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American Journal of Transplantation 2016; 16: 484–496
Wiley Periodicals Inc.

doi: 10.1111/ajt.13488

Molecular Characterization of Acute Cellular Rejection Occurring During Intentional Immunosuppression Withdrawal in Liver Transplantation

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Acute cellular rejection occurs frequently during the first few weeks following liver transplantation. During this period, its molecular phenotype is confounded by peri- and postoperative proinflammatory events. To unambiguously define the molecular profile associated with rejection, we collected sequential biological specimens from 55 patients at least 3 years after liver transplantation who developed rejection during trials of intentional immunosuppression withdrawal. We analyzed liver tissue and blood samples obtained before initiation of drug withdrawal and at rejection, alongside blood samples collected during the weaning process. Gene expression profiling was conducted using whole-genome microarrays and real-time polymerase chain reaction. Rejection resulted in distinct blood and liver tissue transcriptional changes in patients who were either positive or negative for hepatitis C virus (HCV). Gene expression changes were mostly independent from pharmacological immunosuppression, and their magnitude correlated with severity of histological damage. Differential expression of a subset of genes overlapped across all conditions. These were used to define a blood predictive model that accurately identified rejection in HCV-negative, but not HCV-positive, patients. Changes were detectable 1–2 mo before rejection was diagnosed. Our results provide insight into the molecular processes underlying acute cellular rejection in liver transplantation and help clarify the potential utility and limitations of transcriptional biomarkers in this setting.

Abbreviations: ABMR, antibody-mediated rejection; ACR, acute cellular rejection; AKI, acute kidney injury; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; CNI, calcineurin inhibitor; CSA, cyclosporin A; DSA, donor-specific antibody; FC, fold change; FDR, false discovery rate; GGT, γ-glutamyltransferase; GSEA, gene set enrichment analysis; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN-γ, interferon-γ; IS, immunosuppression; MSigDB, Molecular Signatures Database; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin; NA, not available; NES, normalized enrichment score; PCR, polymerase chain reaction; TAC, tacrolimus; TCMR, T cell–mediated rejection; Tx, transplant

Received 26 June 2015, revised 26 July 2015 and accepted for publication 02 August 2015

Introduction

Over the past three decades, liver transplantation has become an accepted and well-standardized treatment for end-stage liver disease. Together with improvements in surgical techniques, intensive care and infection control, the development of efficient immunosuppressant drugs has been instrumental in increasing patient and graft survival rates (1). Acute cellular rejection (ACR) remains a frequent event, particularly early after liver transplantation, with recent series reporting incidence of ≈30% (2–4). Provided diagnosis is made early, ACR can be easily controlled with additional immunosuppressive medication and does not have negative prognostic implications. If unchecked, it can induce irreversible graft damage and thus remains a key consideration in the differential diagnosis of liver allograft dysfunction. This results in the need to closely monitor transplant recipients and to conduct confirmatory liver biopsies, which are cumbersome and incur substantial costs.

In kidney transplantation, molecular profiling techniques have been widely used to clarify the pathophysiology of

ACR and to develop novel diagnostic tools. In contrast, very few studies have attempted to comprehensively characterize ACR in liver transplantation; therefore, the molecular features of clinical liver allograft rejection remain only partially understood.

Molecular profiling studies in liver transplantation are complicated by the fact that ACR overwhelmingly occurs during the first 2–3 weeks after transplantation, during which time (1) allograft function is often abnormal as a consequence of inflammatory liver damage induced by reperfusion injury and surgical complications, and (2) a substantial proportion of liver recipients develop spontaneously resolving subclinical rejection episodes that are not detected unless protocol liver biopsies are conducted. Unambiguously defining rejection-associated molecular changes remains challenging in this scenario. Trials of intentional immunosuppression withdrawal in selected stable liver recipients provide a unique opportunity to thoroughly characterize the immunobiology of rejection because the medical intervention and the initiation of the rejection response can be timed, potential confounders can be adequately controlled, and sequential biological specimens including liver biopsies are typically obtained. In the current study, we analyzed sequential blood and liver tissue specimens collected from adult liver transplant recipients enrolled in two multicenter clinical trials of immunosuppression withdrawal. We were able to identify rejection-specific gene expression markers in these cohorts. Furthermore, we showed that gene expression changes preceded the clinical diagnosis of ACR and could be used as a predictive tool in this clinical scenario.

Patients and Methods

Patient population and study design

A total of 136 stable liver transplant recipients at least 3 years after liver transplantation were enrolled in two prospective multicenter clinical trials of immunosuppression withdrawal. The first trial (ClinicalTrials.gov NCT00647283) (5) enrolled 102 patients, 12 of whom had hepatitis C virus (HCV) infection with detectable HCV RNA. The second trial (ClinicalTrials.gov NCT00668369) enrolled 34 patients, all whom were HCV RNA positive (6). Identical drug withdrawal protocols were used in the two studies and have been described previously (5,6) (Figure 1). Briefly, immunosuppressive drug doses were gradually tapered by reducing approximately one-quarter to half of the doses every 3 weeks until complete discontinuation over a 6- to 9-mo period. Patients were then followed-up for at least 12 additional months. Protocol liver biopsy samples were obtained in all patients at baseline, at 12 mo after successful drug withdrawal in patients who did not reject, or at time of rejection. Blood samples were taken at enrollment, every 3 weeks during the withdrawal period, at the rejection episode, and monthly during the 12 mo after drug cessation or after resolution of the rejection episode. Patients who did not develop rejection were classified as operationally tolerant as long as immunosuppressive drug cessation was maintained for at least 12 mo and no histological evidence of acute and/or chronic rejection was observed. Rejection was diagnosed by the combination of allograft dysfunction and characteristic liver biopsy findings according to Banff criteria (7).

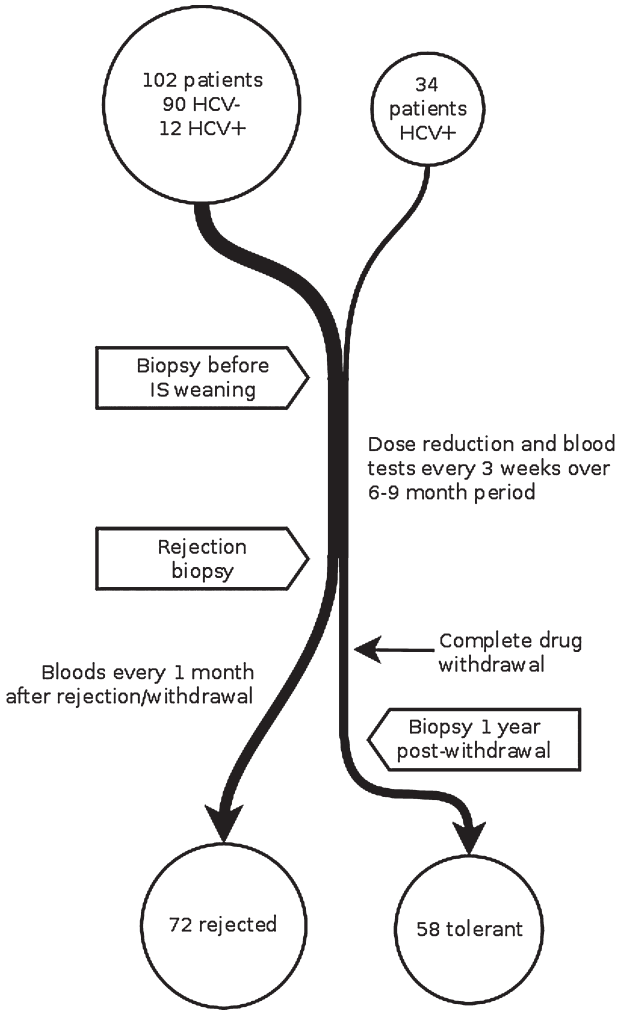

Figure 1: Flow chart showing immunosuppression withdrawal trial design. Data were included from two trials with identical withdrawal protocols (ClinicalTrials.gov NCT00647283 and NCT00668369). A subset of these patients was included in the current study. HCV, hepatitis C virus.

In the first trial, drug withdrawal was successfully achieved in 41 patients, whereas 57 rejected. In the second trial, withdrawal was successful in 17 patients and rejection occurred in 15. Rejection episodes were predominantly mild to moderate and resolved promptly following reinstitution of immunosuppression. No grafts were lost due to rejection. For the current study, we analyzed sequential samples collected from 55 rejecting recipients, 9 of whom were HCV RNA positive (Table 1). In addition, we collected blood samples from a cohort of 86 stable immunosuppressed liver recipients who were matched for age, sex and time from transplantation, with no biochemical evidence of rejection and in whom no attempts at drug withdrawal were performed. Nine early posttransplant rejection biopsies, collected from HCV-negative patients within the first 4 weeks following transplantation and outside the drug-withdrawal setting, were included as a control cohort. Finally, we reanalyzed previously generated gene expression data derived from baseline blood samples from a subset of 25 tolerant recipients.

Table 1: Demographic and clinical characteristics of enrolled patients

Characteristic	Total cohort (n = 55)
Age at transplant (years)	48.23 ± 11.73
Age at weaning start (years)	55.53 ± 11.83
Sex (% male)	54.5
White (%)	100
Transplant indication	
HCV cirrhosis	21
HCV RNA positive at enrolment	(9)
HBV cirrhosis	9
Alcoholic cirrhosis	9
Amyloidotic polyneuropathy	5
Fulminant hepatitis	3
Other causes	8
Time from transplant to weaning start (years)	6.83 ± 3.55
Time from weaning start to rejection (months)	7.82 ± 4.49
Immunosuppressive therapy at weaning start	
Tacrolimus	25
Cyclosporin A	22
Mycophenolate	11
Rapamycine	2
Cyclosporin A + mycophenolate	5
CNI-based immunosuppression at weaning start (%)	85
Immunosuppressive drug trough levels at weaning start (ng/ml)	
Tacrolimus	5.2 ± 2.3
Cyclosporin A	57.4 ± 36
Liver function tests at weaning start	
AST (U/l)	33 ± 22
ALT (U/l)	38 ± 35
GGT (U/l)	40 ± 70
Alkaline phosphatase (U/l)	172 ± 64
Total bilirubin (mg/dl)	0.75 ± 0.3
Rejection severity	
Indeterminate	19
Mild	23
Moderate	7
Severe	6
Banff score*	4 (2–9)
Liver function tests at rejection time	
AST (U/l)	150 ± 137
ALT (U/l)	207 ± 196
GGT (U/l)	188 ± 208
Alkaline phosphatase (U/l)	287 ± 173
Total bilirubin (mg/dl)	1.55 ± 4.51
Rejection treatment	
Baseline IS	23
Baseline IS + prednisone 20 mg/day (4–6 weeks)	26
Baseline IS + prednisone 40–60 mg/day (4–6 weeks)	4
Corticosteroids boluses	2

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CNI, calcineurin inhibitor; GGT, gglutamyltransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; IS, immunosuppression.
*Banff score is expressed as median (range).
Data are expressed as mean ± standard deviation.

Biological specimens

Liver biopsies were taken before the initiation of the immunosuppressive drug withdrawal protocol (baseline) and at the time of rejection. Histological analysis was performed including quantitative measurements obtained for lobular inflammation, central vein perivenulitis, central vein endothelitis, portal inflammation, interface hepatitis, bile duct lesions, ductular reaction, ductopenia, portal vein endothelitis, perisinusoidal fibrosis and portal fibrosis. Blood samples were collected at enrollment, at each drug dose modification (every 3 weeks), at the time rejection was diagnosed, and monthly during the year after rejection or complete drug discontinuation. For the current study, sequential blood and liver tissue specimens were made available from 77 and 33 patients, respectively. RNA was extracted from tissue and blood samples, as described in the Supplementary Methods.

Liver tissue and blood microarray gene expression experiments

Transcriptional profiling of liver tissue samples was conducted using Illumina microarrays. Forty liver tissue RNA samples (preweaning and rejection time points) from 20 patients (13 HCV-negative and 7 HCV-positive patients) were profiled, as described in the Supplementary Methods.

RNA extracted from blood samples was analyzed on a custom Agilent complementary DNA microarray containing probes for 5069 preselected immunology-related transcripts (8). Seventy-four blood samples (37 preweaning blood samples and 37 rejecting blood samples) from 37 nontolerant HCV-negative patients were analyzed. Data acquisition and normalization methods are described in the Supplemental Methods. Analysis for differential expression on a probe basis was done by *limma*, including correction for multiple testing using the false discovery rate (FDR) method.

We defined statistically significant differences in gene expression as those showing fold change (FC) >1.2 and FDR <0.05. To compare expression data across different microarray platforms (i.e. blood vs liver tissue), a lower threshold of significance was used (FC >1.2 and p-value <0.01).

Gene set enrichment analysis

To assess the functional pathways associated with ACR, we used a gene set enrichment analysis (GSEA) application (9,10). This tool compares differentially expressed genes with previously identified gene sets. Three different gene set databases were used for our analyses: (1) the Hallmark gene sets from the Molecular Signatures Database (MSigDB) at the Massachusetts Institute of Technology Broad Institute (10), with 50 gene sets mapped to known biological processes; (2) the HaemAtlas gene set database (11), which contains gene sets preferentially expressed by specific peripheral blood cell subsets (CD4, CD8, CD19, CD14, CD56, CD 66B, EB, MK); and (3) pathogenesis-based transcript gene sets, which have been associated with different subtypes of rejection in both experimental animal models and human kidney and heart allografts (http://atagc.med.ualberta.ca/Research/GeneLists/Pages/default.aspx) (12,13). The full list of gene sets used is shown in Table 3 and Table S2. All analyses were performed using the GSEAPreranked tool, based on t-statistic and a weighted scoring scheme with 1000 permutations.

Correlation between gene expression and liver histopathology

To investigate which histological compartment contributed to the rejection-associated transcriptional changes, we correlated histopathology data with complete liver tissue microarray data. We used a set of 80 biopsy samples from which full quantitative histological data could be uniquely mapped to tissue microarray data. This included 21 samples taken at the point of rejection, 57 biopsies obtained prior to weaning, and two follow-up samples from tolerant patients 1 year after cessation of immunosuppression. Quantitative measurements were available for each of 11 available histological parameters. Each parameter was graded from 0 to 3 for each sample. For each parameter, differentially expressed genes were identified using the limma bioconductor package. A linear regression model was constructed to correlate gene expression with severity of that parameter.

Influence of type of immunosuppression on gene expression patterns

We assessed the influence of immunosuppression (tacrolimus, TAC; cyclosporine A, CSA; mycophenolate mofetil, MMF) on baseline gene expression profiles by constructing linear regression models that explored the impact of each type of immunosuppression on the blood (n = 72) and liver tissue (n = 33) microarray data, using the limma bioconductor package.

Validation real-time polymerase chain reaction experiments in peripheral blood samples

Quantitative real-time polymerase chain reaction (PCR) gene expression experiments were performed using a Fluidigm Biomark HD system, fully described in the Supplementary Methods. The gene expression profiles of 45 target genes plus two housekeeping genes (Table S1) were quantified in 21 paired blood samples (collected at baseline and at the time of rejection) from 14 HCV-negative and 8 HCV-positive patients. An additional group of blood samples collected from 86 stable immunosuppressed liver recipients with no biochemical evidence of rejection was used for comparative purposes. To define a gene expression signature predictive of rejection, we performed a multivariate logistic regression analysis incorporating all Fluidigm gene expression data in the set of paired samples from the group of HCV-negative patients. The best gene model was used to compute the risk probability of rejection in the blood samples collected from the same patients but at different intervals and in all available blood samples from the remaining patients.

In addition to the Fluidigm analyses, we conducted additional real-time PCR experiments as part of the biomarker portfolio studies dictated by the Reprogramming the Immune System for the Establishment of Tolerance (RISET) European consortium. These experiments used an Applied Biosystem real-time PCR platform and quantified the expression of *CD3*, *FOXP3*, *MAN1A1*, *PRF1*, and *TOAG-1* in blood samples collected before the initiation of weaning.

Identification of predictive signatures of rejection

We used multivariate logistic regression to identify gene signatures predictive of rejection among the 45 genes analyzed by Fluidigm real-time PCR. A training set of 14 nontolerant patients from whom Fluidigm data were available both at baseline and at the point of rejection (total samples n = 28) was initially assessed. Given the small number of samples available, the analysis was restricted to signatures containing up to two genes. The regression algorithm used is defined in the varSelec method of the MMIX bioconductor package.

Following variable selection, a generalized linear model was generated using the glm method of the R statistical package (R Foundation for Statistical Computing), using the 28 paired samples to classify a given sample into a rejection or nonrejection (baseline) group. Internal cross-validation was performed using the boot R package. This model was then applied to additional samples to generate a probability of being rejection sample. When applied to samples from patients not exhibiting clinically apparent rejection, we defined the model as giving a ''correct'' result if the probability of rejection was <50% and an ''incorrect'' result if predicted rejection with >50% probability.

Results

Acute cellular rejection is associated with a common transcriptional signature regardless of the presence of underlying liver inflammation

To identify the transcriptional patterns most highly associated with ACR, we conducted a paired microarray analysis comparing baseline (preweaning) liver tissue samples with those obtained at the time of rejection in patients with or without HCV infection (7 and 13, respectively). In HCV-negative recipients, rejection was associated with statistically significant changes in 34 transcripts. The impact of rejection was more substantial in HCV-positive recipients, in whom it significantly modified the expression of 408 genes. HCV-positive and -negative recipients shared a common set of 19 genes (Figure 2, Table 2).

We explored functional pathways more highly associated with ACR using GSEA. We compared our data with Hallmark gene sets from MSigDB to identify high-level functional pathways. Differentially expressed genes were involved in a variety of pathways related to, among others, inflammatory response, interferon signaling, IL-6/JAK/STAT3 signaling and apoptosis (Table S2). We next explored the overrepresentation of pathogenesis-based transcript gene sets, which were previously validated in kidney and heart human allografts (12,14–21). Liver samples with ACR showed significant overlap with gene sets associated with T cell–mediated rejection in kidney and heart transplantation, with infiltrating macrophages and effector T lymphocytes being the principal cell subsets involved. ACR was also associated with enrichment in transcripts related to B cells and donor-specific antibodies (DSAs), although the signal was weaker than for T cell–associated transcripts (Table 3).

To confirm the specificity of our results for ACR, we compared our 13 HCV-negative patients with 9 early posttransplant rejection biopsies all taken from HCV-negative patients within the first 4 weeks after transplantation. Although the two cohorts of rejecting patients (i.e. those enrolled in the weaning study and those developing ACR shortly after transplantation) differed in the expression of 213 genes, no differences were noted in the expression of the 19 genes associated with rejection across all conditions (Figure 2C). Furthermore, GSEA revealed enrichment in similar functional pathways (Table S3).

Histological features of rejection correlate with specific gene expression changes

Liver ACR is characterized by portal and, sometimes, lobular inflammation, portal tract nonsuppurative cholangiolitis and portal and/or central vein subendothelial inflammation. To identify the main histological drivers of transcriptional patterns associated with ACR, we correlated gene expression profiles with quantified histological data (Table 4, Table S4). Table 4 shows the distribution of histological damage in our cohort and the number of genes for which expression was significantly correlated with severity of damage (FC >1.2, FDR <0.05).

We observed a correlation between the magnitude of histological damage in each histological compartment and expression of a set of 423 genes mostly associated with

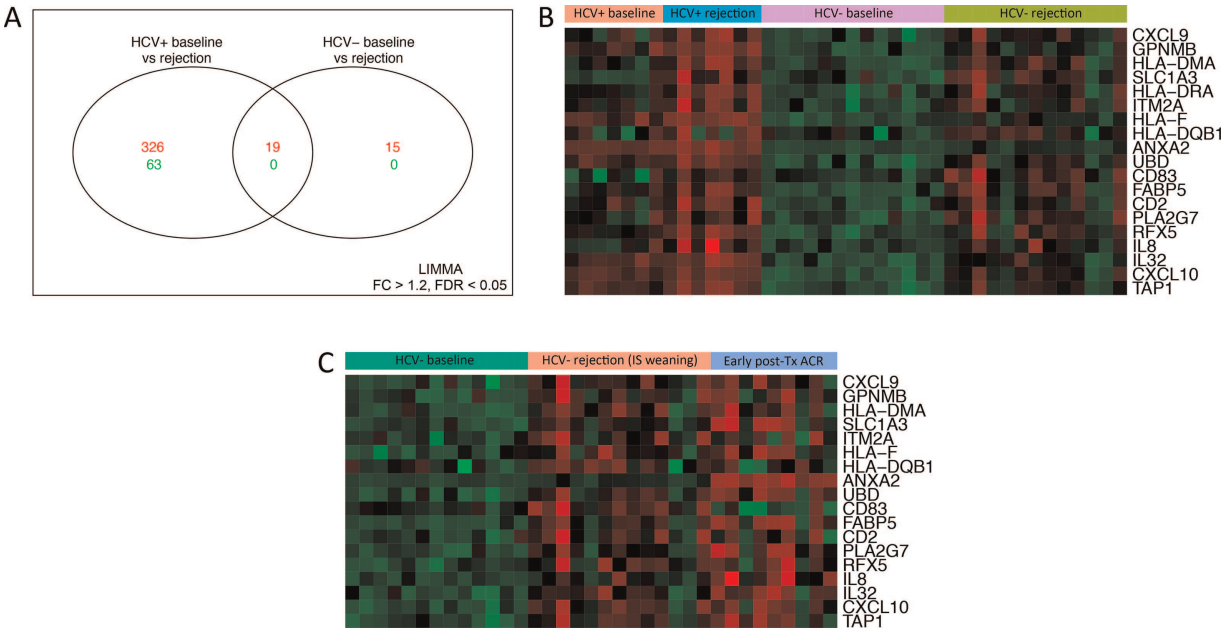

Figure 2: Transcriptional changes associated with rejection in HCV-negative and -positive patients. (A) Venn displaying the number of upregulated (red) or downregulated (green) genes at FDR <5% and FC >1.2 following limma analysis comparing liver tissue samples obtained from HCV-negative and -positive patients at baseline (before IS weaning) and at the time of rejection. (B) Heat map displaying the common set of 19 significantly expressed genes (FC >1.2 and FDR <0.05) comparing baseline (preweaning) liver tissue samples with those collected at time of rejection in HCV-negative and -positive patients. (C) Heat map showing the 19-gene set in HCV-negative patients undergoing weaning and in HCV-negative patients developing rejection shortly after transplantation. *HLA-DRA* is excluded due to poor data quality in the control group. ACR, acute cellular rejection; FC, fold change; FDR, false discovery rate; HCV, hepatitis C virus; IS, immunosuppression; Tx, transplant.

inflammation and rejection. The genes expressed were relatively homogeneous across histological compartments; however, the signal was much stronger in acute inflammatory processes such as lobular inflammation, portal inflammation and interface hepatitis (Figure 3, Table S4).

We used GSEA to investigate the functional pathways driving these histological processes (Table S5). Again, this demonstrated homogeneity across histological compartments, with inflammatory and rejection-associated pathways upregulated across all compartments (with the exception of ductopenia, which was a very infrequent finding and thus could not be adequately explored).

Within the group of rejection biopsies, the overall severity of rejection as defined by the Banff Rejection Activity Index (7) was not associated with significant differential gene expression (based on 20 biopsies with complete rejection activity index data) (data not shown).

Acute cellular rejection results in distinct transcriptional changes in blood that only partially overlap with those observed in the liver allograft

We used the same exploratory approach described above to identify ACR-associated transcriptional patterns in whole blood. This was conducted in paired baseline/rejection samples from 37 HCV-negative patients, with a custom Agilent microarray. Overall, 293 differentially expressed genes were identified with FDR <0.05 and FC >1.2 (Figure 4). Functional analysis using GSEA with the MSigDB Hallmark gene sets showed similar pathways to be upregulated in blood and liver tissue, although the signal was weaker in blood than in tissue (Table 3). Additional analyses using HaemAtlas gene sets (11) revealed that ACR was significantly associated with transcripts preferentially expressed by CD14-positive cells (monocytes). In contrast, transcripts associated with CD8 (cytotoxic T cells) and CD66B (granulocytes) cells were significantly downregulated at the time of rejection. No other blood cell types significantly contributed to the rejection-associated transcriptional pattern. A small group of 22 genes, mostly related to immune response and cell cycle control (e.g. *CXCL9*, *CXCL10*, *CNPM*, *CDC20*, *CCNB2*, and *CD74*), were significantly associated with ACR in both blood and liver tissue samples (Figure 5).

The expression of ACR-associated markers at baseline does not predict the outcome of immunosuppression discontinuation

To determine whether ACR-specific genes were differentially expressed between tolerant and nontolerant liver recipients

Table 2: Liver tissue gene expression markers associated with acute cellular rejection

Gene symbol	Gene name	FC, rejection vs prewean time, HCV−	FDR, HCV−	FC, rejection vs prewean time, HCV+	FDR, HCV+
CXCL9	Chemokine (C-X-C motif) ligand 9	3.214	0.002	3.241	1.42×10^{-6}
GPNMB	Glycoprotein (transmembrane) nmb	1.779	0.006	1.832	0.001
HLA-DMA	Major histocompatibility complex, class II, DM alpha	1.573	0.027	1.764	0.001
SLC1A3	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	1.572	0.027	1.854	0.001
HLA-DRA	Major histocompatibility complex, class II, DR alpha	1.529	0.027	1.734	0.001
ITM2A	Integral membrane protein 2A	1.554	0.027	1.615	0.003
HLA-F	Major histocompatibility complex, class I, F	1.520	0.041	1.605	0.004
HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1	1.615	0.008	1.607	0.005
ANXA2	Annexin A2	1.556	0.032	1.743	0.006
UBD	Ubiquitin D	3.370	0.002	1.678	0.009
CD83	CD83 molecule	1.615	0.008	1.667	0.009
FABP5	Fatty acid binding protein 5 (psoriasis-associated)	2.105	0.002	1.625	0.011
CD2	CD2 molecule	1.480	0.034	1.503	0.013
PLA2G7	Phospholipase A2, group VII	1.631	0.013	1.454	0.022
RFX5	Regulatory factor X, 5 (influences HLA class II expression)	1.419	0.029	1.403	0.026
IL8	Interleukin 8	1.549	0.021	2.194	0.029
IL32	Interleukin 32	1.848	0.006	1.537	0.031
CXCL10	Chemokine (C-X-C motif) ligand 10	3.738	0.002	1.485	0.039
TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	1.651	0.027	1.363	0.047

Table 2 shows the 19 common differentially expressed genes in HCV-negative and -positive recipients when comparing liver samples at rejection time with liver samples collected before the start the IS withdrawal. FC >1.2, FDR <5%, $p < 0.001$. FC, fold change; FDR, false discovery rate; HCV, hepatitis C virus.

before immunosuppression weaning was initiated, we reinterrogated a previously described Affymetrix microarray gene expression database generated with blood samples collected at baseline (before the initiation of weaning) from 43 nontolerant and 25 tolerant liver recipients (22). At baseline, none of the ACR-associated gene expression markers differed between patients who eventually rejected and those who successfully discontinued immunosuppression (data not shown). In addition to the microarray data, we quantified the expression of *CD3*, *FOXP3*, *MAN1A1*, *PRF1*, and *TOAG-1*, which were part of the RISET consortium biomarker portfolio and had been previously assessed in the setting of kidney transplantation (8,23). Foxp3 expression, which was upregulated in tolerant recipients, was the only marker differentially expressed between the tolerant and nontolerant recipients at baseline (Figure 6).

Pharmacological immunosuppression has a minimal impact on rejection-associated gene expression changes in both blood and liver tissue

We explored the transcriptional impact of specific immunosuppressive drugs to investigate the extent to which pharmacological immunosuppression could act as a confounder in the analyses. The type of immunosuppressive agent did not influence liver tissue gene expression. Analyses of blood gene expression patterns revealed no differences between patients on tacrolimus monotherapy versus cyclosporin A monotherapy. In contrast, a set of 17 genes was differentially expressed between patients on calcineurin inhibitor monotherapy and patients on mycophenolate mofetil monotherapy (Table 5), but only eight were included in the set of 293 genes associated with ACR (LOC652494, ENST00000359488, LOC100132941, ENST00000377226, C13ORF33, A_24_P110487, ENST00000322032, SPTA1).

Sequential gene expression profiling in blood samples during the immunosuppression withdrawal process predicts the development of rejection

To investigate whether changes in rejection-associated transcriptional markers precede the clinical diagnosis of ACR and could be used as a predictive tool, we quantified the expression of a set of 45 target genes in sequential blood samples collected from 14 HCV RNA–negative and 8 HCV RNA–positive recipients. A mean of six sequential blood samples collected before the diagnosis of rejection were available per patient. Univariate analysis of blood samples collected at baseline and at time of rejection revealed that eight and two genes were significantly upregulated at the time of rejection in HCV-negative and -positive patients, respectively (Table 6). Using stepwise multivariate logistic regression, we identified *CXCL10* plus *FOXP3* as the best combination of markers discriminating between rejection and baseline blood samples in

Table 3: Pathogenesis-based transcript sets significantly enriched in liver allograft rejection–associated transcriptional patterns (GSEA)

Symbol	Description	Reference	Size[1]	NES[2] HCV−	FDR HCV−	NES HCV+	FDR HCV+
GRIT1	Human orthologs of IFN-γ dependent, rejection associated transcripts defined in mice; expressed in TCMR, especially in association with AMAT1	(17)	19	2.64	0.00*	2.42	0.00*
QCMAT	Macrophage associated transcripts defined in purified cell lines, associated with TCMR in kidney patients	(16)	45	2.54	0.00*	2.34	0.00*
QCAT	Transcripts associated with cytotoxic T lymphocytes, defined in purified cell lines; associated with TCMR in renal transplants, with expression levels correlating with T cell infiltration	(15)	21	2.48	0.00*	2.44	0.00*
IRITD3	Injury and rejection induced transcripts upregulated day 3 after isograft transplant (humanized results from mouse model)	(39)	173	2.30	0.00*	2.60	0.00*
IRITD5	As for IRITD3, measured on day 5.	(39)	133	2.25	0.00*	2.68	0.00*
CIRIT	Cardiac injury- and repair-induced transcripts, expressed following heart isograft transplant; mouse data extrapolated to humans	(40)	174	2.20	0.00*	2.22	0.00*
IRRAT	Injury-repair response associated transcripts, defined in early renal transplants with no rejection, derived as a model for AKI	(39)	22	2.05	0.00*	1.67	0.00*
BAT	B cell–associated transcripts, derived from purified B cells; upregulated in both ABMR and TCMR	(19)	55	1.97	0.00*	2.15	0.00*
DSAST	DSA-positive–specific transcripts derived from comparative analysis of DSA with and without renal biopsies; observed in both ABMR and TCMR with much higher levels in ABMR	(18)	15	1.64	0.01*	1.18	0.29
HTS	Heart-selective transcripts derived from control mice without inflammation present	(40)	385	1.54	0.03*	1.59	0.01*
TCB	T cell–specific transcripts based on purified cell lines	(21)	4	1.47	0.04*	1.66	0.00*
ENDAT	Endothelial cell associated transcripts derived from purified cell lines; increased in ABMR and TCMR with higher levels in ABMR	(20,41)	81	1.46	0.04*	2.23	0.00*
NKB	Natural killer cell–specific transcripts derived from purified cell lines; identified in early TCMR and late ABMR in renal patients	(21)	3	1.43	0.05	1.04	0.43
IGT	Immunoglobulin associated transcripts, observed in both ABMR and TCMR	(19)	4	1.36	0.08	1.57	0.01*
AMAT1	Alternative Macrophage Associated Transcript 1; human orthologs of mouse data; high GRIT1 plus AMAT1 scores correlate with TCMR	(16)	6	1.31	0.12	1.73	0.00*
MCAT	Mast cell associated transcripts, associated with scarring and poor survival in renal transplants	(22)	3	−0.80	0.78	1.13	0.32

ABMR, antibody-mediated rejection; AKI, acute kidney injury; DSA, donor-specific antibody; FDR, false discovery rate; GSEA, gene set enrichment analysis; IFN-γ, interferon-γ; NES, normalized enrichment score; TCMR, T cell–mediated rejection.
*Significance threshold based on FDR <0.05
[1]Size is the number of transcripts in the gene set.
[2]NES as calculated by GSEA software (http://www.broadinstitute.org/gsea/doc/GSEAUserGuideTEXT.htm#_Interpreting_GSEA_Results).

Table 4: Differential gene expression by histological compartment

	No. of patients by severity level				
Histological compartment	0	1	2	3	Gene count
Lobular inflammation	29	34	15	0	354
Portal inflammation	17	33	21	2	223
Interface hepatitis	44	23	10	0	284
Central vein perivenulitis	53	12	4	0	10
Endothelitis central vein	0	3	0	0	1
Endothelitis portal veins	54	16	3	1	4
Bile duct lesions	51	19	3	1	16
Ductular reaction	50	20	0	0	0
Ductopenia	76	3	0	0	4
Perisinusoildal fibrosis	52	8	5	0	0
Portal fibrosis	29	24	21	2	31

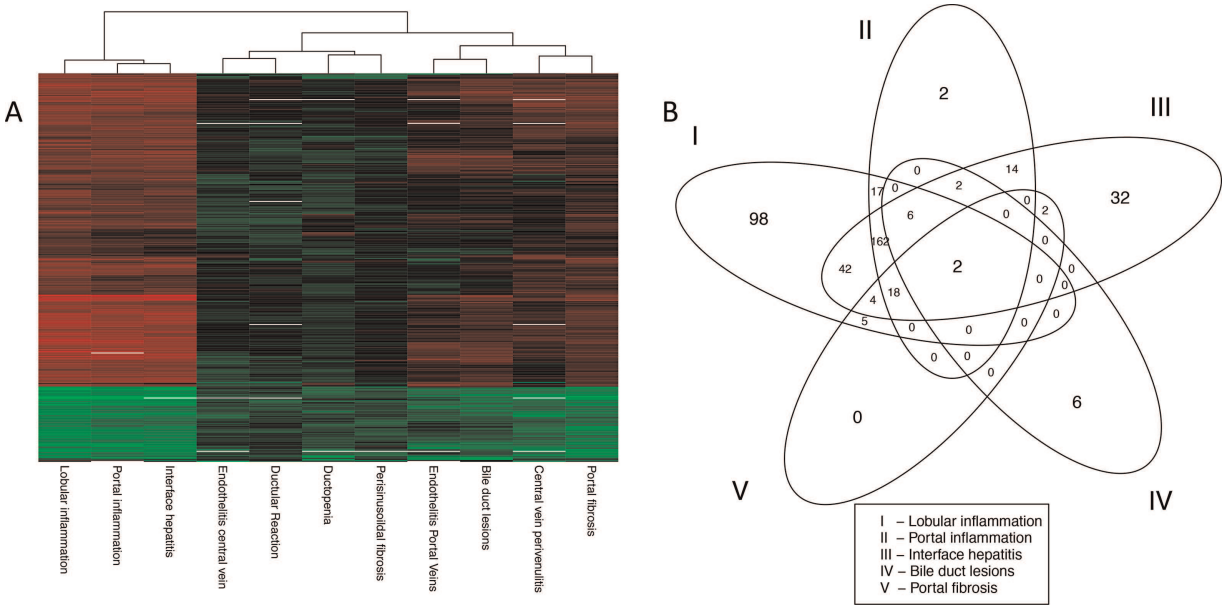

Figure 3: Differentially expressed genes across histological compartments. (A) Clustered heat map demonstrating all genes significantly differentially expressed across histological compartments (fold change >1.2 and false discovery rate <0.05 in at least one compartment). Analysis performed in limma comparing gene expression against quantified severity score for each histological parameter. Red indicates upregulation, green indicates downregulation. (B) Venn diagram showing the number of genes differentially expressed in each histological compartment and demonstrating overlap between compartments.

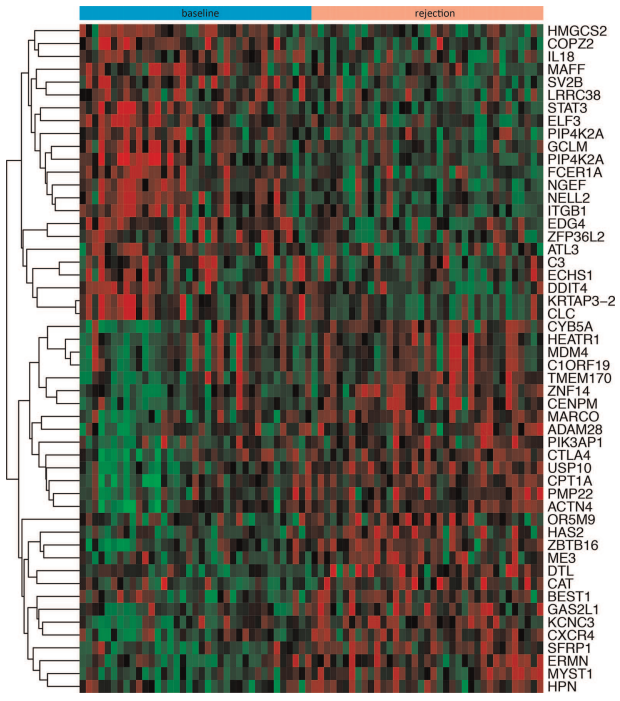

Figure 4: Differentially expressed genes in whole blood. Heat map of the top 50 genes differentially expressed in whole blood based on t-statistic comparing paired baseline (preweaning) and rejection samples. All patients were negative for hepatitis C virus.

HCV-negative patients (cross-validated area under the curve [AUC] 0.82). When using this signature in all sequentially collected blood samples, we observed that the probability of ACR progressively increased with time, with a peak that coincided with the time of the diagnosis of biopsy-proven ACR and a rapid decline following reinstitution of robust immunosuppression (Figure 7A). *CXCL10* plus *FOXP3* expression correctly predicted the absence of rejection in 81% of 86 liver recipients with stable graft function under immunosuppression 3–10 years after transplantation. In contrast, this model failed to correctly classify 70% of 57 blood samples sequentially collected from seven tolerant liver recipients who were successfully weaned from immunosuppression. Given the well-known influence of calcineurin inhibitors on *FOXP3* expression (24), we repeated the logistic regression analysis excluding *FOXP3*. This resulted in *CXCL10* alone being selected as the most robust predictor. *CXCL10* accurately classified rejecting samples with a cross-validated AUC of 0.76 and correctly predicted absence of rejection in 79% of stable patients and 67% of samples from tolerant patients (Figure 7C). The probability of rejection as predicted by *CXCL10* expression increased 1–2 mo prior to rejection being clinically apparent and a liver biopsy considered indicated. A detailed analysis of the kinetics of liver function tests, however, revealed that the changes in *CXCL10* expression mirrored mild but gradually increasing changes in γ-glutamyltransferase, aspartate aminotransferase and alanine aminotransferase occurring over the same time period (Figure 7D). In contrast to these results observed in

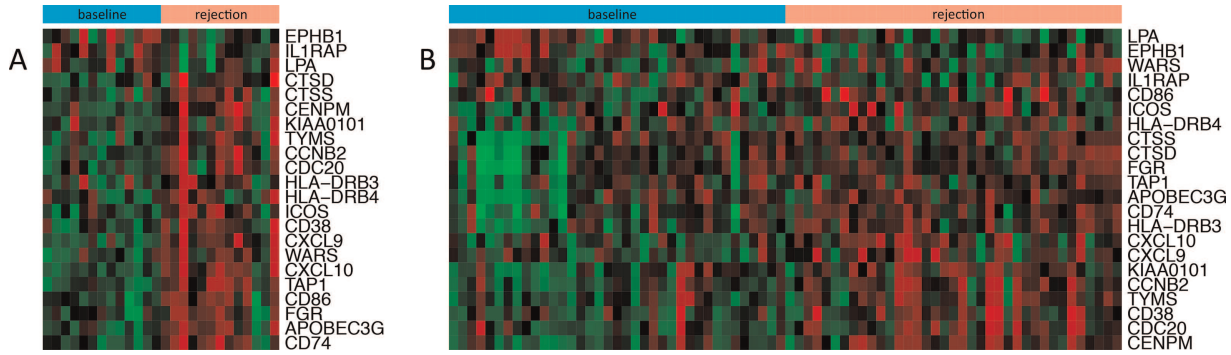

Figure 5: Significantly expressed genes in liver tissues and in PBMC of HCV-negative patients. Heat maps displaying the common set of 22 significantly expressed genes (fold change >1.2 and false discovery rate <0.05) when comparing baseline (preweaning) liver tissue samples (A) with those collected at the time of rejection in HCV-negative patients and in PBMC samples (B) at the same time points for the same patient groups. HCV, hepatitis C virus; PBMC, peripheral blood mononuclear cells.

HCV-negative recipients, a gene expression model with adequate diagnostic performance could not be generated from the 45-gene PCR expression data set in the cohort of seven recipients with active HCV infection (data not shown).

Discussion

The identification of reproducible noninvasive biomarkers of graft rejection remains an important area of research in organ transplantation. In kidney transplantation, the molecular profiles associated with allograft rejection have been comprehensively defined, and results from single- and multicenter studies have shown that specific biomarkers, in particular, mRNAs, can be useful diagnostic tools. In contrast, in liver transplantation, the field is much less developed, and our understanding of the predictive accuracy and specificity of molecular biomarkers in different biological specimens is incomplete.

Similar to other organ transplantation settings, our microarray experiments revealed that there are transcriptional differences between rejecting and nonrejecting liver allografts. Many genes identified in our study are well studied in the context of transplant rejection and correspond to functional networks known to be associated with rejection, such as nuclear factor-κB, STAT1/interferon-γ, tumor necrosis factor-α, chemokine receptor networks and immune effector networks (25). In particular, the set of 19 genes that we identified in tissue samples in both HCV-positive and -negative patients includes genes previously associated with rejection in kidney (*CXCL9, HLA-DMA, IL32, CXCL10, HLA-F, CD2*) (26,27), in lung (*ITM2A, IL8, IL32*) (28), and in heart transplantation (*CXCL9, HLA-F, CXCL10*) (29). A meta-analysis by Spivey *et al* (25) identified 330 genes known to be differentially expressed in rejection tissue samples across a variety of transplanted organs, including liver, kidney, heart and lung. From our set of 19 genes, 12 were previously identified (*CXCL9, HLA-DMA, HLA-DRA, ITM2A, HLA-F, HLA-DQB1, UBD, CD2, IL8,*

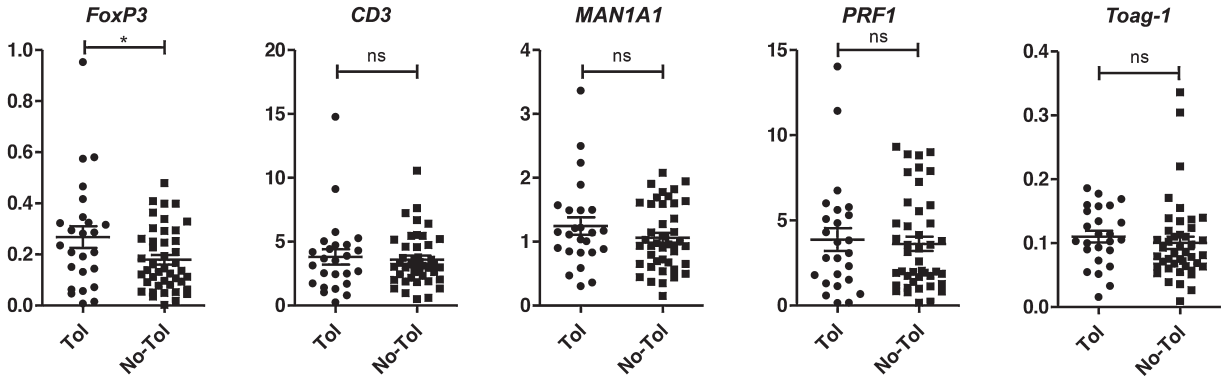

Figure 6: Baseline acute cellular rejection–associated gene expression in nontolerant and tolerant patients. Graphics showing the quantitative gene expression of five genes included in the Reprogramming the Immune System for the Establishment of Tolerance (RISET) consortium biomarker portfolio performed at baseline (preweaning) in 43 nontolerant and 25 tolerant patients. No-Tol, nontolerant; Tol, tolerant. *$p < 0.05$.

Table 5: Differential gene expression by baseline immunosuppression

	Tissue		Blood (microarray)		Blood (Fluidigm)	
Immunosuppression	n	Genes expressed	n	Genes expressed	n	Genes expressed
TAC	18	0	29	0	12	0
CSA	14		23		11	
CNI monotherapy	32	0	52	17[1]	23	NA
MMF monotherapy	6		12		0	
CNI monotherapy	32	0	52	1[2]	23	3[3]
CNI + MMF	6		8		6	
mTOR inhibitor	2	13[4]	0	NA	2	3[5]
Other therapies	44		72		29	

CNI, calcineurin inhibitor; CSA, cyclosporin A; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin; NA, not available; TAC, tacrolimus.
[1]*FA2H, SPTA1, TFPI, ENST00000322032, C13ORF33, LOC653513;LOC727893; LOC727927;LOC727942;LOC728802;PDE4DIP;XXYAC-YX155B6.2, CCNF, POGK, SLFN12, LOC652494, ENST00000359488, LOC100132941, ENST00000377226, IGJ, A_24_P110487, LOC644538, PTPRF.*
[2]*IFNA2.*
[3]*CENPM, EZH2, PARVG.*
[4]*TIMD4, GDF15, FOS, IL8, CXCR7, MELK, CETP, JUN, EZH2, CIDEC, STAB2, FOLR2, ZGPAT.*
[5]*CD74, TOP2A, HLA-DMA.*

IL32, CXCL10, TAP1), whereas seven were unique to our study (*GPNMB, SLC1A3, ANXA2, CD83, FABP5, PLA2G7, RFX5*).

Specific to the liver, Sreekumar et al identified a gene signature associated with ACR in HCV-positive patients (30). This study highlighted the difficulties of differentiating ACR from HCV histopathologically. Our HCV-positive results implicate many of the same pathways (e.g. MHC, ubiquitin), if not exactly the same genes. The large number of differentially expressed genes in HCV-positive patients relative to HCV-negative patients in our study supports previous data showing a significant interaction between HCV infection and alloimmune responses at a molecular level (6). The identification of ACR-specific genes independent of HCV infection, however, supports the conclusion of Sreekumar et al that molecular profiling could be useful to aid in this difficult histopathological diagnosis.

T cell–related transcripts were more highly differentially expressed in the liver at the time of ACR. These findings indicate that at the transcriptional level, liver allografts respond to T cell–mediated rejection (TCMR) similarly to what has been described for other organs, particularly kidneys. A significant association, however, was also found with genes known to be overexpressed in kidney allografts exhibiting antibody-mediated rejection (ABMR; i.e. B cells and natural killer cells). This is not entirely surprising, given that the transcriptional differences between TCMR and ABMR in kidney transplantation are more quantitative than qualitative (i.e. pathways described as being specific for ABMR are also observed, albeit with a weaker signal, in the setting of TCMR). This observation will need to be considered if transcriptional profiling is ever used to provide pathogenic guidance in the analyses of liver allograft biopsies exhibiting mixed or unclear histological phenotypes. In contrast to the liver tissue transcriptome results, T cell–related transcripts were decreased in blood at the time of ACR. We hypothesize that this is due to the well-known homing of T cells, particularly $CD8^+$ T cells, to the transplanted organ at the time of allograft rejection (31).

Table 6: Significantly differentially expressed genes at baseline vs rejection based on univariate analysis of Fluidigm data

Gene	p-value (HCV+)	p-value (HCV−)
ABCB1	0.441	0.048*
APOL3	0.383	0.942
CCL19	0.570	0.369
CCN2B	0.053	0.799
CD52	0.083	0.144
CD74	0.567	0.742
CD8A	0.587	0.011*
CDC20	0.777	0.559
CECR1	0.141	0.911
CENPM	0.634	0.505
CXCL10	0.019*	0.794
CXCL9	0.010*	0.809
DHRS9	0.046*	0.933
DOCK11	0.412	0.511
EMILIN2	0.096	0.604
EZH2	0.329	0.263
FOXP3	0.002*	0.321
GBP1	0.689	0.078
GBP2	0.680	0.610
GPNMB	0.856	0.233
GZMB	0.149	0.234
GZMK	0.518	0.053
HLA.DMA	0.718	0.927
HLA.DMB	0.935	0.624
HMMR	0.935	0.254
IL15	0.335	0.436
IL18BP	0.592	0.761
IL32	0.586	0.136
IRF1	0.084	0.532
LYZ	0.062	0.952
ME2	0.327	0.554
MMP9	0.836	0.366
PARVG	0.353	0.354
PLEKHG1	0.788	0.873
PRF1	0.670	0.068
RFX5	0.604	0.731
STAT1	0.024*	0.839
TAP1	0.154	0.937
TGFb1	0.166	0.098
TK1	0.019*	0.608
TLR8	0.978	0.748
TOP2A	0.203	0.572
TYMS	0.013*	0.720
UBD	0.002*	0.209
ZWINT	0.144	0.368

HCV, hepatitis C virus.
*Significant difference between baseline and rejection ($p < 0.05$).

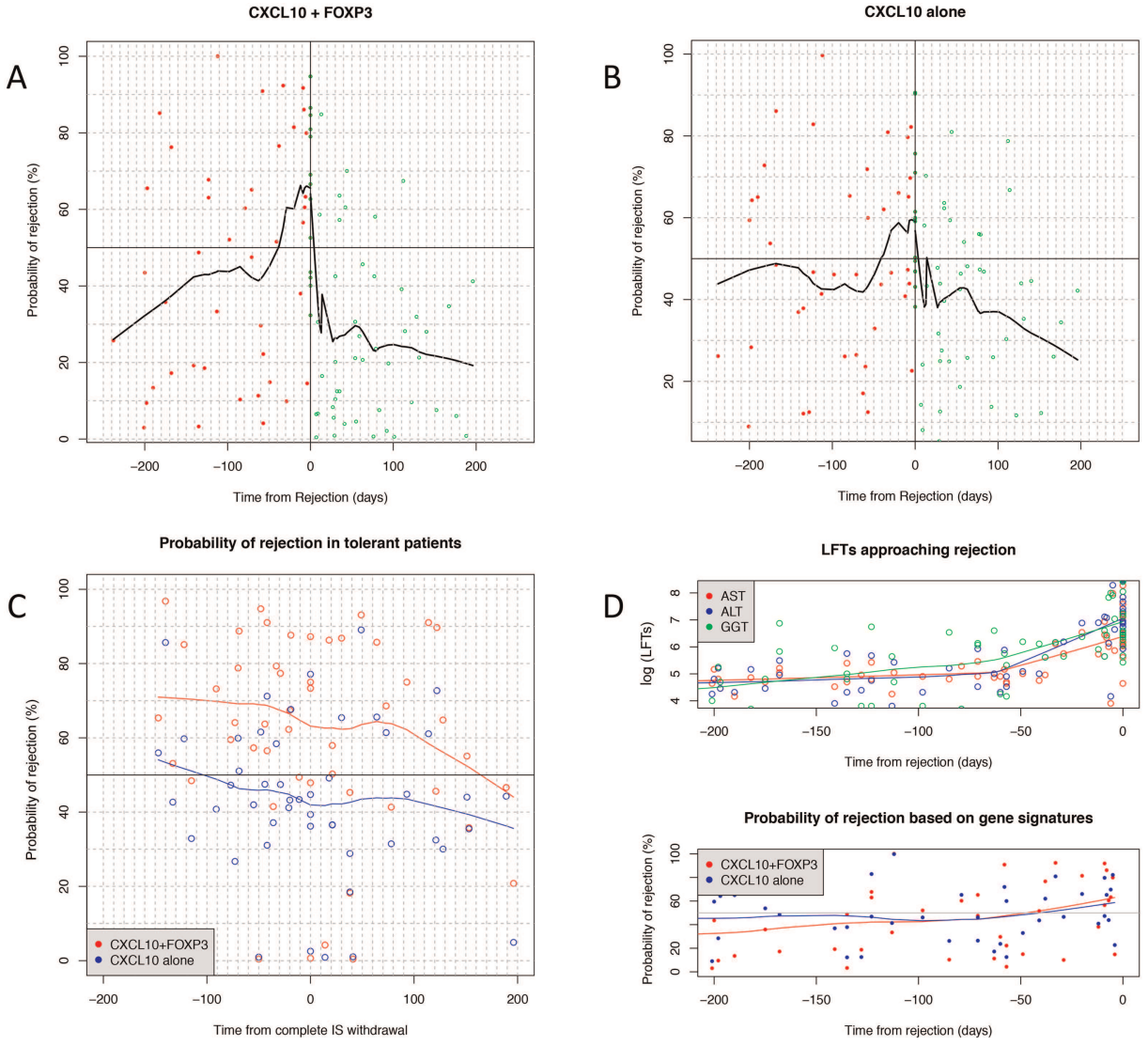

Figure 7: Prediction of rejection by sequential gene expression in blood samples. (A, B) Time evolution of risk probabilities based on gene signatures for *CXCL10* plus *FOXP3* and *CXCL10* alone, respectively. Both models show a peak coinciding with acute cellular rejection biopsy-proven diagnosis (time 0), with a rapid reduction following reinstitution or reinforcement of IS. (C) Evolution of risk probabilities in operationally tolerant patients up to and after complete withdrawal of immunosuppression (time 0), showing that *CXCL10* plus *FOXP3*, but not *CXCL10* alone, misclassifies as rejecting 70% of blood samples. (D) Sequential risk probabilities based on gene signature models alongside conventional LFTs (logarithmic scale). Risk increases gradually from ≈1–2 mo prior to rejection, mirroring a rise in LFTs. IS, immunosuppression; LFT, liver function test.

In comparing ACR signatures in tissue and blood, we acknowledge that the use of different microarray platforms presents challenges in drawing firm conclusions. A relatively low significance threshold was required for between-platform analyses to extract meaningful data. We note, however, that functional analyses were similar between tissue and blood, supporting our findings of overlapping transcriptional profiles.

Our histological analysis demonstrates a clear correlation between severity of inflammatory histological damage and expression of genes associated with inflammatory and rejection processes. This is a homogeneous finding across the different histological compartments, but the signal is particularly striking in lobular and portal inflammation and in interface hepatitis. Similar results have been reported in renal patients, in whom expression of inflammatory response genes correlates with the Banff scoring system for histological rejection (32). It should be acknowledged that the number of patients exhibiting severe central vein endothelitis or ductopenia was small (Table 4). This is because patients were very closely followed, and rejection

was diagnosed as soon as allograft dysfunction occurred. Consequently, our ability to detect transcriptional signatures for the most severe forms of rejection was limited.

CXCL10 and *FOXP3* whole-blood expression warrants further investigation as a potential biomarker for rejection, although its utility in the setting of immunosuppression withdrawal is questionable, given the influence of CNI on *FOXP3* expression. In this setting, *CXCL10* alone is a more robust marker, although it is noteworthy that this model misclassified as rejecting 33% of blood samples collected from tolerant liver recipients exhibiting no clinical signs of rejection. We hypothesize that some of these misclassifications may reflect subclinical inflammation caused by the withdrawal process that was not sufficiently severe to lead to a diagnosis of rejection.

The clinical applicability of transcriptional biomarkers of rejection in blood will need to be established in larger clinical trials in which gene expression data are combined with sequential liver function test measurements. We hope to improve on diagnosis based on liver function tests alone, which are neither sensitive nor specific for rejection (33). A note of caution is warranted, however, considering that blood gene expression was not sensitive enough to detect rejection in patients with chronic HCV infection. Further work is required to identify other potential confounding factors.

Our study provides insight into the processes underlying ACR in liver transplant patients. We observed significant overlap with T cell–mediated rejection processes that are well characterized in renal patients and identified characteristic tissue and blood signatures specific to the liver. Clinically, we demonstrated the potential utility of transcriptional markers in peripheral blood as a predictor for rejection. Although further work is required, these markers could lead to more accurate diagnosis and reduce the need for invasive biopsies.

Acknowledgments

The research was funded by Roche Organ Transplantation Research Foundation (ROTRF) and Instituto de Salud Carlos III Spain (FISS 1881/2011). In addition, the investigator team was supported by the Medical Research Council (MRC) Centre for Transplantation (MRC grant no. MR/J006742/1) and by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' National Health Service (NHS) Foundation Trust and King's College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health. F.B. was supported by a research fellowship of the Deutsche Forschungsgemeinschaft (DfG reference BO 3370/1-1). CIBEREHD is funded by the Instituto de Salud Carlos III Spain.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Supplementary Methods

Table S1: List of gene expression markers assessed using Fluidigm quantitative polymerase chain reaction experiments.

Table S2: Functional pathways significantly overrepresented in liver allograft rejection–associated transcriptional patterns (Hallmark pathways, Molecular Signatures Database; gene set enrichment analysis).

Table S3: Comparison of functional pathways associated with rejection in patients enrolled in withdrawal trials against early posttransplant rejection outside of the trial setting.

Table S4: Full list of genes differentially expressed in different histological compartments, showing t-statistic.

Table S5: Functional pathways associated with severity of different histological parameters. Analysis performed using gene set enrichment analysis software against Hallmark gene sets from the Molecular Signatures Database and pathogenesis-based transcript sets from the University of Alberta. Normalized enrichment score and false discovery rate (FDR) are shown for each parameter. Significantly enriched pathways (FDR <0.05) are highlighted.