arteritis with minimal tubulointerstitial inflammation (TI). Although the Banff classification assesses IV as T cell-mediated rejection (TCMR), clinical, and prognostic significance of early IV (early IV, eIV) with negative C4d and donor-specific antibodies (DSA) remains unclear. To help resolve if such eIV truly represents acute rejection, a molecular study was performed. The transcriptome of eIV ($n=6$), T cell-mediated vascular rejection with rich TI (T cell-mediated vascular rejection, TCMRV, $n=4$) and non-rejection histologic findings ($n=8$) was compared using microarrays. A total of 310 genes were identified to be deregulated in TCMRV compared with eIV. Gene enrichment analysis categorized deregulated genes to be associated primarily with T-cells associated biological processes involved in an innate and adaptive immune and inflammatory response. Comparison of deregulated gene lists between the study groups and controls showed only a 1.7% gene overlap. Unsupervised hierarchical cluster analysis revealed clear distinction of eIV from TCMRV and showed similarity with a control group. Up-regulation of immune response genes in TCMRV was validated using RT-qPCR in a different set of eIV ($n=12$) and TCMRV ($n=8$) samples. The transcriptome of early IV (< 1 month) with negative C4d and DSA is associated with a weak immune signature compared with TCMRV and shows similarity with normal findings. Such eIV may feature non-rejection origin and reflect an injury distinct from an alloimmune response. The present study supports use of molecular methods when interpreting kidney allograft biopsy findings.

Introduction

Intimal arteritis, also called v-lesion, is known to be a negative prognostic factor for kidney allograft survival [1–3]. According to the Banff classification, intimal arteritis is characterized by infiltration of mononuclear cells beneath the arterial endothelium. It is considered pathognomonic of acute rejection, either T cell- (TCMR) or antibody-mediated rejection (ABMR) [4,5].

Isolated v-lesion (IV), defined as intimal arteritis that occurs with minimal concurrent tubulointerstitial inflammation (TI), poses a challenge of unclear clinical and prognostic significance. According to the Banff classification, arterial involvement is sufficient to classify IV as type II or III acute TCMR regardless of interstitial inflammation (i) and tubulitis (t). IV could also be considered as ABMR in the presence of donor-specific antibodies (DSA) and evidence of antibody interaction with the endothelium (C4d positive staining, moderate microvascular inflammation (MI) or gene expression markers of endothelial injury in the biopsy tissue) [6].

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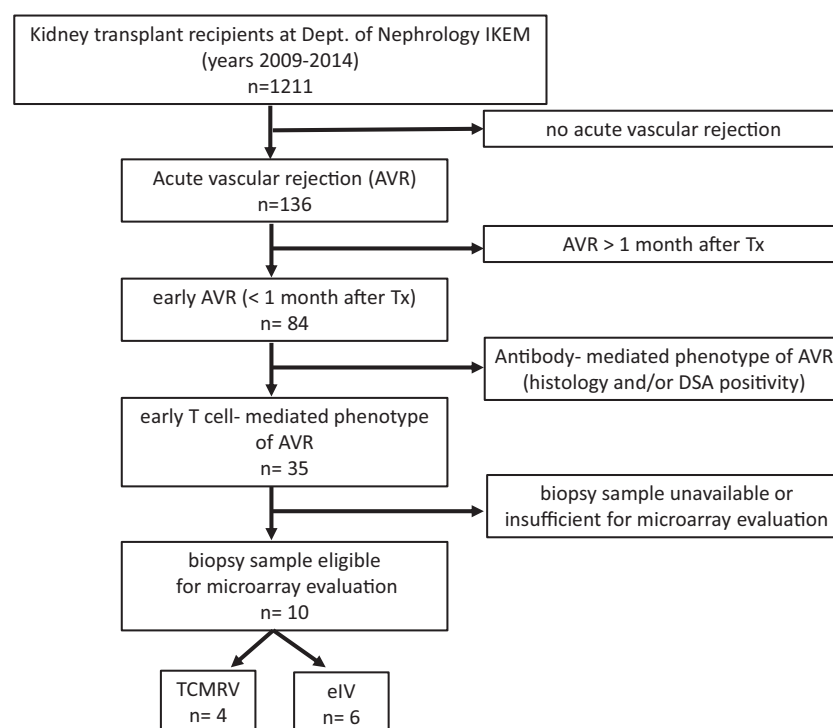


Figure 1. Study flowchart of the training set (cross-sectional screening)

All patients after kidney transplantation at our department in the years 2009–2014 were considered for inclusion in the study. Abbreviations: AVR, acute vascular rejection; DSA, donor specific antibodies; eIV, early isolated v-lesion; IKEM, Institute for Clinical and Experimental Medicine; TCMRV, T cell-mediated vascular rejection; Tx, transplantation.

However, the role of the immune response in the pathophysiology of IV remains unclear. Recent studies have reported diverse findings on IV response to antirejection treatment, clinical and prognostic significance [7–12]. While some authors believe in hidden ischemic/reperfusion injury, others are convinced of its rejection origin.

In response to the limitations of conventional histologic assessment, molecular technologies are being explored in the quest for greater precision [13]. In the transplantation field, a few genome-wide transcriptional studies have aimed to elucidate the origin of IV [14–16]. However, inclusion of heterogeneous samples regardless of DSA and time after transplantation, brought no clear findings, particularly on the non-antibody-associated phenotype of IV.

Therefore, this retrospective study was conducted to thoroughly evaluate the transcriptome of early v-lesion within the first month after kidney transplantation with no signs of ABMR (negative DSA, absence of C4d, and MI) and minimal (eIV) or rich (T cell-mediated vascular rejection, TCMRV) tubulointerstitial inflammation.

Materials and methods

Study design and patients

An observational, nested case–control study within a large single-center retrospective cohort study was performed to compare the expression profiles of renal allograft biopsies with histologic findings of eIV and TCMRV. eIV was defined as intimal arteritis ($v > 0$) with minimal tubulitis ($t \leq 1$) and interstitial inflammation ($i \leq 1$), no MI [glomerulitis (g) + peritubular capillaritis (ptc) = 0], with C4d and DSA negativity. TCMRV was defined as the presence of intimal arteritis with significant TI ($t > 1$, $i > 1$) and negative C4d, MI and DSA and served as a positive control. Only indication kidney allograft biopsies performed within the first month of post-transplant were included. As a negative control group, normal histologic findings from 3-month protocol biopsies without a history of previous rejection were included.

For a training set, 18 patients undergoing kidney transplantation at the Prague-based Institute for Clinical and Experimental Medicine (IKEM) from 2009 to 2014, with eIV ($n=6$), TCMRV ($n=4$), or normal histology ($n=8$) and sufficient biopsy samples for microarray evaluation were selected. A flowchart of the training set is provided in Figure 1, patients' baseline characteristics are shown in Table 1.

Table 1 Characteristics of patients in the training set

	TCMRV (n=4)	eIV (n=6)	normal (n=8)	P
Donor characteristics				
Age, years	44.5 (40.3; 57)	58.5 (51.3; 68.3)	54 (31; 58)	0.186
Gender (female)	2 (50)	5 (83.3) ²	1 (14.3) ²	0.045
Type of donor (deceased)	3 (75)	6 (100)	8 (100)	0.157
ECD	1 (15)	5 (83.3)	4 (57)	0.175
Hypertension	1 (15)	5 (83.3)	3 (43)	0.125
Recipient characteristics				
Age, years	55.2 (44; 66)	59.5 (53.3; 66.3)	62.5 (56.5; 66.2)	0.792
Gender (female)	1 (25)	1 (16.7)	2 (25)	0.923
Dialysis vintage, months	39.7 (24.7; 45)	30 (11; 43)	21.6 (15.6; 30)	0.678
PRA max, %	3 (0.5; 32.5)	22 (6.5; 34.75)	1 (0; 3.5)	0.062
HLA mismatch	4.5 (4; 5)	3 (2.75; 4.25)	3 (2; 4.75)	0.22
HLA at biopsy (neg./pos./not known)	2/0/2	6/0/0 ²	0/0/8 ²	0.001
DSA at biopsy (neg./pos./not known)	2/0/2	6/0/0 ²	0/0/8 ²	0.001
Transplantation characteristics				
Retransplantation	0	0	0	N/A
Cold ischemia, hours	15.25 (6.5; 19.8)	15 (14; 16)	18.1 (7.4; 19.5)	0.696
Delayed graft function	1 (15)	2 (33)	3 (43)	0.908
Biopsy characteristics				
Postoperative day	6.5 (6; 8) ³	7.5 (7; 10) ²	97 (93.5; 101) ^{2,3}	0.001
Clinical characteristics				
Induction therapy				0.005
None	3 (75) ¹	0 ¹	8 (100)	
Basiliximab	1 (25)	5 (83.3) ²	0 ²	
Thymoglobulin	0	1 (16.7)	0	
Maintenance immunosuppression				0.245
TAC/MMF/steroids	3 (75)	6	5 (62.5)	
CsA/MMF/steroids	1 (25)	0	3 (37.5)	
TAC level at the biopsy (μg/l)	14.6 (10.3; 17.2)	10.95 (8; 16.6)	8.7 (8.3; 10.4)	0.646
CsA level at the biopsy (μg/l)	231	NA	191 (176; 278)	1
Anti-rejection treatment				0.3745
Steroids	2 (50)	5 (83)	N/A	
Thymoglobulin	1 (25)	1 (17)	N/A	
Steroids + Thymoglobulin	1 (25)	0	N/A	
Serum creatinine (μmol/l)				
At biopsy	378 (307; 448) ³	441 (226; 514) ²	115 (97; 154) ^{2,3}	0.002
12 months	119 (100; 121)	161 (151; 172)	115 (93; 148)	0.171
24 months	114 (100; 121)	186 (158; 187) ²	106 (99; 144) ²	0.017
Proteinuria (g/24 h)				
At biopsy	0.21 (0.105; 1.37) ¹	1.52 (1.25; 2.27) ^{1,2}	0.36 (0.27; 0.55) ²	0.038
12 months	0 (0; 0.11)	0.26 (0.23; 0.26)	0.26 (0.18; 0.40)	0.144
24 months	0.12 (0.06; 0.54)	0.29 (0.09; 0.3)	0.19 (0.10; 0.33)	0.913
Banff scores in diagnostic biopsy (grade)				
mm	0 (0; 0)	0 (0; 0)	0 (0; 0)	0.535
g	0 (0; 0)	0 (0; 0)	0 (0; 0)	1
cg	0 (0; 0)	0 (0; 0)	0 (0; 0)	1
i	2 (2; 2) ^{1,3}	0 (0; 0) ¹	0 (0; 0) ³	0.0001
t	2.5 (2; 3) ^{1,3}	0 (0; 0) ¹	0 (0; 0) ²	0.001
v	1 (1; 1.75) ³	1 (1; 1.25) ²	0 (0; 0) ^{2,3}	0.0001
ptc	0 (0; 0)	0 (0; 0)	0 (0; 0)	1
ti	2 (2; 2) ³	0 (0; 1) ²	0 (0; 0) ^{2,3}	0.001
ci	0 (0; 0.75)	1 (0.75; 1) ²	0 (0; 0) ²	0.027
ct	0.5 (0; 1)	0 (0; 1)	1 (0; 1)	0.871
ah	1 (1; 1)	1.5 (1; 1.25) ²	0 (0; 0.75) ²	0.004
cv	1 (0.25; 1)	2 (1; 3) ²	0 (0; 0) ²	0.002
C4d positivity, n	0	0	0	N/A

Continued over

Table 1 Characteristics of patients in the training set (Continued)

	TCMRV (n=4)	eIV (n=6)	normal (n=8)	P
Pathologic diagnosis				
pure TCMR	3 ³	4 ²	0 ^{2,3}	0.0012
TCMR + ATN	1	2	0	
Normal	0 ³	0 ²	8 ^{2,3}	
Number of glomeruli	8.5 (7.25; 9)	12 (7; 15)	8 (7; 12.25)	0.522

Data are presented as medians (interquartile (IQ) range) or n (%). Differences were calculated by the Kruskal–Wallis test or χ^2 Fisher exact test and significant results of *post hoc* comparisons were adjusted by the Bonferroni correction for multiple tests (¹TCMRV compared with eIV, ²eIV compared with control, ³TCMRV compared with control). Abbreviations: ah, arteriolar hyaline thickening; ATN, acute tubular necrosis; cg, transplant glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, vascular intimal fibrosis; CsA, cyclosporine A; ECD, expanded criteria donor; g, glomerulitis; HLA, human leukocyte antigen; i, interstitial inflammation; mm, mesangial matrix expansion; MMF, mycophenolate mofetil; ptc, peritubular capillaritis; PRA, panel reactive antibody; t, tubulitis; TAC, tacrolimus; ti, total interstitial inflammation; v, intimal arteritis.

For a validation set, 12 eIV and 8 TCMRV biopsy samples from patients transplanted between 2010 and 2016 were retrospectively identified and validated by RT-qPCR (Table 2). The Institutional Review Board (IRB) of IKEM approved the study protocol (G09-12-20), and all patients provided informed consent to participate in the study.

Induction immunosuppression was based on the immunological risk of recipients according to panel reactive antibodies, DSA, dialysis vintage, and transplantation history. High-risk patients received depletive induction treatment with Thymoglobulin, medium-risk patients received basiliximab, and low-risk patients received no induction treatment. Maintenance immunosuppression was based on standard triple immunosuppression regimen with tacrolimus in the majority of patients. T cell-mediated rejection was treated by steroid pulses. Thymoglobulin was administered in the case of type 2B TCMR or in steroid-resistant rejections.

Biopsy of kidney allografts

All the kidney allograft biopsies were obtained under ultrasound guidance using a 16-gauge Tru-Cut needle (Somatex®; Medical Technologies GmbH, Germany). A small portion (2 mm) of the cortical zone of the biopsy specimen was immediately placed in the RNA later Stabilization Reagent (Qiagen) and stored at -20 or -80°C until RNA extraction. The remaining part of each particular biopsy was sent for histological examination and found representative.

Microarray analysis

RNA preparation and hybridization

Total RNA was isolated from 16-gauge renal biopsies using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Samples with RNA integrity number < 6 (measured using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.)) were excluded from analysis. A total of 200 ng of purified RNA served as a template for the amplification and biotinylation of cRNA using the Illumina® TotalPrep™ RNA Amplification Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) according to manufacturer's instructions. Yields of labeled cRNA were determined using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). Labeled cRNA (750 ng) was hybridized at 58°C for 16 h to Illumina HumanHT-12 v4.0 Expression BeadChips (Illumina, Inc., San Diego, CA, U.S.A.), and then washed and Cy3-stained according to manufacturer's instructions.

Scanning and microarray data analysis

After hybridization, the chip was scanned using the iScan System (Illumina), and raw data were extracted with the BeadStudio Data Analysis Software (Illumina). The lumi package in R software was used to process the raw data. The quantile method was used for normalization [17]. The given workflow represents one of the standard preprocessing pipelines for Illumina microarrays with verified applicability under similar conditions [18].

Only probes with detectable signal intensity in at least five samples were included in the analysis. After normalization, probes with log of average expression intensity less than 3.5 were removed from further analysis. Differentially expressed genes were chosen as those with a fold change > 2 and adjusted $P < 0.05$ (Benjamini–Hochberg method). The affected genes were functionally annotated, and the deregulated pathways were identified using the David database (<http://david.abcc.ncifcrf.gov>). For comparing the lists of deregulated genes, an interactive online

Table 2 Characteristics of patients in the validation set

	TCMRV (n=8)	eIV (n=12)	P
Donor characteristics			
Age, years	46 (41; 62)	57 (50; 66)	0.082
Gender (female)	4 (50)	7 (51.3)	0.535
Type of donor (deceased)	5 (62.5)	10 (83.2)	0.296
ECD	3 (37.5)	6 (50)	0.465
Recipient characteristics			
Age, years	52 (43; 62)	58 (54; 60)	0.3841
Gender (female)	1 (12.5)	2 (16.7)	0.656
Dialysis vintage, months	41 (17.5; 51)	20.4 (7.1; 25)	0.698
PRA max, %	3 (0; 10)	2 (0.5; 32.3)	0.973
HLA mismatch	5 (4; 5)	3 (3; 4.75)	0.39
HLA at biopsy (neg./pos./not known)	1/1/6	4/1/7	0.571
DSA at biopsy (neg./pos./not known)	2/0/6	5/0/7	0.392
CKD diagnosis, n (%)			0.339
Diabetes	1 (12.5)	5 (41.7)	
Glomerulonephritis	2 (25)	2 (16.7)	
Polycystosis	1 (12.5)	2 (16.7)	
TIN	2 (25)	0	
Hypertension	1 (12.5)	2 (16.7)	
Ischemic nephropathy	1 (12.5)	0	
Other	0	1 (8.3)	
Transplantation characteristics			
Retransplantation	1 (12.5)	1 (8.3)	0.653
Cold ischemia, hours	14 (1.25; 14)	15 (11.7; 18.2)	1
Delayed graft function	4 (50)	6 (50)	0.675
Biopsy characteristics			
Postoperative day	6.5 (6; 18)	7 (6; 13)	1
Clinical characteristics			
Induction therapy			0.966
None	1 (12.5)	2 (16.7)	
Basiliximab	5 (62.5)	7 (58.3)	
Thymoglobulin	2 (25)	3 (25)	
Maintenance immunosuppression			1
TAC/MMF/steroids	8 (100)	11 (100)	
TAC level at the biopsy (μg/l)	11.6 (8.3; 15.1)	10.3 (7.55;14.7)	0.629
Rejection treatment			0.4177
Steroids	4(50)	8 (67)	
Thymoglobulin	3 (37.5)	4 (33)	
Steroids + Thymoglobulin	1 (12.5)	0	
Serum creatinine (μmol/l)			
At biopsy	378 (166; 470)	464 (231; 609)	0.305
12 months	154 (112; 167)	154 (135; 188)	0.571
24 months	142 (118; 172)	144 (113; 184)	1.0
Banff scores in diagnostic biopsy (grade)			
mm	0 (0; 0)	0 (0; 0)	1
g	0 (0; 0)	0 (0; 0)	1
cg	0 (0; 0)	0 (0; 0)	1
i	2 (2; 2)	0 (0; 0.75)	0.000016
t	2 (1.25; 3)	0 (0; 1)	0.0002
v	1 (1; 1.75)	1 (1; 2)	0.734
ptc	0 (0; 0)	0 (0; 0)	1
ti	2 (2; 2)	0 (0; 1)	0.000016
ci	0 (0; 0.75)	0.5 (0; 1)	0.384
ct	0.5 (0; 1)	1 (0.25; 1)	0.384
ah	1 (1; 1)	1 (1; 2)	0.181
cv	1 (1; 1)	2 (1; 2)	0.02

Continued over

Table 2 Characteristics of patients in the validation set (Continued)

	TCMRV (<i>n</i> =8)	eIV (<i>n</i> =12)	<i>P</i>
C4d positivity, <i>n</i>	0	0	1
Pathologic diagnosis			0.2421
Pure TCMR	8	9	
TCMR + ATN	0	3	
Number of glomeruli	9 (7–19)	12 (7.5–14)	0.851

Data are presented as medians (interquartile (IQ) range) or *n* (%). Differences were calculated by the Mann–Whitney test or χ^2 Fisher exact test. Abbreviations: ah, arteriolar hyaline thickening; ATN, acute tubular necrosis; cg, transplant glomerulopathy; ci, interstitial fibrosis; CKD, chronic kidney disease; ct, tubular atrophy; cv, vascular intimal fibrosis; ECD, expanded criteria donor; g, glomerulitis; HLA, human leukocyte antigen; i, interstitial inflammation; mm, mesangial matrix expansion; MMF, mycophenolate mofetil; ptc, peritubular capillaritis; PRA, panel reactive antibody; t, tubulitis; TAC, tacrolimus; TCMR, T cell-mediated rejection; ti, total interstitial inflammation; TIN, tubulointerstitial nephritis; v, intimal arteritis.

tool for Venn diagrams <http://bioinformatics.psb.ugent.be/webtools/Venn/> was used. Complete raw and normalized data were deposited in the NCBI Gene Expression Omnibus (GEO) database [19] and are accessible through the GEO Series accession number GSE114712 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114712>).

Validation of microarray data

In order to validate the microarray results, internal validation and validation by RT-qPCR analysis was performed.

Internal validation

To identify the most relevant genes that distinguish between the TCMRV and eIV groups, the well-known support vector machine (SVM) classification algorithm [20], specifically e1071 R library implementation was applied [21]. The negative effect of overfitting caused by the small number of samples and the large number of evaluated genes was minimized by the recursive feature elimination (RFE) algorithm [22]. We first trained the classifiers on microarray data. Performance of SVM–RFE was evaluated by leave-one-out cross validation (LOOCV) [23]. Since the group sizes were not perfectly balanced in their size, the usual measure of classification accuracy (ACC) was complemented by another measure robust to skewed groups, the area under the ROC curve (AUC) [24]. The LOOCV demonstrated that TCMRV and eIV groups can be perfectly differentiated by a large scale of gene sets of various sizes, i.e., the groups can be separated by a small number of genes as well as by much larger gene sets. The gene set size ranges from 5 to 500 genes and showed absolute 100% ACC and maximum AUC (Supplementary Figure S1). However, the single small set of genes that separate the study groups was not easy to determine due to the large number of differentially expressed microarray genes. SVM–RFE perfectly split the groups with a large scale of potentially different gene sets of various sizes. For this reason, the final selection of genes for external validation was not induced directly from SVM–RFE as described below.

Validation by RT-qPCR analysis

Using a custom-made Taqman low-density array (Applied Biosystems, Foster City, CA, U.S.A.) on a validation set of patients (*n*=20), RT-qPCR was employed to analyze 38 genes identified by microarray to be deregulated between TCMRV and eIV and found to be of biological interest in the rejection process (Supplementary Table S1). Quantitative RT-qPCR based on TLDA technology was carried out as described elsewhere [25]. Real-time RT-qPCR data were quantified using the SDS 2.4 software package (Applied Biosystems) and relative gene expression values were determined using the comparative $2^{-\Delta\Delta C_t}$ method of the relative quantification (RQ) Manager Software v 1.2.1 (Applied Biosystems) with normalization to an endogenous control (HPRT1). The endogenous control was chosen from three candidate housekeeping genes (GAPDH-Hs99999905_m1, PGK1-Hs99999906_m1, HPRT1-Hs01003267_m1) using NormFinder (www.mdL.dk) as the gene with the most stable expression (HPRT1 with a stability value of 0.003). As a calibrator, one of the samples with a good expression profile on all of the target genes was used. All investigated mRNAs were measured in triplicate for each sample.

Risk of overfitting

In our study, we deal with the well-known $n < P$ problem (the large number of variables and the small number of samples) that represents a specific case of ill-posed problem and may result in overfitting [26,27]. This risk is minimized by careful handling with the train, test and validation datasets. First, we employ LOOCV to split between train

and test sets. Both gene selection and classifier construction are performed solely on train sets, while the corresponding test sets serve for their evaluation. In particular, the SVM–RFE procedure for gene selection was re-performed with each iteration of the LOOCV procedure, so that the features are selected from each train set and applied independently to each test set. In general, this train-test split allows us to detect overfitting and avoid complex biomarkers that heavily overfit the data used for model construction. It enables to propose simple biomarkers and to smoothly distinguish between them in terms of their performance. Second, we work with the independent RT-qPCR data set that serves to validate the selected biomarkers, remove the selection bias and get an unbiased estimate of their classification accuracy (expressed in terms of AUC to compensate for unbalanced classes) [27,28].

Statistical methods

Normality of the data was tested using the Kolmogorov–Smirnov test. Nonparametric values are presented as median and interquartile range. Two groups were compared by the two-tailed Mann–Whitney U-test and three groups by the Kruskal–Wallis test with *post hoc* adjustment by the Bonferroni correction for multiple tests. For comparison of categorical data, the χ^2 Fisher exact test was used. Two-sided *P*-values considered statistically significant for a *P* < 0.05.

In order to visualize different transcriptomes of studied samples, principal component analysis (PCA) and unsupervised hierarchical clustering (HC) were performed using R software. Both visualization tools were constructed using all genes evaluated by microarray to provide independent evidence of potential phenotype separation and to exclude circular misleading conclusion by choosing only genes deregulated between the study groups. In PCA analysis, a 3D scatter plot was produced based on the whole microarray expression profile of each sample. Calculation was done by the singular value decomposition of the centered expression matrix; no further scaling of the normalized expressions was performed.

In unsupervised HC, the Euclidean distance was selected as a similarity measure between the normalized expression profiles. The agglomerative complete-linkage HC was performed. The function *hclust* from R stats package was used for HC construction.

Results

Training set (microarray analysis)

Distinct microarray profiles of TCMRV and eIV

Using microarray-based gene expression profiling in the training set of patients, we identified 310 differentially regulated genes in early TCMRV compared with eIV (Supplementary Table S2). The volcano plot demonstrates deregulated genes between both groups after correction for multiple testing and shows most genes being up-regulated in TCMRV (*n*=288, 92.9%) compared with eIV (Figure 2A).

To get an insight into relevant biological processes, we used the DAVID database that allows gene annotation to gene ontology (GO) terms. We found that the genes regulated differentially between TCMRV and eIV were primarily associated with the innate and adaptive immune and inflammatory responses. In detail, TCMRV samples showed up-regulation of T-cell activation, costimulation, differentiation, receptor signaling pathway, positive regulation of T-cell proliferation, antigen processing, and presentation as well as B-cell activation and receptor signaling pathway, interferon- γ - and tumor necrosis factor-mediated signaling pathway and its positive regulation etc. Details of top 25 out of 65 enriched GO terms for biological processes are shown in Table 3. Activated biological processes indicate higher activation of immunity and inflammation in early TCMRV biopsies compared with eIV. The Circos plot was created based on 15 most significantly up-regulated genes between TCMRV and eIV to visualize gene association with significantly enriched pathways and GO terms that play a role in the immune response (Figure 3).

Analysis of the molecular relationships between differentially expressed genes (TCMRV compared with eIV) revealed involvement of GO terms for molecular functions highly associated with the rejection process (GO:0032395 MHC class II receptor activity, adjusted *P*=2.63E-07; GO:0004872 receptor activity, *P*=1.19E-04, GO:0005164 tumor necrosis factor receptor binding, *P*=2.87E-04; GO:0042605 peptide antigen binding, *P*=3.08E-04; GO:0045028 G-protein coupled purinergic nucleotide receptor activity, *P*=2.83E-03; GO:0004896 cytokine receptor activity, *P*=9.79E-03; GO:0019976 interleukin-2 binding, *P*=3.24E-02; GO:0004911 interleukin-2 receptor activity, 3.24E-02).

Comparison of the study groups with normal histologic findings

By comparing eIV with normal histologic findings, 28 up- and 22 down-regulated genes were identified (Figure 2B, Supplementary Table S3). Comparison of eIV with normal indicates involvement of down-regulated genes in GO terms for molecular functions highly associated with ion binding (GO:0046872, metal ion binding, *P*=6.0E-3;

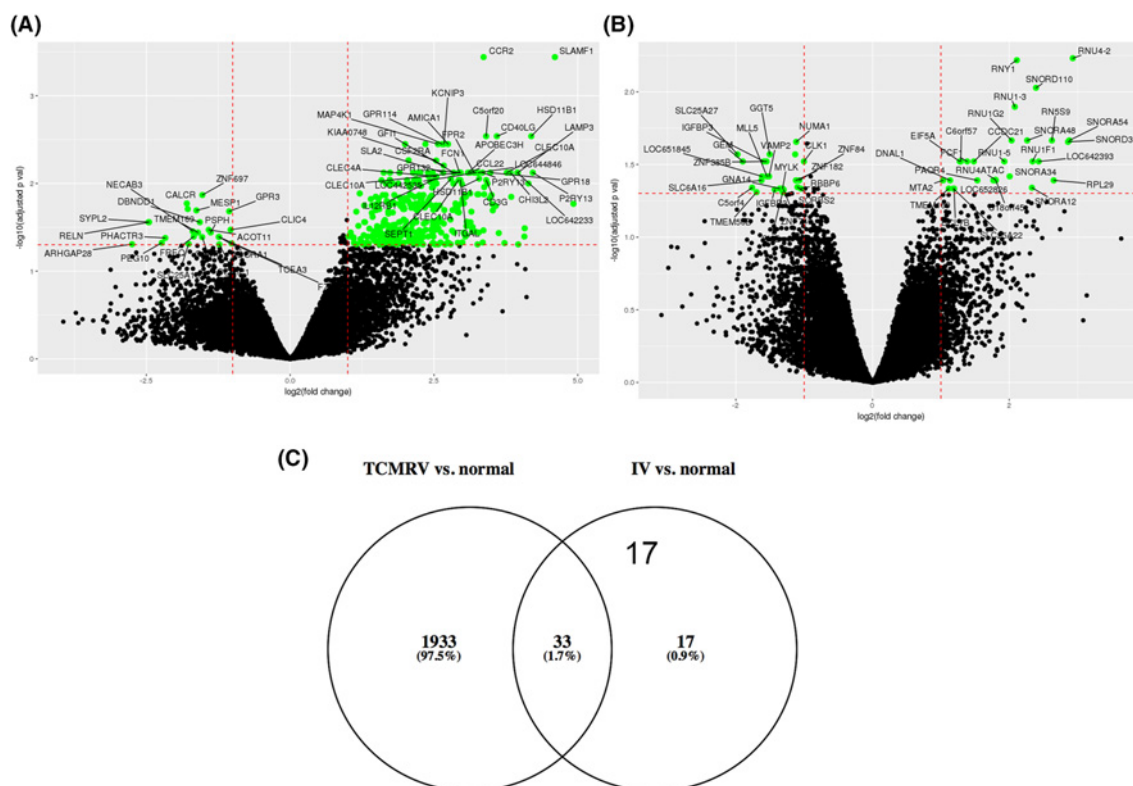


Figure 2. Genes with different mRNA expression in studied groups.

The volcano plot analysis showing differences in mRNA expression values between TCMRV and eIV (A) and between eIV and normal (B) considering an adjusted P -value cut-off = 0.05 and a fold-change cut-off = 2. The data for all genes are plotted as log₂-fold change compared with the -log₁₀ of the p -value. Thresholds are shown as dashed lines. (C) Venn diagram of shared deregulated genes between TCMRV compared with normal and eIV compared with normal. Comparison of deregulated gene lists between the study groups and controls shows a 1.7% ($n=33$) gene overlap with no association with any gene ontology (GO) term.

GO:0043169, cation binding, $P=6.4E-3$; GO:0043167, ion binding, $P=7.2E-3$; GO:0046914, transition metal ion binding, $P=26.1E-3$; GO:0008270, zinc ion binding, $P=36.7E-3$, unadjusted P -values shown). Genes up-regulated in eIV compared with normal histology showed association with translation (GO:0006412, unadjusted $P=8.39E-03$) and RNA binding (GO:0003723, unadjusted $P=3.95E-02$) reflecting the early post-transplant time period of eIV findings (median 8 days in eIV compared with 97 days in control group).

On the other hand, comparison of TCMRV to the control group revealed a large number of significantly deregulated genes ($n=1966$). Gene enrichment analysis found 239 GO terms for the biological process, predominantly from an immune response, to be significantly associated with deregulated genes (Supplementary Table S4) and confirmed true rejection origin of TCMRV. Comparison of deregulated gene lists between the study groups and controls using a Venn diagram showed only a 1.7% ($n=33$) gene overlap (Figure 2C, Supplementary Table S5). The genes shared between those two comparisons (TCMRV compared with normal and eIV compared with normal) were not significantly associated with any GO term. Taken together, the transcriptome of eIV showed relative similarity with the control group but was dramatically different from that of TCMRV.

Visualization of microarray data

The 3D PCA analysis applied to the whole transcriptome data demonstrated clear separation of TCMRV from eIV kidney allografts (Figure 4). Control samples were spread among eIVs demonstrating similarity of both groups' transcriptome. In addition, unsupervised HC analysis was applied to the whole microarray data and a similar pattern was obtained (Figure 5). Three main subclusters were formed. The first cluster (left) was formed by an individual eIV patient (eIV_5) undergoing successful steroid treatment of early IV but a low level of DSA class I (MFI 1100) was detected retrospectively with no impact on graft function or later development of ABMR. The second cluster

Table 3 Top 25 GO terms for biological process enriched in TCMRV compared with eIV

	GO term	Count	P
GO:0006955	Immune response	54	4.28E-31
GO:0031295	T-cell costimulation	21	7.03E-17
GO:0050853	T-cell receptor signaling pathway	25	8.95E-16
GO:0006954	Inflammatory response	36	9.93E-16
GO:0002250	Adaptive immune response	21	1.61E-11
GO:0042110	T-cell activation	14	2.44E-11
GO:0045087	Innate immune response	31	3.24E-10
GO:0007165	Signal transduction	50	3.01E-09
GO:0060333	Interferon- γ -mediated signaling pathway	14	4.92E-09
GO:0002504	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	8	3.39E-07
GO:0050776	Regulation of the immune response	17	1.17E-06
GO:0032729	Positive regulation of interferon- γ production	10	2.31E-06
GO:0007166	Cell surface receptor signaling pathway	20	2.84E-06
GO:0033209	Tumor necrosis factor-mediated signaling pathway	13	1.77E-05
GO:0042102	Positive regulation of T-cell proliferation	10	1.94E-05
GO:0030217	T-cell differentiation	8	2.01E-05
GO:0006935	Chemotaxis	13	2.10E-05
GO:0006915	Apoptotic process	27	2.53E-05
GO:0030168	Platelet activation	12	7.54E-05
GO:0050853	B-cell receptor signaling pathway	9	7.81E-05
GO:0043547	Positive regulation of GTPase activity	26	7.88E-05
GO:0001816	Cytokine production	7	8.71E-05
GO:0050900	Leukocyte migration	12	1.18E-04
GO:0042113	B-cell activation	7	1.96E-04
GO:0006968	Cellular defense response	9	2.01E-04

The genes with different expression in TCMRV and eIV after correction for multiple comparisons ($n=310$) were entered into the DAVID gene ontology database. Top 25 out of 65 GO terms are shown.

included exclusively TCMRV samples. In the third cluster, we were not able to distinguish between eIV and control samples mirroring the groups' transcriptome similarity. Taken together, unsupervised HC analysis distinguished TCMRV from eIV samples but failed to distinguish between eIV and controls.

Validation set (RT-qPCR analysis)

RT-qPCR analysis of the validation set confirmed up-regulation of the immune response in TCMRV compared with eIV

We verified these findings in a validation set of patients ($n=20$) using a different technique. The clinical characteristics of the validation cohort are similar to that of the training set (Table 2). A total of 38 genes were selected for RT-qPCR validation (Supplementary Table S1). These genes exhibited at least two-fold change and adjusted P -value <0.05 in microarray expression and were of biological interest for TCMRV and eIV comparison.

RT-qPCR analysis confirmed that early indication biopsy tissues of TCMRV had statistically significantly higher expression of *BCL11B*, *BTLA*, *CCL17*, *CCR7*, *CD2*, *CTLA4*, *CXCL13*, *GIMAP5*, *IL21R*, *KLRG1*, *LAX1*, *LCK*, *LTA*, *LTB*, *SLA2*, *SLAMF1*, *TNFRSF4* and *ZAP70* compared with eIV (Figure 6). The validated genes are significantly involved in regulation of immune system process, T-cell differentiation, activation, proliferation, B-cell activation, overall lymphocyte and leukocyte activation, immune response-regulating cell signal transduction, and apoptosis.

Agreement between microarray and RT-qPCR data

Validation of reference genes in the validation set was defined as both qualitative (direction) and quantitative agreement between microarray and RT-qPCR measurements. The direction of RT-qPCR gene expressions agreed with the microarray technique in 100% of validated genes. Quantitative agreement between microarray and RT-qPCR was confirmed by a significant correlation of normalized data (Pearson $r = 0.663$, $P=0.00006$) (Supplementary Figure

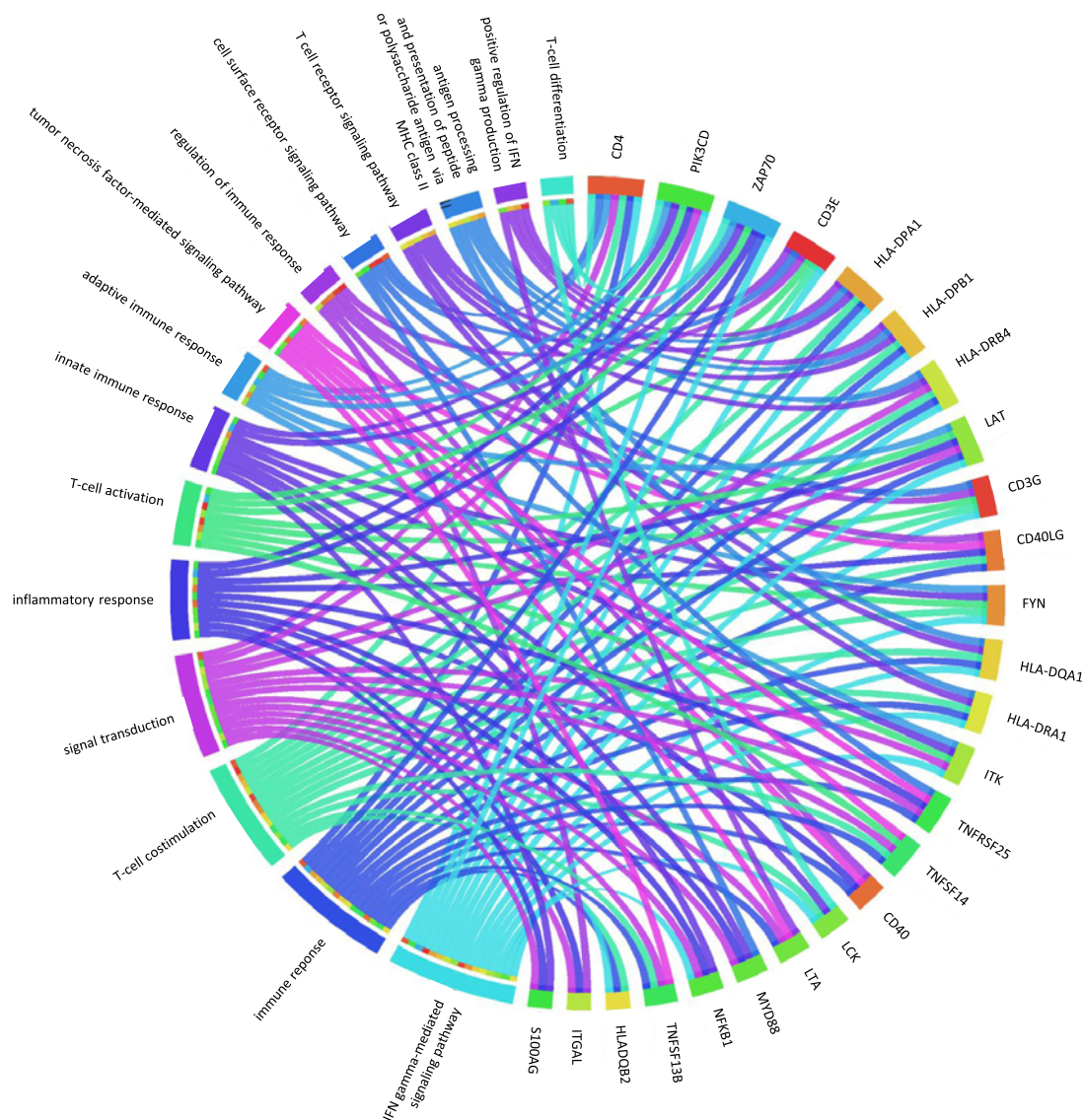


Figure 3. The Circos plot represents significantly enriched pathways and GO terms for biological process associated with the 15 most significant up-regulated genes between TCMRV compared with eIV samples, detected using the DAVID database

Outside the circle, dysregulated genes and significantly enriched pathways together with GO terms are indicated.

S2). To further validate differences in the transcriptome of the study groups, the SVM–RFE classifiers were trained on RT-qPCR data. LOOCV confirmed that the genes selected for validation from microarray data showed around 80% accuracy (ACC) and a 0.75 area under the curve (AUC) (Supplementary Figure S3) thus confirming reasonable gene selection for external RT-qPCR validation.

Discussion

In the present study, we investigated the transcriptome of eIV with paucity of TI and TCMRV with rich TI. Our main results are that the transcriptome of eIV revealed a weak immunologic signature compared with TCMRV and showed similarity with non-rejection 3-month protocol biopsy. Based on our results, eIV may feature a non-rejection phenotype and reflect peritransplant injury. As the current Banff histopathological criteria consider intimal arteritis (after exclusion of ABMR) to be at least type II of TCMR irrespective of TI, our results agree with calls for reassessment of the current approach in histology interpretation. Furthermore, difference in non-rejection phenotype of DSA- and

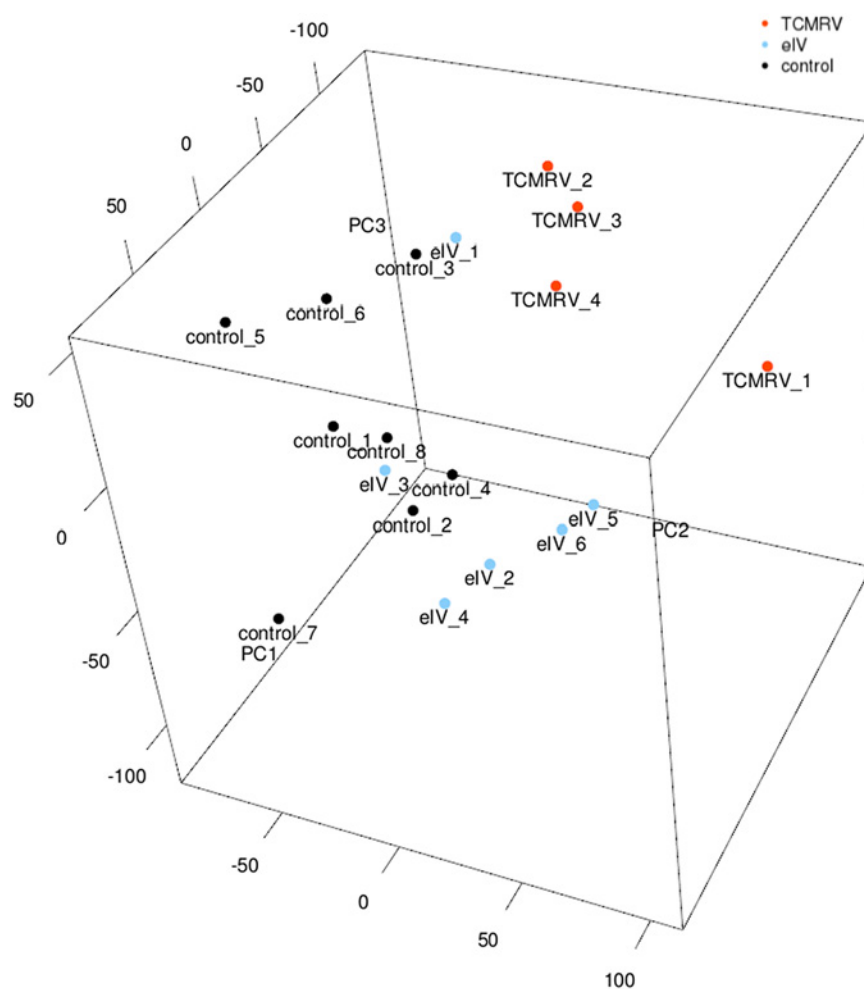


Figure 4. 3D PCA applied to the whole transcriptome of TCMRV, eIV, and control samples (training set)

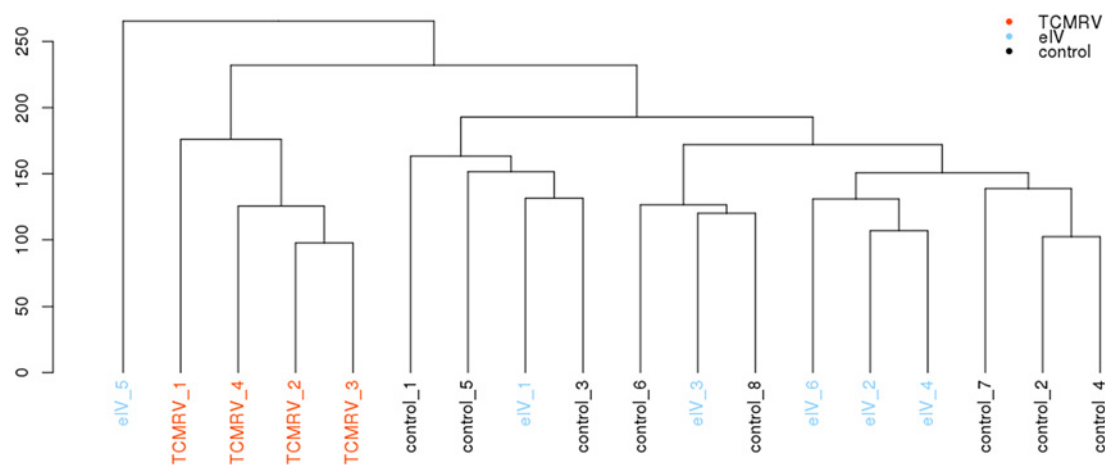


Figure 5. Unsupervised hierarchical cluster analysis applied to the whole transcriptome of TCMRV, eIV and control samples (training set)

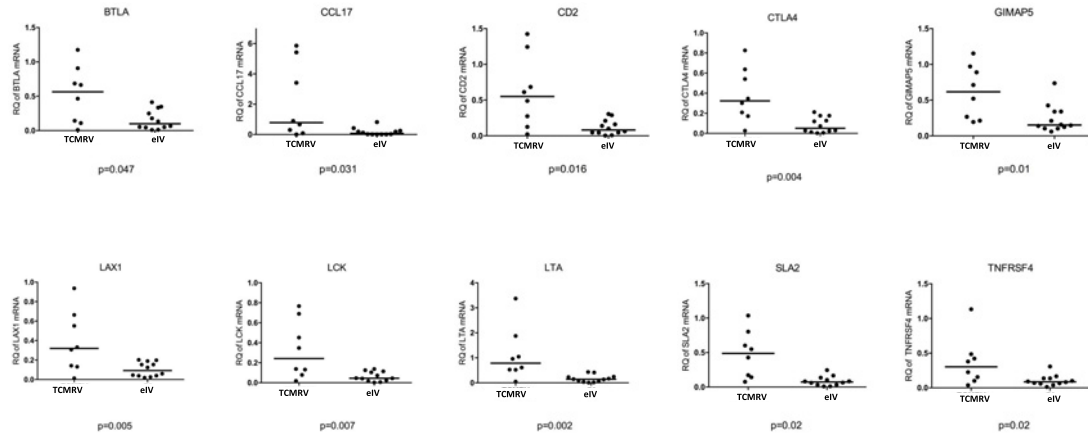


Figure 6. Validation of microarray analysis by RT-qPCR of early indication biopsy samples
Scatter plots show top 10 deregulated genes between TCMRV and eIV.

C4d-negative eIV described in our study and rejection phenotype of late and often DSA positive IV [16] supports use of a molecular microscope in the case of an IV finding as recommended by the last Banff Kidney Meeting report [6].

Our first goal was to find out whether eIV represents a true TCMR. To do so, we included early intimal arteritis with minimal TI (eIV) and rich TI (TCMRV) and excluded cases with any MI, and C4d or DSA positivity. With these inclusion/exclusion criteria, only not anti-HLA-antibody-mediated intimal arteritis was evaluated. We used microarray technology to allow for investigation at the molecular level. Because the gene expression patterns change over time after transplantation and may vary widely, even among tissues with similar morphological changes [29], we decided to include only early indication kidney allograft biopsies into the study groups.

We found that the transcriptome of eIV was significantly different from that of TCMRV. In general, the genes deregulated between TCMRV and eIV were annotated to GO terms associated with the innate and adaptive immune and inflammatory responses. This is in accordance with other studies which identified relevant biological processes for acute rejection to be up-regulated in T cell-mediated rejection and led to development of molecular test for T cell-mediated rejection (TCMR score) [30,31]. In detail, up-regulated genes in our TCMRV samples were associated with T-cell activation, differentiation, costimulation, T-cell receptor signaling pathway, signal transduction, interferon- γ - and tumor necrosis factor-mediated signaling pathway, antigen processing and presentation, apoptosis, and others as listed in Table 3. Vice versa, eIV revealed significant down-regulation of T cell-mediated processes similarly to recent study showing negative molecular TCMR score in majority of IV in contrast to positive TCMR score in 95% of v-lesions with rich TI and proposing that IV in absence of DSA should not be used to diagnose TCMR [16].

Another interesting finding of our study is that PCA and HC indicate a rather clear distinction of TCMRV from eIV and controls at the molecular level. The separation is not perfect as all microarray probes, not only the deregulated ones, were used. The standalone eIV.5 patient had retrospectively detected DSA and his separation might reflect the fact that molecular changes precede the histologic ones.

The Venn diagram comparing deregulated genes in TCMRV and eIV compared with controls demonstrated that the shared genes were not annotated to GO terms associated with rejection or the immune response. Moreover, eIV samples revealed relative similarity to normal histologic findings. So, if TCMRV represents true tubulointerstitial rejection, then eIV with a distinct transcriptome profile is probably of non-rejection origin.

Interestingly, by comparison of eIV and controls only 50 genes were found to be differentially regulated and found to be associated with down-regulation of zinc ion binding in early IV. It is well known that zinc plays an important role in ischemia/reperfusion injury and has potential antioxidant properties [32]. Recent work shows that oral zinc chloride supplementation mitigates renal ischemia/reperfusion injury in rats by modulating oxidative stress, endoplasmic reticulum stress, and autophagy [33]. Therefore, we speculate that down-regulation of zinc ion binding genes in eIV might reflect exhaustion of zinc metabolism in early post-transplant biopsies with potential ongoing ischemia/reperfusion injury compared with quiescent 3-month protocol biopsies with normal histology and no signs of acute kidney injury.

Another important finding in the search of IV origin was the identification of several potential target molecules that could have important roles in understanding of IV process. Therefore, we evaluated set of deregulated genes from

microarray study with potential biological interest in a validation set of samples using different molecular diagnostic technique. RT-qPCR analysis revealed significant up-regulation of several genes with an important role in rejection process in TCMRV compared with IV, such as regulation of immune system process, T-cell differentiation, activation, proliferation, B-cell activation, overall lymphocyte and leukocyte activation, immune response-regulating cell signal transduction and apoptosis and confirmed stronger activation of an innate and adaptive immune responses in TCMRV compared with eIV.

Our findings agree with other microarray studies which showed a discrepancy between conventional histologic and molecular assessment of TCMR [14–16,31,34]. The discrepancy was particularly marked in IV, where most cases exhibited low TCMR scores [31]. Intimal arteritis has been shown to have less importance in the diagnostics of TCMR than tubulitis and interstitial infiltrate [15]. Salazar reported that only a minority of IV exhibited the molecular signature of TCMR. Particularly early biopsies performed in the first year post-transplant in DSA-negative patients, usually did not reflect rejection, while late IV often associated with DSA was indicative of ABMR. Taken together, current diagnostic conventions for v-lesion tend to overdiagnose rejections as many biopsies with no molecular TCMR score are traditionally assessed as being TCMR [16].

Our and others' results suggest that early post-transplant IV might reflect mechanisms different from rejection, i.e., injury-repair response to implantation stresses, endothelial injury related to ischemia-reperfusion [7]. The presence of intimal arteritis in early non-rejection biopsies might be explainable by increased vascular permeability and facilitated extravasation of leukocytes by interrupting the integrity of the renal vascular endothelium triggered by renal injury [35,36].

However, our conclusions differ from those of the few clinical studies analyzing IV. Wu et al. [37] reported intimal arteritis to be an independent histologic risk factor of long-term graft loss regardless of the timing of finding and the extent of TI and considered IV to be an acute rejection of kidney allograft with a poor long-term outcome. Sis provided clinical evidence that IV behaves as true acute rejection because it is associated with functional improvement after anti-rejection treatment and represents an independent risk factor for allograft failure [38]. Such a putative discrepancy might be explainable by few facts. First, these studies analyzed the clinical course of IV at different time points after transplantation ignoring the fact that it is the time of biopsy that is critical for understanding IV. Nevertheless, most early IV specimens have no signature of molecular rejection and are DSA negative, whereas late IV ones are often DSA positive and show molecular ABMR [16]. Second, all discrepant studies were based on the clinical presentation of intimal arteritis, not on transcriptome analysis and evaluated the response to anti-rejection treatment in a very heterogeneous cohort. Third, clinical studies reporting the severity of IV analyzed biopsies were from earlier eras when aggressive TCMR was common and ABMR poorly recognized. Historically, IV was believed to actually imply rejection and had a negative effect on kidney graft survival. However, the nature and significance of IV have changed over time and v-lesions from a decade ago must be reinterpreted in the current era [39]. Nowadays, IV represents a rare finding in indication biopsies. At our center, 2.5% of kidney allografts show IV, mostly during the first 3 months post-transplant, with a mild clinical course and a favorable outcome [40].

Potential limitations of our study include its retrospective design and small sample size. However, the study limitations reflect IV in the real-life setting where IV is a rare finding, with heterogeneous interpretation and treatment. We realize that most of the samples in our study had mild to moderate arteritis (v1) and were found in early indication biopsies within the first month post-transplant. Therefore, our study results indicating a potential non-rejection origin of eIV are applicable exclusively to this specific cohort. We are also aware that all findings of intimal arteritis were treated by steroids or Thymoglobulin according to our historical standards of care for TCMR, which might have influenced graft prognosis. However, the renal allograft transcriptome was analyzed before initiating any anti-rejection treatment. Moreover, the question whether eIV patients should be given anti-rejection treatment, augmentation of immunosuppression or whether they should be followed by periodic surveillance biopsies, cannot be answered by the present study and calls for appropriate randomized controlled clinical trials. We are aware of fact that 2 eIV and 1 TCMRV kidney allografts exhibited intimal arteritis in addition to acute tubular necrosis which may have affected mRNA expression and graft function development. Additionally, the eIV group showed higher rates of anti-IL2 blockade (basiliximab) which may have had a potential impact on milder histologic finding and the molecular phenotype of eIV. While, admittedly, mRNA expression evaluation using laser capture microdissection would be of relevance in molecular analysis of highly focal changes, this approach is limited by the tendency of laser capture to damage the RNA, high costs and complexity, making it technically challenging [41]. Moreover, molecular assessments are able to assess an ongoing pathological process as rejection or injury even in the absence of glomeruli and arteries, are independent of cortex proportion and, although histology cannot assess the medulla, rejection does occur in the medulla as well as cortex [42]. Therefore, in our opinion, it still seems reasonable to evaluate vascular rejection from non-dissected tissue.

The strength of the present study is careful selection of AVR samples with the histology of the T cell-mediated phenotype and exclusion of ABMR according to the updated Banff classification [4]. Our study is further strengthened by the uniform approach of a high-volume transplant center and well-selected study groups, where only patients early after kidney transplantation within the first month post-transplant, with no rejection history, no surgical complications, similar immunological risk, and incidence of delayed graft function were enrolled into study groups. We included only indication biopsies and excluded subclinical, late, and DSA positive findings. Furthermore, microarray analysis was performed based on recent recommendations to avoid common errors in the implementation and interpretation of microarray studies [43]. Only genes that passed through correction for multiple testing were judged as significantly deregulated. To avoid circular logic, PCA and cluster analyses were based on the whole microarray data, not only on deregulated genes between the two groups, which allow to show the real separation of the distinct transcriptomes of eIV and TCMRV.

Our microarray study of DSA- and C4d-negative eIV revealed a weaker immune signature compared with TCMRV and showed similarity with normal histologic findings. Our results suggest that mild eIV occurring early after kidney transplantation in a not highly sensitized population may feature non-rejection origin. It seems more likely that inflammation seen in eIV might reflect endothelial injury from the transplantation process distinct from an alloimmune response. The evaluation of problematic biopsies with IV should combine histology and serologic HLA antibody assessment with use of molecular techniques as microarray to ensure correct diagnosis assignment. This is important particularly in case of IV which often show a discrepancy between conventional histology and microarrays. Using current conventions, many cases of IV are interpreted by pathologists as T-cell mediated rejection but exhibit low molecular TCMR score by molecular assessment, especially if DSA negative and observed early after kidney transplantation. Incorporation of molecular microscope into clinical praxis of IV on top of conventional histology and HLA antibody assessment might lower the rate of false positive rejections and help with therapeutic approach. Further prospective studies are warranted to delineate the pathogenesis and prognosis of this rare and heterogeneous entity before any reassessment of guidelines for diagnostic interpretation and treatment of IV.

Clinical perspectives

- **Background as to why the study was undertaken:** IV with minimal TI represents a challenging clinical situation after kidney transplantation, especially when occurring early and in the absence of DSA and C4d. To find out if early IV (eIV) represents a true rejection, the present study compared the transcriptome of eIV and T-cell mediated vascular rejection (TCMRV).
- **A brief summary of the results:** The transcriptome of early IV (<1 month) with negative C4d and DSA is associated with a weak immune signature compared with TCMRV and shows similarity with normal findings. Such eIV may feature non-rejection origin and reflect an injury distinct from an alloimmune response.
- **The potential significance of the results to human health and disease:** Although intimal arteritis has been known to have a deleterious impact on kidney graft survival regardless of TI, our results agree with recent studies impeaching the rejection origin and negative prognosis of eIV. The present study supports reassessment of the current approach in interpretation of kidney allograft biopsy and supports use of molecular methods.

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Competing interests

The author declares that there are no competing interests associated with the manuscript.

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Author contribution

M.W. participated in design and performance of the research, data analysis, writing of the paper. P.H. participated in processing of tissue samples. J.K. and V.S. contributed to statistical analysis. M.N. participated in performance of the research. E.H. participated in histologic assessment. Z.K. participated in microarray evaluation. P.V. participated in immunogenetic examination. O.V. contributed to the research design and writing of the paper

Abbreviations

ABMR, antibody-mediated rejection; ACC, classification accuracy; ah, arteriolar hyaline thickening; ATN, acute tubular necrosis; AUC, area under the ROC curve; AVR, acute vascular rejection; DSA, donor-specific antibody; ECD, expanded criteria donor; eIV, early isolated v-lesion; GEO, Gene Expression Omnibus; GO, gene ontology; HC, hierarchical clustering; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IKEM, Institute for Clinical and Experimental Medicine; IV, isolated v-lesion; LOOCV, leave-one-out cross validation; MI, microvascular inflammation; PCA, principal component analysis; PRA, panel reactive antibodies; RFE, recursive feature elimination; ROC, receiver operating characteristic; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SVM, support vector machine; TCMR, T cell-mediated rejection; TCMRV, T cell-mediated vascular rejection; TI, tubulointerstitial inflammation; TLDA, TaqMan Low Density Array.

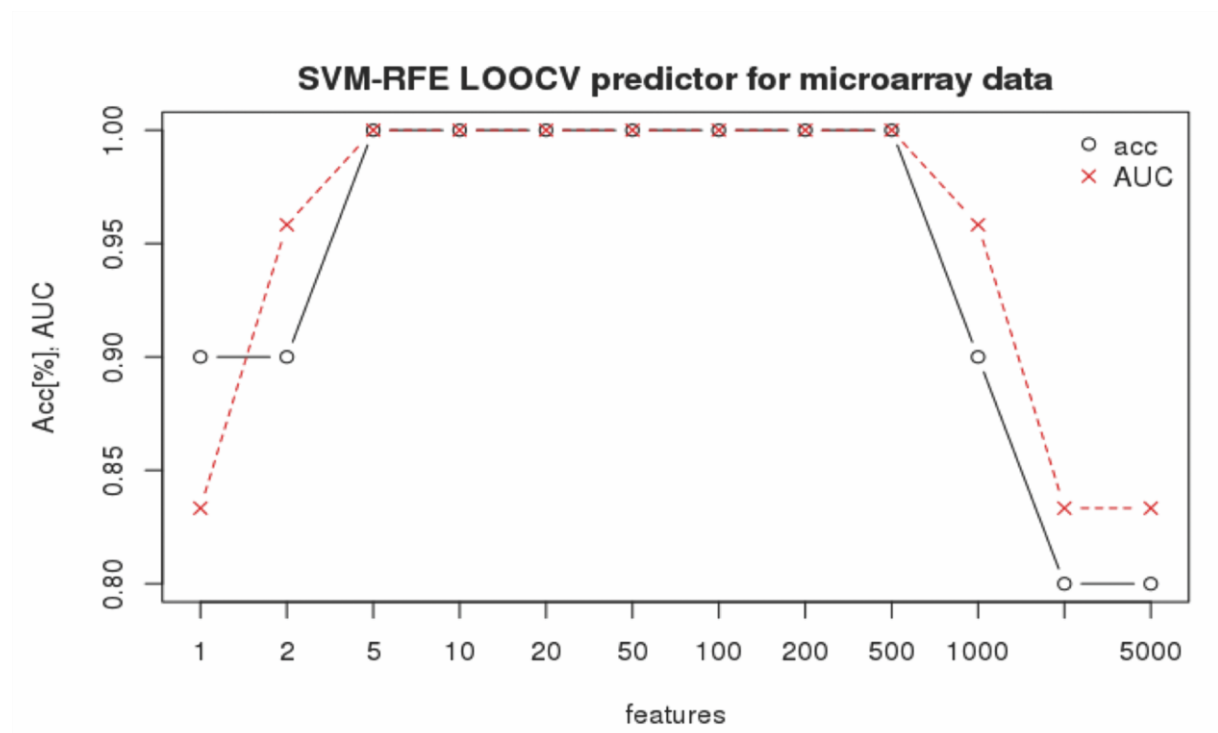
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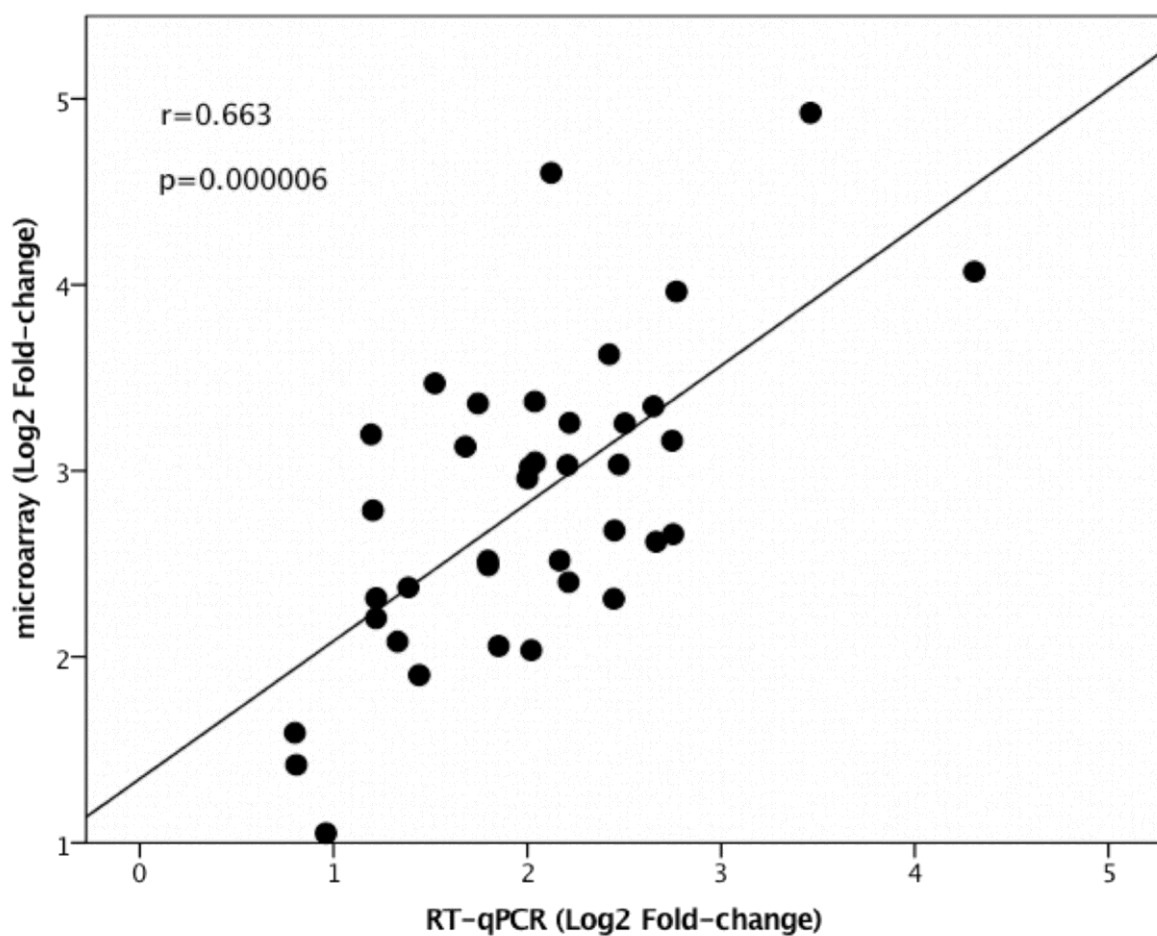
Supplement Figure 1. The development of leave-one-out cross validation (LOOCV) support vector machine (SVM) classification accuracy (ACC) and the area under the ROC curve (AUC) with different RFE parametrizations using microarray data on the training set of patients (n=10).

The figure shows that microarray-based SVM perfectly separates the TCMRV and IV groups for reasonably sized gene sets. For fewer than 5 and more than 500 genes, the classifier tends to underfit / overfit the data.



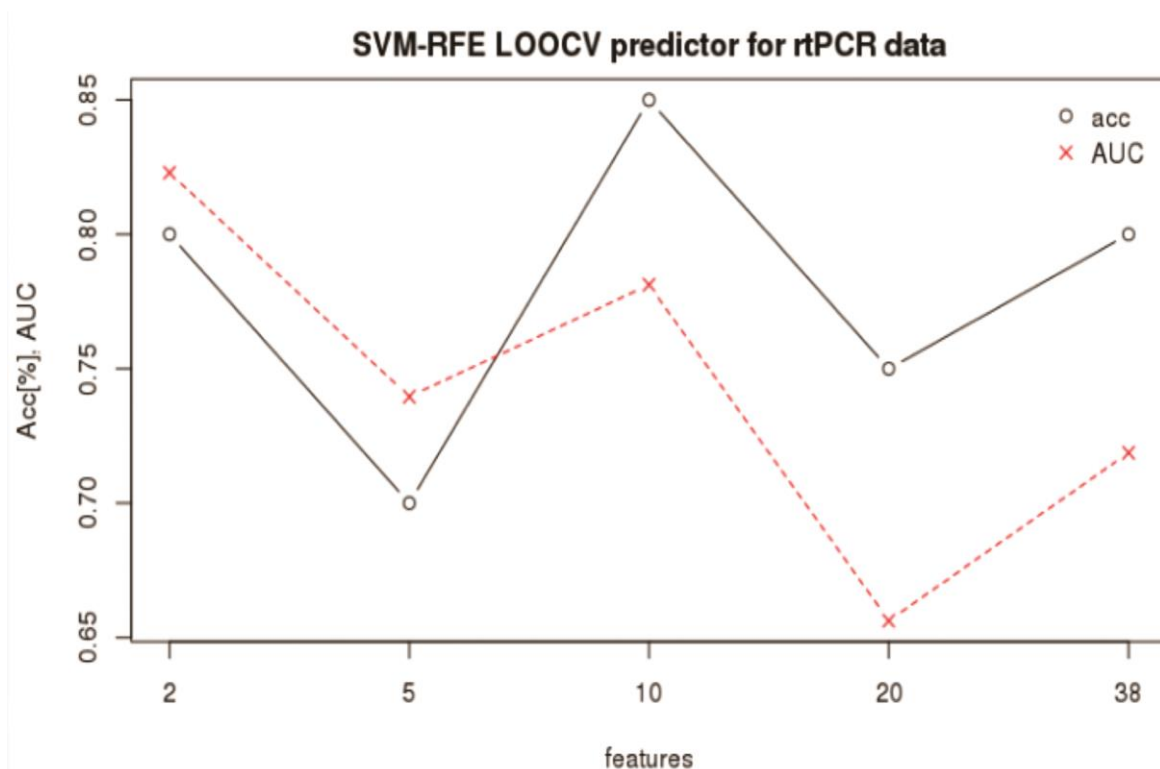
Supplement Figure 2. Correlation of microarray and real-time RT-qPCR

analyses of selected genes. Pearson's correlations between \log_2 -fold change of the gene expression values from microarrays and RT-qPCR experiments ($r=0.663$, $p=0.000006$). Scatter plot shows correlation of the median fold changes from the RT-qPCR and microarray data set for a validated set of genes ($n=38$). A complete list of validated genes is shown in Suppl. Table 1.



Supplement Figure 3. The development of the leave-one-out cross validation (LOOCV) support vector machine (SVM) classification accuracy (ACC) and the area under the ROC curve (AUC) with different RFE parametrizations using RT-qPCR data on the validation set of patients (n=20).

The figure shows that RT-qPCR-based SVM working with 38 validated genes selected in the microarray phase reasonably separates TCMRV and IV samples. The plot shows the association of the area under the ROC curve with the number of validated genes and demonstrates maximal classification accuracy (ACC) and AUC for 10 target genes. Each point represents one ROC curve for a given number of validated genes.



Gene symbol	Gene Name	PCR FC	Microarray F
BCL11B	B-cell CLL/lymphoma 11B	4.65	9.56
BTLA	B and T lymphocyte associated	5.67	9.55
CARD9	Caspase recruitment domain family member 9	2.61	5.18
CCL17	C-C motif chemokine ligand 17	11.01	30.38
CCR2	C-C motif chemokine receptor 2	3.35	10.29
CCR7	C-C motif chemokine receptor 7	6.82	15.61
CD19	CD19 molecule	6.34	6.14
CD2	CD2 molecule	6.71	8.95
CD3E	CD3e molecule	4.49	5.73
CD4	CD4 molecule	4.64	5.28
CD40	CD40 molecule	2.33	4.97
CD79A	CD79a molecule	5.46	6.41
CTLA4	Cytotoxic T-lymphocyte associated protein 4	6.28	10.18
CXCL13	C-X-C motif chemokine ligand 13	19.80	16.81
FPR2	Formyl peptide receptor 2	2.29	9.17
GIMAP5	GTPase, IMAP family member 5	4.06	4.10
ICOS	Inducible T-cell costimulatory	4.04	8.11
IKZF1	IKAROS family zinc finger 1	3.20	8.75
IL21R	Interleukin 21 receptor	4.62	8.17
IL27RA	Interleukin 27 receptor subunit alpha	2.51	4.23
ITGAL	Integrin subunit alpha L	4.00	7.78
ITK	IL2 inducible T-cell kinase	4.11	8.26
KLRG1	Killer cell lectin-like receptor G1	2.33	4.62
LAT	Linker for activation of T-cells	2.72	3.74
LAX1	Lymphocyte transmembrane adaptor 1	3.47	5.73
LCK	LCK proto-oncogene, Src family tyrosine kinase	5.45	4.96
LCP2	Lymphocyte cytosolic protein 2	3.61	4.16
LTA	Lymphotoxin alpha	5.55	8.18
LTB	Lymphotoxin beta	5.36	12.35
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	1.74	3.01
MYD88	Myeloid differentiation primary response 88	1.75	2.67
NFKB1	Nuclear factor kappa B subunit 1	1.95	2.07
SLA2	Src-like adaptor 2	6.74	6.32
SLAMF1	Signaling lymphocytic activation molecule family member 1	4.36	24.29
TLR8	Toll-like receptor 8	2.87	11.08
TNFRSF4	TNF receptor superfamily member 4	3.48	5.63
TNFSF14	Tumor necrosis factor superfamily member 14	2.30	6.90
ZAP70	Zeta chain of T cell receptor associated protein kinase 70	4.11	10.36

Supplement table 1. List of 38 validated genes showing a fold change in gene

expression between TCMRV and eIV for RT-qPCR and the microarray technique.

Entrez Gene ID	Gene symbol	Gene name	FC	Adjusted p-value (BH)
6504	SLAMF1	Signaling lymphocytic activation molecule family member 1	24.29	3.63E-04
1231	CCR2	Chemokine (C-C motif) receptor 2	10.29	3.63E-04
3290	HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	18.27	2.90E-03
959	CD40LG	CD40 ligand	12.06	2.90E-03
140947	C5orf20	TRAF-interacting protein with forkhead-associated domain, family member B; chromosome 5 open reading frame 20	10.60	2.90E-03
120425	AMICA1	Adhesion molecule, interacts with CXADR antigen 1	6.70	3.56E-03
30818	KCNIP3	Kv channel interacting protein 3, calsenilin	6.38	3.56E-03
221188	GPR114	G protein-coupled receptor 114	6.24	3.56E-03
7128	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	5.10	3.56E-03
91523	FAM113B	Family with sequence similarity 113, member B	3.99	3.56E-03
3937	LCP2	Lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)	4.16	5.44E-03
9840	KIAA0748	Thymocyte expressed, positive selection associated 1	5.81	5.48E-03
1438	CSF2RA	Colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)	6.36	6.25E-03
53829	P2RY13	Purinergic receptor P2Y, G-protein coupled, 13	18.60	7.53E-03
27074	LAMP3	Lysosomal-associated membrane protein 3	15.53	7.53E-03
643733	LOC642233	Hypothetical LOC643733	15.43	7.53E-03
1117	CHI3L2	Chitinase 3-like 2	14.22	7.53E-03
10462	CLEC10A	C-type lectin domain family 10, member A	13.61	7.53E-03
2841	GPR18	G protein-coupled receptor 18	11.16	7.53E-03
644846	LOC644846	Hypothetical protein LOC644846 (LOC644846), mRNA.	9.64	7.53E-03
442535	LOC442535	Similar to T-cell receptor gamma chain V region PT-gamma-1/2 precursor (LOC442535), mRNA.	9.42	7.53E-03
2358	FPR2	Formyl peptide receptor 2	9.17	7.53E-03
6367	CCL22	Chemokine (C-C motif) ligand 22	8.72	7.53E-03
164668	APOBEC3H	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3H	8.52	7.53E-03
2219	FCN1	Ficolin (collagen/fibrinogen domain containing) 1	7.69	7.53E-03
29933	GPR132	G protein-coupled receptor 132	7.65	7.53E-03
2672	GFI1	Growth factor independent 1 transcription repressor	7.17	7.53E-03
84174	SLA2	Src-like adaptor 2	6.32	7.53E-03
6252	RTN1	Reticulon 1	5.56	7.53E-03
11151	CORO1A	Coronin, actin binding protein, 1A	5.26	7.53E-03
64386	MMP25	Matrix metalloproteinase 25	4.81	7.53E-03
81030	ZBP1	Z-DNA binding protein 1	4.67	7.53E-03
81793	TLR10	Toll-like receptor 10	4.40	7.53E-03
27040	LAT	Linker for activation of T cells	3.74	7.53E-03
79713	TMEM149	Transmembrane protein 149	3.33	7.53E-03
83593	RASSF5	Ras association (RalGDS/AF-6) domain family member 5	3.09	7.53E-03
50856	CLEC4A	C-type lectin domain family 4, member A	5.82	8.35E-03
497189	TIFAB	TRAF-interacting protein with forkhead-associated domain, family member B; chromosome 5 open reading frame 20	5.31	8.35E-03
1731	SEPT1	Septin 1	6.93	8.89E-03

3594	IL12RB1	Interleukin 12 receptor, beta 1	5.72	8.89E-03
4068	SH2D1A	SH2 domain protein 1A	4.56	8.89E-03
960	CD44	CD44 molecule (Indian blood group)	3.32	9.08E-03
917	CD3G	CD3g molecule, gamma (CD3-TCR complex)	10.62	9.16E-03
3683	ITGAL	Integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	7.78	9.16E-03
3117	HLA-DQA1	Similar to hCG2042724; similar to HLA class II histocompatibility antigen, DQ(1) alpha chain precursor (DC-4 alpha chain); major histocompatibility complex, class II, DQ alpha 1	4.46	9.16E-03
240	ALOX5	Arachidonate 5-lipoxygenase	4.72	9.19E-03
117289	TAGAP	T-cell activation RhoGTPase activating protein	4.09	9.19E-03
5293	PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	3.00	9.19E-03
3112	HLA-DOB	Major histocompatibility complex, class II, DO beta	5.40	9.58E-03
10538	BATF	Basic leucine zipper transcription factor, ATF-like	10.78	1.00E-02
100133678	LOC100133678	Similar to hCG2042724; similar to HLA class II histocompatibility antigen, DQ(1) alpha chain precursor (DC-4 alpha chain); major histocompatibility complex, class II, DQ alpha 1	4.37	1.00E-02
168537	GIMAP7	GTPase, IMAP family member 7	3.68	1.00E-02
5696	PSMB8	Proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	3.44	1.00E-02
7409	VAV1	Vav 1 guanine nucleotide exchange factor	3.16	1.00E-02
27334	P2RY10	Purinergic receptor P2Y, G-protein coupled, 10	7.17	1.00E-02
8698	S1PR4	Sphingosine-1-phosphate receptor 4	5.83	1.00E-02
7293	TNFRSF4	Tumor necrosis factor receptor superfamily, member 4	5.63	1.01E-02
4069	LYZ	Lysozyme (renal amyloidosis)	4.73	1.01E-02
1178	CLC	Charcot-Leyden crystal protein	10.36	1.10E-02
606724	LOC606724	Coronin, actin binding protein, 1A pseudogene	5.59	1.10E-02
5880	RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	4.23	1.10E-02
10320	IKZF1	IKAROS family zinc finger 1 (Ikaros)	8.75	1.30E-02
7535	ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa	10.36	1.33E-02
4332	MNDA	Myeloid cell nuclear differentiation antigen	9.68	1.33E-02
151888	BTLA	B and T lymphocyte associated	9.55	1.33E-02
9466	IL27RA	Interleukin 27 receptor, alpha	4.23	1.33E-02
921	CD5	CD5 molecule	11.32	1.36E-02
51176	LEF1	Lymphoid enhancer-binding factor 1	4.98	1.36E-02
149628	PYHIN1	Pyrin and HIN domain family, member 1	4.29	1.36E-02
1437	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	4.24	1.36E-02
64789	DEM1	Defects in morphology 1 homolog (S. cerevisiae)	3.51	1.36E-02
79958	DENND1C	DENN/MADD domain containing 1C	3.16	1.36E-02
7185	TRAF1	TNF receptor-associated factor 1	3.06	1.36E-02
90874	ZNF697	Hypothetical LOC100130667; zinc finger protein 697	-2.88	1.36E-02
257106	ARHGAP30	Rho GTPase activating protein 30	5.50	1.38E-02
3560	IL2RB	Interleukin 2 receptor, beta	4.48	1.42E-02
3120	HLA-DQB2	Major histocompatibility complex, class II, DQ beta 2	14.35	1.42E-02
3559	IL2RA	Interleukin 2 receptor, alpha	8.31	1.42E-02
4049	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	8.18	1.42E-02
4261	CIITA	Class II, major histocompatibility complex, transactivator	4.84	1.42E-02
3394	IRF8	Interferon regulatory factor 8	2.81	1.42E-02

56829	ZC3HAV1	Zinc finger CCCH-type, antiviral 1	2.81	1.42E-02
115004	C6orf150	Chromosome 6 open reading frame 150	5.39	1.44E-02
9938	ARHGAP25	Rho GTPase activating protein 25	3.75	1.44E-02
100133583	LOC100133583	Major histocompatibility complex, class II, DQ beta 1; similar to major histocompatibility complex, class II, DQ beta 1	4.80	1.47E-02
100130229	LOC100130229	Hypothetical protein LOC100130229 (LOC100130229), mRNA.	4.00	1.47E-02
51311	TLR8	Toll-like receptor 8	11.08	1.48E-02
80709	AKNA	AT-hook transcription factor	4.89	1.48E-02
143689	PIWIL4	Piwi-like 4 (Drosophila)	2.91	1.48E-02
643	CXCR5	Chemokine (C-X-C motif) receptor 5	7.98	1.51E-02
9267	PSCD1	Cytohesin 1	2.64	1.51E-02
645432	ARRDC5	Arrestin domain containing 5	4.15	1.58E-02
7454	WAS	Wiskott-Aldrich syndrome (eczema-thrombocytopenia)	3.95	1.58E-02
11064	CEP110	Centrosomal protein 110kDa	3.20	1.58E-02
5778	PTPN7	Protein tyrosine phosphatase, non-receptor type 7	3.45	1.62E-02
27163	NAAA	N-acyl ethanolamine acid amidase	3.24	1.65E-02
387763	LOC387763	Hypothetical LOC387763 (LOC387763), mRNA.	2.90	1.65E-02
29851	ICOS	Inducible T-cell co-stimulator	8.11	1.69E-02
55423	SIRPG	Signal-regulatory protein gamma	7.87	1.69E-02
51316	PLAC8	Placenta-specific 8	7.33	1.69E-02
64926	RASAL3	RAS protein activator-like 3	4.96	1.69E-02
5292	PIM1	Pim-1 oncogene	4.66	1.69E-02
7187	TRAF3	TNF receptor-associated factor 3	3.50	1.69E-02
266747	RGL4	Ral guanine nucleotide dissociation stimulator-like 4	3.32	1.69E-02
799	CALCR	Calcitonin receptor	-3.47	1.69E-02
283234	CCDC88B	Coiled-coil domain containing 88B	3.53	1.69E-02
50619	DEF6	Differentially expressed in FDCP 6 homolog (mouse)	3.75	1.70E-02
6361	CCL17	Chemokine (C-C motif) ligand 17	30.38	1.71E-02
64005	MYO1G	Myosin IG	4.25	1.71E-02
10225	CD96	CD96 molecule	11.44	1.73E-02
64170	CARD9	Caspase recruitment domain family, member 9	5.18	1.73E-02
129293	C2orf89	Hypothetical protein LOC129293	5.39	1.75E-02
387841	LOC387841	Similar to ribosomal protein L13a, transcript variant 2 (LOC387841), mRNA	2.78	1.75E-02
3904	LAIR2	Leukocyte-associated immunoglobulin-like receptor 2	7.37	1.79E-02
84230	LRRC8C	Leucine rich repeat containing 8 family, member C	6.06	1.79E-02
100131967	LOC100131967	Misc_RNA (LOC100131967), miscRNA	5.81	1.79E-02
387751	GVIN1	GTPase, very large interferon inducible 1	4.52	1.79E-02
79961	DENND2D	DENN/MADD domain containing 2D	3.31	1.79E-02
4615	MYD88	Myeloid differentiation primary response gene (88)	2.67	1.79E-02
54900	LAX1	Lymphocyte transmembrane adaptor 1	5.73	1.79E-02
910	CD1B	CD1b molecule	11.91	1.80E-02
50852	TRAT1	T cell receptor associated transmembrane adaptor 1	5.61	1.80E-02
10346	TRIM22	Tripartite motif-containing 22	3.78	1.80E-02

2534	FYN	FYN oncogene related to SRC, FGR, YES	3.93	1.86E-02
6648	SOD2	Superoxide dismutase 2, mitochondrial	4.43	1.89E-02
10219	KLRG1	Killer cell lectin-like receptor subfamily G, member 1	4.62	1.91E-02
3932	LCK	Lymphocyte-specific protein tyrosine kinase	4.68	1.91E-02
653361	NCF1	Neutrophil cytosolic factor 1; neutrophil cytosolic factor 1C pseudogene	5.81	1.94E-02
80342	TRAF3IP3	TRAF3 interacting protein 3	7.52	1.95E-02
3431	SP110	SP110 nuclear body protein	2.92	1.95E-02
388325	C17orf87	Chromosome 17 open reading frame 87	4.55	1.96E-02
114836	SLAMF6	SLAM family member 6	3.96	1.96E-02
5724	PTAFR	Platelet-activating factor receptor	3.90	1.96E-02
3695	ITGB7	Integrin, beta 7	2.93	1.96E-02
6775	STAT4	Signal transducer and activator of transcription 4	5.61	2.00E-02
973	CD79A	CD79a molecule, immunoglobulin-associated alpha	6.41	2.01E-02
9051	PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1	7.08	2.01E-02
11322	TMC6	Transmembrane channel-like 6	2.82	2.01E-02
25797	QPCT	Glutaminy-peptide cyclotransferase	2.80	2.01E-02
53347	UBASH3A	Similar to ubiquitin associated and SH3 domain containing, A; ubiquitin associated and SH3 domain containing, A	11.49	2.03E-02
5579	PRKCB1	Protein kinase C, beta 1	4.62	2.03E-02
55897	MESP1	Mesoderm posterior 1 homolog (mouse)	-3.09	2.03E-02
2827	GPR3	G protein-coupled receptor 3	-2.09	2.08E-02
11040	PIM2	Pim-2 oncogene	3.26	2.09E-02
1890	TYMP	Thymidine phosphorylase	7.48	2.10E-02
11184	MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1	8.88	2.10E-02
11182	SLC2A6	Solute carrier family 2 (facilitated glucose transporter), member 6	7.64	2.14E-02
389386	LOC389386	Similar to LAP3 protein	3.44	2.14E-02
93058	COQ10A	Coenzyme Q10 homolog A (S. cerevisiae)	2.68	2.17E-02
8869	ST3GAL5	ST3 beta-galactoside alpha-2,3-sialyltransferase 5	3.27	2.21E-02
56833	SLAMF8	SLAM family member 8	6.01	2.50E-02
8740	TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14	6.90	2.58E-02
26002	MOXD1	Monoxygenase, DBH-like 1	3.93	2.58E-02
51314	TXNDC3	Thioredoxin domain containing 3 (spermatozoa)	3.13	2.62E-02
64805	P2RY12	Purinergic receptor P2Y, G-protein coupled, 12	4.53	2.63E-02
60489	APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	5.57	2.70E-02
2533	FYB	FYN binding protein (FYB-120/130)	4.03	2.70E-02
6402	SELL	Selectin L	8.40	2.73E-02
5791	PTPRE	Protein tyrosine phosphatase, receptor type, E	3.76	2.75E-02
930	CD19	CD19 molecule	6.14	2.75E-02
374403	TBC1D10C	TBC1 domain family, member 10C	5.78	2.76E-02
1050	CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	2.44	2.76E-02
284612	SYPL2	Synaptophysin-like 2	-5.48	2.76E-02
9363	RAB33A	RAB33A, member RAS oncogene family	4.39	2.76E-02
330	BIRC3	Baculoviral IAP repeat-containing 3	8.79	2.77E-02

7940	LST1	Leukocyte-specific transcript 1	5.15	2.77E-02
11262	SP140	SP140 nuclear body protein	4.57	2.77E-02
3122	HLA-DRA	Major histocompatibility complex, class II, DR alpha	2.46	2.77E-02
79007	DBNDD1	Dysbindin (dystrobrevin binding protein 1) domain containing 1	-2.97	2.77E-02
5649	RELN	Reelin	-5.51	2.77E-02
197259	MLKL	Mixed lineage kinase domain-like	3.10	2.79E-02
3601	IL15RA	Interleukin 15 receptor, alpha	6.37	2.87E-02
963	CD53	CD53 molecule	5.00	2.87E-02
474344	GIMAP6	GTPase, IMAP family member 6	3.53	2.96E-02
5777	PTPN6	Protein tyrosine phosphatase, non-receptor type 6	3.24	2.98E-02
10673	TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13b	5.58	2.99E-02
3126	HLA-DRB4	Major histocompatibility complex, class II, DR beta 4; major histocompatibility complex, class II, DR beta 1	3.45	2.99E-02
3561	IL2RG	Interleukin 2 receptor, gamma (severe combined immunodeficiency)	3.06	3.05E-02
145864	HAPLN3	Hyaluronan and proteoglycan link protein 3	8.37	3.07E-02
6279	S100A8	S100 calcium binding protein A8	5.84	3.07E-02
55303	GIMAP4	GTPase, IMAP family member 4	2.85	3.07E-02
79037	PVRIG	Poliovirus receptor related immunoglobulin domain containing	9.01	3.14E-02
57559	STAMBPL1	STAM binding protein-like 1	3.51	3.14E-02
27299	ADAMDEC1	ADAM-like, decysin 1	16.88	3.24E-02
914	CD2	CD2 molecule	8.95	3.24E-02
951	CD37	CD37 molecule	5.97	3.24E-02
162979	ZNF296	Zinc finger protein 296	2.60	3.28E-02
10385	BTN2A2	Butyrophilin, subfamily 2, member A2	3.38	3.29E-02
5142	PDE4B	Phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	3.79	3.31E-02
339541	MGC33556	Chromosome 1 open reading frame 22	5.83	3.36E-02
63940	GPSM3	G-protein signaling modulator 3 (AGS3-like, C. elegans)	4.31	3.36E-02
5698	PSMB9	Proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	4.29	3.36E-02
4001	LMNB1	Lamin B1	5.61	3.37E-02
10261	IGSF6	Immunoglobulin superfamily, member 6	5.46	3.37E-02
5579	PRKCB	Protein kinase C, beta	5.12	3.37E-02
6892	TAPBP	TAP binding protein (tapasin)	2.94	3.37E-02
25932	CLIC4	Chloride intracellular channel 4	-2.05	3.37E-02
26027	ACOT11	Acyl-CoA thioesterase 11	-2.67	3.37E-02
2357	FPR1	Formyl peptide receptor 1	10.27	3.43E-02
10859	LILRB1	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1	4.74	3.43E-02
80149	ZC3H12A	Zinc finger CCCH-type containing 12A	4.30	3.43E-02
79626	TNFAIP8L2	Tumor necrosis factor, alpha-induced protein 8-like 2	7.04	3.45E-02
10148	EBI3	Epstein-Barr virus induced 3	8.95	3.52E-02
3055	HCK	Hemopoietic cell kinase	4.79	3.52E-02
9262	STK17B	Serine/threonine kinase 17b	4.76	3.52E-02
3115	HLA-DPB1	Major histocompatibility complex, class II, DP beta 1	4.30	3.52E-02
1235	CCR6	Cyclin L2; chemokine (C-C motif) receptor 6	4.23	3.52E-02

55340	GIMAP5	GTPase, IMAP family member 5	4.10	3.52E-02
6891	TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	3.94	3.52E-02
3134	HLA-F	Major histocompatibility complex, class I, F	3.80	3.52E-02
100129104	LOC100129104	Similar to hydroxyproline-rich glycoprotein VSP-3	3.40	3.52E-02
201294	UNC13D	unc-13 homolog D (C. elegans)	3.38	3.52E-02
55843	ARHGAP15	Rho GTPase activating protein 15	2.92	3.52E-02
10287	RGS19	Regulator of G-protein signaling 19	2.76	3.52E-02
63941	NECAB3	N-terminal EF-hand calcium binding protein 3	-3.23	3.52E-02
864	RUNX3	Runt-related transcription factor 3	3.26	3.55E-02
340061	TMEM173	Transmembrane protein 173	2.74	3.61E-02
5723	PSPH	Phosphoserine phosphatase-like; phosphoserine phosphatase	-2.61	3.63E-02
160364	CLEC12A	C-type lectin domain family 12, member A	7.21	3.63E-02
924	CD7	CD7 molecule	6.17	3.63E-02
1606	DGKA	Diacylglycerol kinase, alpha 80kDa	5.56	3.63E-02
2833	CXCR3	Chemokine (C-X-C motif) receptor 3	4.54	3.63E-02
4790	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	2.07	3.63E-02
92691	TMEM169	Transmembrane protein 169	-3.04	3.63E-02
154075	SAMD3	Sterile alpha motif domain containing 3	8.09	3.65E-02
150223	LOC150223	Hypothetical protein LOC150223 (LOC150223), transcript variant 2, mRNA.	3.29	3.65E-02
923	CD6	CD6 molecule	8.17	3.65E-02
4050	LTB	Lymphotoxin beta (TNF superfamily, member 3)	12.35	3.68E-02
3111	HLA-DOA	Major histocompatibility complex, class II, DO alpha	2.89	3.73E-02
648470	LOC648470	Similar to Caspase-4 precursor (CASP-4) (ICH-2 protease) (TX protease) (ICE(rel)-II) (LOC648470), mRNA.	3.22	3.74E-02
26270	FBXO6	F-box protein 6	2.74	3.79E-02
155038	GIMAP8	GTPase, IMAP family member 8	2.98	3.79E-02
199	AIF1	Allograft inflammatory factor 1	2.92	3.79E-02
50615	IL21R	Interleukin 21 receptor	8.17	3.81E-02
57514	CDGAP	Rho GTPase Activating Protein 31	3.79	3.82E-02
9770	RASSF2	Ras association (RalGDS/AF-6) domain family member 2	3.72	3.83E-02
8556	CDC14A	CDC14 cell division cycle 14 homolog A (S. cerevisiae)	3.84	3.89E-02
440836	ODF3B	Outer dense fiber of sperm tails 3B	2.72	3.89E-02
202309	GAPT	GRB2-binding adaptor protein, transmembrane	4.12	3.89E-02
51735	RAPGEF6	Rap guanine nucleotide exchange factor (GEF) 6	2.43	3.89E-02
8718	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	4.10	3.90E-02
11118	BTN3A2	Butyrophilin, subfamily 3, member A2	3.47	3.92E-02
643866	CBLN3	Cerebellin 3 precursor	3.22	3.92E-02
10563	CXCL13	Chemokine (C-X-C motif) ligand 13	16.81	4.03E-02
3702	ITK	IL2-inducible T-cell kinase	8.26	4.03E-02
51738	GHRL	Ghrelin/obestatin prepropeptide	6.54	4.03E-02
920	CD4	CD4 molecule	5.28	4.03E-02
6280	S100A9	S100 calcium-binding protein A9	4.67	4.03E-02
55016	MARCH1	Membrane-associated ring finger (C3HC4) 1	4.61	4.03E-02

653820	LOC653820	Similar to family with sequence similarity 72, member A, transcript variant 1 (LOC653820), mRNA.	3.04	4.03E-02
976	CD97	CD97 molecule	2.96	4.03E-02
391	RHOG	ras homolog gene family, member G (rho G)	2.55	4.03E-02
80063	ATF7IP2	Activating transcription factor 7 interacting protein 2	2.02	4.03E-02
23413	FREQ	Neuronal Calcium Sensor 1	-3.20	4.03E-02
958	CD40	CD40 molecule, TNF receptor superfamily member 5	4.97	4.07E-02
8807	IL18RAP	Interleukin 18 receptor accessory protein	4.92	4.07E-02
6920	TCEA3	Transcription elongation factor A (SII), 3	-2.36	4.07E-02
134	ADORA1	Adenosine A1 receptor	-2.35	4.08E-02
2593	GAMT	Guanidinoacetate N-methyltransferase	-2.89	4.09E-02
54149	C21orf91	Chromosome 21 open reading frame 91	2.14	4.11E-02
29125	C11orf21	Chromosome 11 open reading frame 21	3.39	4.12E-02
1043	CD52	CD52 molecule	4.36	4.18E-02
730415	LOC730415	Hypothetical LOC730415, transcript variant 2 (LOC730415), mRNA.	3.08	4.18E-02
116154	PHACTR3	Phosphatase and actin regulator 3	-4.50	4.18E-02
2323	FLT3LG	fms-related tyrosine kinase 3 ligand	4.20	4.19E-02
9535	GMFG	Glia maturation factor, gamma	2.71	4.19E-02
5699	PSMB10	Proteasome (prosome, macropain) subunit, beta type, 10	2.33	4.30E-02
170954	KIAA1949	Phostensin	3.70	4.34E-02
962	CD48	CD48 molecule	4.79	4.44E-02
5771	PTPN2	Protein tyrosine phosphatase, non-receptor type 2	3.31	4.48E-02
9404	LPXN	Leupaxin	2.77	4.48E-02
5168	ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	2.35	4.56E-02
80714	PBX4	Pre-B-cell leukemia homeobox 4	4.38	4.56E-02
4791	NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	2.88	4.61E-02
7052	TGM2	Transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	2.84	4.67E-02
259307	IL4I1	Interleukin 4 induced 1	7.97	4.69E-02
64333	ARHGAP9	Rho GTPase activating protein 9	6.68	4.69E-02
8651	SOCS1	Suppressor of cytokine signaling 1	3.16	4.69E-02
1493	CTLA4	Cytotoxic T-lymphocyte-associated protein 4	10.18	4.71E-02
2634	GBP2	Guanylate binding protein 2, interferon-inducible	2.82	4.71E-02
10068	IL18BP	Interleukin 18 binding protein	3.94	4.72E-02
9744	ACAP1	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1	7.54	4.75E-02
23616	SH3BP1	SH3-domain binding protein 1	5.60	4.75E-02
9934	P2RY14	Purinergic receptor P2Y, G-protein coupled, 14	4.86	4.75E-02
114614	MIR155HG	microRNA host gene 2 (non-protein coding); microRNA 155	4.46	4.75E-02
10870	HCST	Hematopoietic cell signal transducer	3.68	4.75E-02
57121	LPAR5	Lysophosphatidic acid receptor 5	2.16	4.75E-02
23089	PEG10	Paternally expressed 10	-4.70	4.75E-02
118932	ANKRD22	Ankyrin repeat domain 22	13.23	4.78E-02
1326	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	3.01	4.78E-02
9246	UBE2L6	Ubiquitin-conjugating enzyme E2L 6	2.91	4.81E-02

8322	FZD4	Frizzled homolog 4 (Drosophila)	-2.03	4.82E-02
10077	TSPAN32	Tetraspanin 32	5.55	4.82E-02
3045	HBD	Hemoglobin, delta	3.32	4.82E-02
201799	TMEM154	Transmembrane protein 154	2.58	4.82E-02
26191	PTPN22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	3.54	4.84E-02
54435	HCG4	HLA complex group 4	2.56	4.89E-02
10166	SLC25A15	Solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15	-3.40	4.89E-02
387357	C6orf190	Thymocyte Selection Associated	6.91	4.92E-02
197358	NLRC3	NLR family, CARD domain containing 3	3.66	4.92E-02
653907	LOC653907	Similar to complement receptor related protein isoform 1 (LOC653907), mRNA.	3.60	4.92E-02
10695	CNPY3	Canopy 3 homolog (zebrafish)	2.22	4.92E-02
79822	ARHGAP28	Rho GTPase activating protein 28	-6.72	4.93E-02
1236	CCR7	Chemokine (C-C motif) receptor 7	15.61	4.95E-02
64919	BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein)	9.56	4.95E-02
4689	NCF4	Neutrophil cytosolic factor 4, 40kDa	6.47	4.95E-02
728650	LOC728650	Hypothetical LOC728650	5.96	4.95E-02
916	CD3E	CD3e molecule, epsilon (CD3-TCR complex)	5.73	4.95E-02
3113	HLA-DPA1	Major histocompatibility complex, class II, DP alpha 1	2.93	4.95E-02
29970	SCHIP1	Schwannomin interacting protein 1	-2.35	4.95E-02

Supplement table 2. List of 310 deregulated genes in early T cell-mediated vascular rejection vs. early isolated v-lesion. BH; Benjamini-Hochberg

Genes down-regulated in eIV vs. normal				
Entrez Gene ID	Gene symbol	Gene name	FC	Adjusted p-value (BH)
4926	NUMA1	nuclear mitotic apparatus protein 1	2.17	2.21E-02
2687	GGT5	gamma-glutamyltransferase 5	2.84	2.69E-02
9481	SLC25A27	solute carrier family 25 member 27	3.93	2.69E-02
2669	GEM	GTP binding protein overexpressed in skeletal muscle	3.73	3.01E-02
1195	CLK1	CDC like kinase 1	2.01	3.01E-02
151126	ZNF385B	zinc finger protein 385B	2.93	3.01E-02
3486	IGFBP3	insulin-like growth factor-binding protein 3	3.01	3.01E-02
651845	LOC651845	similar to HLA class II histocompatibility antigen	3.07	3.82E-02
6844	VAMP2	vesicle associated membrane protein 2	2.86	3.82E-02
55904	MLL5	Myeloid/Lymphoid or Mixed-Lineage Leukemia 5	2.92	3.82E-02
7569	ZNF182	zinc finger protein 182	2.18	4.06E-02
7637	ZNF84	zinc finger protein 84	2.10	4.06E-02
28968	SLC6A16	solute carrier family 6 member 16	3.08	4.06E-02
8470	SORBS2	sorbin and SH3 domain containing 2	2.14	4.52E-02
10826	C5orf4	fatty acid hydroxylase domain containing 2	3.39	4.57E-02
5930	RBBP6	RB binding protein 6, ubiquitin ligase	2.07	4.63E-02
4638	MYLK	myosin light chain kinase	2.49	4.63E-02
9630	GNA14	G protein subunit alpha 14	2.56	4.63E-02
3486	IGFBP3	insulin like growth factor binding protein 3	2.70	4.71E-02
283337	ZNF740	zinc finger protein 740	2.46	4.71E-02
126669	SHE	Src homology 2 domain containing E	2.49	4.90E-02
757	TMEM50B	transmembrane protein 50B	3.24	4.90E-02
Genes up-regulated in eIV vs. normal				
Entrez Gene ID	Gene symbol	Gene name	FC	Adjusted p-value (BH)
26834	RNU4-2	RNA, U4 small nuclear 2	7.62	5.9E-3
6084	RNY1	RNA, Ro-Associated Y1	4.32	6.1E-3
692213	SNORD110	small nucleolar RNA, C/D Box 110	5.25	9.4E-3
26869	RNU1-3	RNA, U1 small nuclear 3	4.23	12.7E-3
677833	SNORA54	small nucleolar RNA, H/ACA Box 54	7.35	21.6E-3
26864	RNU1G2	RNA, U1 small nuclear 4	4.10	21.6E-3
100169760	RN5S9	RNA, 5S Ribosomal 9	6.17	21.6E-3
652965	SNORA48	small nucleolar RNA, H/ACA box 48	4.80	21.6E-3
26818	SNORD33	small nucleolar RNA, C/D Box 33	7.26	22.1E-3
26863	RNU1-5	RNA, U1 small nuclear 5	3.81	30.1E-3
64793	CCDC21	coiled-coil domain containing 21	2.79	30.1E-3
51077	FCF1	FCF1 small subunit (SSU) processome component homolog	2.61	30.1E-3
1984	EIF5A	eukaryotic translation initiation factor 5A; eukaryotic translation initiation factor 5A-like 1	2.42	30.1E-3

26866	RNU1F1	RNA, U1 small nuclear 4	5.07	30.1E-3
135154	C6orf57	chromosome 6 open reading frame 57	2.45	30.1E-3
642393	LOC642393	similar to mitochondrial ribosomal protein L20; mitochondrial ribosomal protein L20	5.39	30.1E-3
677815	SNORA34	small nucleolar RNA, H/ACA Box 2C	4.02	38.2E-3
100151683	RNU4ATAC	small nuclear RNA U4atac (U12-Dependent Splicing)	3.48	40.2E-3
85019	C18orf45	chromosome 18 open reading frame 45	3.49	40.6E-3
9219	MTA2	metastasis associated 1 family, member 2	2.04	40.6E-3
6159	RPL29	ribosomal protein L29 pseudogene 9	6.28	40.6E-3
124222	PAQR4	progesterin and adipoQ receptor family member IV	2.90	40.6E-3
652826	LOC652826	similar to 26S protease regulatory subunit 6B (MIP224)	3.41	40.6E-3
83544	DNAL1	dynein, axonemal, light chain 1	2.20	40.6E-3
677800	SNORA12	small nucleolar RNA, H/ACA Box 12	5.04	45.7E-3
92691	TMEM169	transmembrane protein 169	2.17	46.3E-3
79751	SLC25A22	solute carrier family 25 (mitochondrial carrier: glutamate), member 22	2.27	46.3E-3
9275	BCL7B	B-cell CLL/lymphoma 7B	2.29	46.3E-3

Supplement table 3. List of 22 down-regulated and 28 up-regulated genes in eIV vs. normal. BH; Benjamini-Hochberg

	Term	Count	Adjusted p-value (BH)
GO:000695	Immune response	197	2.61E-36
GO:0006952	Defense response	155	1.45E-21
GO:0006954	Inflammatory response	97	5.37E-18
GO:0009611	Response to wounding	131	2.88E-17
GO:0002684	Positive regulation of immune system process	77	3.20E-16
GO:0050778	Positive regulation of immune response	51	1.36E-11
GO:0001775	Cell activation	77	2.91E-11
GO:0048584	Positive regulation of response to stimulus	66	2.55E-10
GO:0045321	Leukocyte activation	67	2.47E-10
GO:0051249	Regulation of lymphocyte activation	49	3.73E-10
GO:0050865	Regulation of cell activation	53	1.76E-09
GO:0002694	Regulation of leukocyte activation	51	2.38E-09
GO:0042110	T cell activation	43	2.78E-09
GO:0006935	Chemotaxis	49	6.31E-09
GO:0042330	Taxis	49	6.31E-09
GO:0046649	Lymphocyte activation	56	7.41E-09
GO:0002443	Leukocyte mediated immunity	33	2.68E-08
GO:0002460	Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	31	2.55E-08
GO:0002250	Adaptive immune response	31	2.55E-08
GO:0050863	Regulation of T cell activation	39	4.68E-08
GO:0002252	Immune effector process	42	6.89E-08
GO:0002253	Activation of immune response	34	6.58E-08
GO:0002764	Immune response-regulating signal transduction	25	1.52E-07
GO:0007067	Mitosis	56	3.06E-07
GO:0000280	Nuclear division	56	3.06E-07
GO:0051301	Cell division	68	3.93E-07
GO:0001817	Regulation of cytokine production	49	3.86E-07
GO:0000087	M phase of mitotic cell cycle	56	5.44E-07
GO:0000278	Mitotic cell cycle	79	6.39E-07
GO:0002757	Immune response-activating signal transduction	23	8.09E-07
GO:0002449	Lymphocyte mediated immunity	27	9.03E-07
GO:0048285	Organelle fission	56	1.09E-06
GO:0050867	Positive regulation of cell activation	35	1.37E-06
GO:0051251	Positive regulation of lymphocyte activation	32	1.93E-06
GO:0046651	Lymphocyte proliferation	20	1.99E-06
GO:0007049	Cell cycle	135	1.98E-06
GO:0002819	Regulation of adaptive immune response	23	3.32E-06
GO:0001819	Positive regulation of cytokine production	30	4.06E-06
GO:0032943	Mononuclear cell proliferation	20	4.51E-06
GO:0070661	Leukocyte proliferation	20	4.51E-06
GO:0002696	Positive regulation of leukocyte activation	33	4.42E-06
GO:0050870	Positive regulation of T cell activation	27	4.81E-06
GO:0022402	Cell cycle process	104	5.54E-06
GO:0046635	Positive regulation of alpha-beta T cell activation	15	8.08E-06
GO:0009615	Response to virus	33	8.28E-06

GO:0002822	Regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	22	9.95E-06
GO:0002768	Immune response-regulating cell surface receptor signaling pathway	19	9.93E-06
GO:0045087	Innate immune response	38	1.06E-05
GO:0042098	T cell proliferation	15	1.34E-05
GO:0006968	Cellular defense response	23	1.48E-05

Supplement Table 4. Top 50 GO terms for the biological process enriched in TCMRV versus control group

The genes with different expression in TCMRV and control group after multiple comparisons (n=1966) were entered into the David gene ontology database. Top 50 out of 239 GO terms for the biological process are shown. BH; Benjamini-Hochberg

Entrez Gene ID	Gene symbol	Gene name
9275	BCL7B	B-cell CLL/lymphoma 7B
10826	C5orf4	chromosome 5 open reading frame 4
135154	C6orf57	chromosome 6 open reading frame 57
64793	CCDC21	coiled-coil domain containing 21
1984	EIF5A	eukaryotic translation initiation factor 5A
51077	FCF1	FCF1 small subunit (SSU) processome component homolog
642393	LOC642393	similar to mitochondrial ribosomal protein L20
652826	LOC652826	similar to 26S protease regulatory subunit 6B (MIP224) (MB67-interacting protein) (TAT-binding protein 7) (TBP-7)
9219	MTA2	metastasis associated 1 family, member 2
4638	MYLK	myosin light chain kinase
124222	PAQR4	progesterone and adipoQ receptor family member IV
100169760	RN5S9	RNA, 5S Ribosomal 9
26869	RNU1-3	RNA, U1 Small Nuclear 3
26863	RNU1-5	RNA, U1 Small Nuclear 5
26866	RNU1F1	RNA, U1 Small Nuclear 4
26864	RNU1G2	RNA, U1G2 Small Nuclear
26834	RNU4-2	RNA, U4 Small Nuclear 2
6084	RNY1	RNA, Ro-Associated Y1
6159	RPL29	ribosomal protein L29 pseudogene 9
79751	SLC25A22	solute carrier family 25 (mitochondrial carrier: glutamate), member 22
9481	SLC25A27	solute carrier family 25, member 27
28968	SLC6A16	solute carrier family 6, member 16
677800	SNORA12	small nucleolar RNA, H/ACA Box 12
677815	SNORA34	small nucleolar RNA, H/ACA Box 34
652965	SNORA48	small nucleolar RNA, H/ACA box 48
677833	SNORA54	small nucleolar RNA, H/ACA Box 54
692213	SNORD110	small nucleolar RNA, C/D Box 110
26818	SNORD33	small nucleolar RNA, C/D Box 33
757	TMEM50B	transmembrane protein 50B
6844	VAMP2	vesicle-associated membrane protein 2 (synaptobrevin 2)
151126	ZNF385B	zinc finger protein 385B
283337	ZNF740	zinc finger protein 740
7637	ZNF84	zinc finger protein 84

Supplement table 5. The list of 33 genes shared between comparisons of TCMRV vs. normal and eIV vs. normal.