# Transcriptional Signals of T-cell and Corticosteroid-sensitive Genes Are Associated With Future Acute Cellular Rejection in Cardiac Allografts

Mandeep R. Mehra, MD,[a] Jon A. Kobashigawa, MD,[b] Mario C. Deng, MD,[c] Kenneth C. Fang, MD,[d] Tod M. Klingler, PhD,[d] Preeti G. Lal, PhD,[d] Steven Rosenberg, PhD,[d] Patricia A. Uber, PharmD,[a] Randall C. Starling, MD,[e] Srinivas Murali, MD,[f] Daniel F. Pauly, MD,[g] Russell Dedrick, PhD,[d] Michael G. Walker, PhD,[d] Adriana Zeevi, PhD,[h] and Howard J. Eisen, MD,[i] for the CARGO Investigators

**Background:** Profiling mRNA levels of 11 informative genes expressed by circulating immune effector cells identifies cardiac allograft recipients at low risk for current moderate–severe acute cellular rejection (ACR).

**Methods:** We conducted a nested case–control study of 104 cardiac allograft recipients to investigate the association of transcriptional profiles of blood samples with either a future rejection episode within 12 weeks of a baseline clinical sample or persistent histologic quiescence for the same time period.

**Results:** The transcription profile yielded a score (0 to 40 scale) of 27.4 ± 6.3 for future rejectors ($n = 39$) and 23.9 ± 7.1 for controls ($n = 65$) ($p = 0.01$). In patients who were ≤180 days post-transplant, the gene expression score was 28.4 ± 4.9 for rejectors ($n = 28$) and 22.4 ± 7.5 for controls ($n = 48$) ($p < 0.001$). In this period, no samples from patients who went on to reject within 12 weeks had gene expression scores of <20. Differential expression of the gene IL1R2 was significantly associated with future events. Of 33 additional genes profiled, 5 supported corticosteroid-sensitive constituents (IL1R2 and FLT3), whereas 6 supported T-cell activation (PDCD1).

**Conclusions:** These data suggest that pathways regulating T-cell homeostasis and corticosteroid sensitivity are associated with future ACR in cardiac allografts and suggest that these signals are evident before histologically detectable rejection. J Heart Lung Transplant 2007;26:1255–63. Copyright © 2007 by the International Society for Heart and Lung Transplantation.

The need for balance between risk of rejection and over-immunosuppression highlights the need for diagnostic options that might enable earlier detection of rejection.[1,2] Endomyocardial biopsy provides assignment of rejection grades based on the extent and distribution of leukocyte infiltrates, and evidence of accompanying myocyte damage.[3–5] No compelling data exist to demonstrate the utility of endomyocardial biopsy in predicting rejection of a future biopsy sample. The development of a molecular diagnostic assay for cardiac allograft rejection led to the identification of a panel of genes whose expression in peripheral blood mononuclear cells (PBMC) correlates with histologic acute cellular rejection (ACR).[6] Subsequent studies have described the use of this molecular classifier in managing cardiac allograft patients,[7] suggesting links to the chronic condition of coronary artery vasculopathy.[8,9] The classifier is comprised of 11 informative genes (plus 9 control genes) that participate in the regulation of activation, trafficking and morphology of immune effector cells, platelet activation, hematopoiesis and corticosteroid sensitivity.[6]

The purpose of the present investigation was 2-fold. First, we tested if the gene expression–based molecular classifier developed for current rejection was also associated with future rejection when assayed weeks to months before the event. Second, we sought to identify the relative contribution of individual genes and their representative pathways to future rejection risk.

From the [a]University of Maryland School of Medicine, Baltimore, Maryland; [b]University of California at Los Angeles, Los Angeles, California; [c]Columbia University, New York, New York; [d]XDx, Inc., Brisbane, California; [e]Cleveland Clinic Foundation, Cleveland, Ohio; [f]Allegheny General Hospital, Pittsburgh, Pennyslvania; [g]University of Florida, Gainesville, Florida; [h]University of Pittsburgh, Pittsburgh, Pennsylvania; and [i]Drexel University College of Medicine, Philadelphia, Pennsylvania.

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Reprint requests: Mandeep R. Mehra, MD, Division of Cardiology, University of Maryland School of Medicine, 22 South Greene Street, Room S3B06, Baltimore, MD 21201. Telephone: 410-328-7716. Fax: 410-328-4352. E-mail: mmehra@medicine.umaryland.edu

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## METHODS

### Study Objectives

A nested case–control study was used to examine the hypothesis that a 20-gene classifier for current moderate-to-severe ACR (Grade ≥3A) is also associated with the future absence or presence of Grade ≥3A rejection. The primary study objective was to determine the ability of the gene expression score, used to assay current rejection, to distinguish stable patients who remain rejection-free from those patients who develop moderate to severe (Grade ≥3A) rejection within the ensuing 12 weeks. Secondary study objectives included: (1) characterizing the associations with rejection according to time post-transplant, specifically the period within ≤180 days post-transplant; (2) identifying the individual classifier genes associated with future rejection risk; and (3) substantiating the significance of these genes by exploring their representative pathways and functions.

### Study Population

Study patients were selected from the 629 subjects enrolled in the CARGO study between September 2001 and June 2003 in protocols approved by the institutional review boards at each of the participating study sites.[6] To be included in the study, patients had to meet the following criteria: (1) time since transplant surgery ≥30 days; (2) absence of signs or symptoms of current ACR; (3) absence of graft dysfunction assessed either by pulmonary artery catheterization or echocardiography; (4) prior 30-day period of clinical stability, defined as the absence of Grade ≥3A rejection, graft dysfunction or administration of rejection therapy; and (5) not used for development of original classifier. The control group included patients whose endomyocardial biopsy sample indicated Grade 0 or 1A rejection at baseline visit and who remained free of Grade ≥2 rejection for at least 12 weeks, whereas the rejection case group included all those who developed Grade ≥3A rejection during the same period. Patients in the control group were matched as a group with those in the rejection case group based on demographic and clinical parameters.

### Study Conduct

The execution of this study was performed according to a prospective protocol, and both elective analysis of patient demographics and sample availability were performed to assess feasibility before finalization of the study protocol.

Clinical management at the CARGO study sites included the following: performance of endomyocardial biopsy weekly in the first 30 days post-transplant; once every 2 weeks between 31 and 90 days; monthly between 91 and 180 days; and bi-monthly between 181 and 365 days; and usually quarterly thereafter. All patients received a regimen of triple immunosuppression, including center-specific use of corticosteroids, cyclosporine or tacrolimus, and either mycophenolate mofetil or sirolimus.[6]

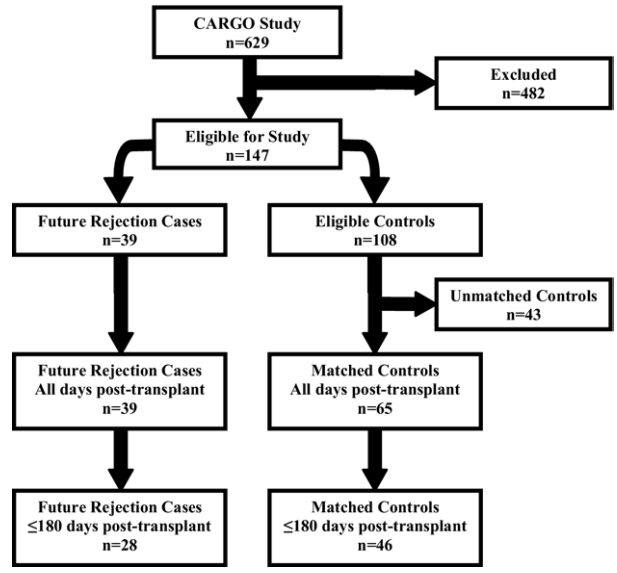


**Figure 1.** Study design. One hundred four patients enrolled in the CARGO study satisfied the inclusion criteria and provided blood samples for time-dependent gene expression profiling in a retrospective case–control study. Patients in the control group included those who were clinically stable (defined as the absence of rejection with a biopsy of Grade 0 or 1A) and who remained free of Grade '3A rejection within the ensuing 12 weeks, whereas those in the rejection group had an episode of Grade ≥3A rejection during the same time interval. Sixty-five of 108 eligible patients were enrolled into the control group based on matching of the encounters with the 39 patients in the rejection group according to demographic and clinical features. Bioinformatic and statistical analyses were performed on both algorithm-derived gene expression scores and expression levels of individual genes to define molecular profiles associated with Grade ≥3A rejection. Messenger RNA quantification by qRT-PCR was performed for the 11 informative rejection algorithm genes and 33 additional non-algorithm genes in related pathways. Analyses were performed on the entire study population of 104 patients and on the sub-group of 74 patients within 180 days post-transplant.

Gene expression profiling was performed on PBMC isolated from blood samples obtained from individual patients from the CARGO study.[6] All blood samples were drawn during the same visit at which the corresponding endomyocardial biopsy was performed. Utilizing the 1990 pathology criteria,[3] all samples were required to be without rejection, defined as an accompanying endomyocardial biopsy assigned a grade of 0 or 1A. Patients who had an episode of rejection (Grade ≥3A) within the following 12 weeks were assigned to the case group, whereas those who remained free of rejection (Grade 0 or 1A) for the same period were assigned to the control group. The details of the

**Table 1.** Demographic and Treatment Profiles of the Rejection and Control Groups

| | All days post-transplant | | | ≤180 days post-transplant | | |
|---|---|---|---|---|---|---|
| | Rejection ($n$ = 39) | Control ($n$ = 65) | $p$-value | Rejection ($n$ = 28) | Control ($n$ = 46) | $p$-value |
| Age, years [Mean (SD)] | 56 (11.0) | 54 (15) | 0.58[a] | 55 (11) | 54 (16) | 0.73[a] |
| Gender, male [no. (%)] | 32 (82.1) | 54 (83.1) | 0.99[b] | 22 (78.6) | 41 (53.6) | 0.31[b] |
| Race [no. (%)] | | | 0.33[b] | | | 0.025[b] |
| White | 23 (59.0) | 47 (72.3) | | 15 (53.6) | 38 (82.6) | |
| Black | 10 (25.6) | 10 (15.6) | | 8 (28.6) | 5 (10.9) | |
| Other | 6 (15.4) | 8 (12.1) | | 5 (17.8) | 3 (6.5) | |
| Immunosuppression, [no. (%)] | | | 0.32[b] | | | 0.29[b] |
| Cyclosporine/mycophenolate mofetil | 20 (51.3) | 37 (56.9) | | 15 (53.6) | 28 (60.9) | |
| Cyclosporine/sirolimus | 1 (2.6) | 2 (3.1) | | 1 (3.6) | 2 (4.3) | |
| Tacrolimus/mycophenolate mofetil | 10 (25.6) | 19 (29.2) | | 6 (21.4) | 12 (26.1) | |
| Tacrolimus/sirolimus | 6 (15.4) | 3 (4.6) | | 5 (17.9) | 2 (4.3) | |
| Other | 2 (5.1) | 4 (6.2) | | 1 (3.6) | 2 (4.3) | |
| Steroid dose, mg/day (mean) | 11 (7.0) | 12 (10) | 0.62[a] | 14 (7) | 15 (11) | 0.75[a] |
| Days post-transplant (mean) | 156 (109) | 137 (76) | 0.30[a] | 103 (51) | 96 (46) | 0.54[a] |
| Days to rejection | 38 (16) | NA | | 35 (14) | NA | |
| ISHLT biopsy [no. (%)] | | | 0.0006[b] | | | 0.008[b] |
| Grade 0 | 12 (30.8) | 43 (66.2) | | 9 (32.1) | 30 (65.2) | |
| Grade 1A | 27 (69.2) | 22 (34.4) | | 19 (67.9) | 16 (34.8) | |

No differences were observed between the rejection and control groups with respect to age, gender, immunosuppression medication regimens, mean daily steroid dose, survival time post-transplant, or the interval of progression to rejection. In the sub-group of patients ≤180 days post-transplant, the rejection group included a higher proportion of black patients. The rejection group also consisted of a higher percentage of biopsies assigned Grade 1A compared with Grade 0.

[a]2-tailed independent $t$-test.

[b]Fisher exact test.

molecular classifier are described in the supplementary materials of the publication explaining its initial development and validation.[6] To substantiate the biologic and statistical relevance of the associated genes, further transcriptional profiling was performed on genes in related molecular pathways.

### Endomyocardial Biopsy Pathology

The CARGO study clinical database included the biopsy pathology grades assigned by the pathologist at the participating study center and those assigned by each of the three members of the study pathology panel of expert cardiac transplant pathologists who were blinded to patient-identifying and clinical data or outcomes.[10] For inclusion in this study, any biopsy diagnosed as moderate to severe acute cellular rejection required assignment of Grade ≥3A by at least two of the four pathologists. The absence of rejection (defined as Grades 0 and 1A) required agreement by at least three pathologists.[6]

### Gene Expression Profiling

Transcriptional profiling was performed on the genes comprising AlloMap molecular expression testing (XDx, Inc., Brisbane, CA).[6] Additional genes were derived using searches in the literature (PubMed, National Center for Biotechnology Information) and public databases (GenBank, NCBI) and used for transcriptional profiling and further analyses. Gene expression profiling was performed as described elsewhere.[6]

### Statistical Analysis

We tested the hypothesis that the gene expression score distinguishes stable patients who have a future episode of Grade ≥3A ACR from patients who remain

**Table 2.** Multivariate Analysis of Gene Expression Score and Demographics

| | | All times post-transplant: rejection ($n$ = 39)/control ($n$ = 65) | | ≤180 days post-transplant: rejection ($n$ = 28)/control ($n$ = 46) | |
|---|---|---|---|---|---|
| Effect | $df$ | Wald chi-square | $p$ (> chi-square) | Wald chi-square | $p$ (> chi-square) |
| Race | 3 | 1.81 | 0.61 | 7.40 | 0.06 |
| Biopsy grade | 1 | 10.48 | 0.001 | 5.05 | 0.025 |
| Gene expression test score | 1 | 5.77 | 0.016 | 11.83 | 0.0006 |

Multivariate logistic regression of the study group (rejection case or control) vs race and biopsy grade was used to assess the association of the gene expression score with future rejection in the context of significant clinical variables.

**Table 3.** Multivariate Analysis of Clinical Variables

| | All times post-transplant | | | ≤180 days post-transplant | | |
|---|---|---|---|---|---|---|
| Sub-group | Rejection | Control | *t*-test (*p*-value) | Rejection | Control | *t*-test (*p*-value) |
| Biopsy Grades 0 + 1A | 27.4 ± 6.3 ($n = 39$) | 23.9 ± 7.1 ($n = 65$) | 0.01 | 28.4 ± 4.9 ($n = 28$) | 22.4 ± 7.5 ($n = 46$) | 0.0004 |
| Biopsy Grades 0 only | 27.2 ± 6.0 ($n = 12$) | 24.2 ± 6.7 ($n = 43$) | 0.17 | 28.4 ± 4.8 ($n = 9$) | 22.7 ± 7.2 ($n = 30$) | 0.03 |
| Biopsy Grades 1A only | 27.6 ± 6.5 ($n = 27$) | 23.3 ± 7.9 ($n = 22$) | 0.04 | 28.4 ± 5.1 ($n = 19$) | 21.9 ± 8.4 ($n = 16$) | 0.008 |

Sub-group analysis of the study group (rejection case or control) by ISHLT biopsy Grade 0 only and Grade 1A only. Analysis was performed for all time-points post-transplant as well as for the period of ≤180 days post-transplant.

stable using a 2-tailed, 2-sample *t*-test as well as a non-parametric Wilcoxon test (Mann–Whitney *U*-test). This analysis was repeated with the patient sub-set ≤180 days post-transplant. The 2-tailed, 2-sample *t*-test was also used to assess differences in expression levels of individual genes between the case and control group patients, initially with molecular classifier genes only, and then subsequently with the extended set of related genes. To account for multiple testing of individual genes, the false discovery rate (FDR) was calculated using the method of Benjamini and Hochberg.[11]

Multivariate logistic regression, including demographic and clinical variables that had distributions significantly different between the case and controls groups, was used to analyze the significance of the independent association of gene expression profile score with future moderate/severe rejection. The independent variables considered were age, race, gender, biopsy grade, panel-reactive antibody, cytomegalovirus mismatch, cyclosporine or tacrolimus, days post-transplant, steroid dose and gene profile score. The relationship of biopsy grade and gene profile score, and their association with future rejection, was further analyzed using sub-group analysis and factorial analysis of variance (ANOVA) with gene profile score as the dependent variable and group (rejection/control) and ISHLT rejection grade as categorical independent variables.

All *p*-values are for 2-tailed, 2-sample *t*-tests and all errors are standard deviations except when indicated otherwise. All statistical calculations were performed using SAS software (SAS Institute, Inc., Cary, NC).

## RESULTS

### Patient Demographics

One hundred forty-seven of the 629 CARGO study patients satisfied the inclusion criteria for this study. The rejection case group included *all* 39 patients who were clinically stable at baseline (with a current biopsy Grade 0 or 1A) who had an episode of Grade ≥3A rejection within the following 12 weeks of sample collection. Sixty-five of the 108 eligible patients who remained clinically stable (with a current biopsy Grade

**Table 4.** Time-dependent Differential Expression of the 11 Informative Algorithm Genes Associated With Future Grade ≥3A Rejection

| | | All times post-transplant: rejection ($n = 39$)/ control ($n = 65$) | | ≤180 days post-transplant: rejection ($n = 28$)/ control ($n = 46$) | |
|---|---|---|---|---|---|
| Gene | Description | Fold change | *t*-test *p*-value | Fold change | *t*-test *p*-value |
| ILIR2 | Interleukin-1 receptor, Type II | 0.62 | 0.009 | 0.44 | 0.0003 |
| FLT3 | fms-related tyrosine kinase 3 | 0.76 | 0.11 | 0.62 | 0.02 |
| PDCD1 | Programmed cell death 1 | 1.32 | 0.03 | 1.32 | 0.06 |
| ITGAM | Integrin-$\alpha$ M | 0.93 | 0.22 | 0.81 | 0.07 |
| SEMA7A | Semaphorin 7A | 1.07 | 0.31 | 1.15 | 0.16 |
| RHOU | ras homolog gene family U | 1.07 | 0.41 | 1.07 | 0.24 |
| PF4 | Platelet factor 4 | 0.87 | 0.18 | 0.87 | 0.27 |
| ITGA4 | Integrin-$\alpha$ 4 | 1.00 | 0.47 | 1.07 | 0.31 |
| G6b | G6b protein (C6orf25) | 0.87 | 0.46 | 0.93 | 0.72 |
| MIR | Membrane-associated ring finger 8 | 0.93 | 0.82 | 1.00 | 0.85 |
| WDR40A | WD repeat domain 40A | 0.93 | 0.68 | 1.07 | 0.88 |

The mRNA levels of the 11 informative algorithm genes were measured using qRT-PCR on PBMC samples from the study patients. Analyses were performed on the entire study population of 104 patients and on the sub-group of 74 patients within 180 days post-transplant. Genes were ordered by *t*-test *p*-value in the group at ≤180 days post-transplant. Similar significance levels were obtained using the Mann–Whitney non-parametric test. Differential expression of genes is presented as fold change, calculated as $2^{(\text{mean control Ct} - \text{mean rejection Ct})}$. Genes with mRNA levels demonstrating a fold change of >1 are up-regulated (increased) in patients with Grade ≥3A rejection relative to the control group, whereas those with a fold change of <1 were down-regulated (decreased). The remaining 9 genes in the test panel are: ERCC5, GPI, LPPR2, GNPDA1, RPLP1 and 18s (normalization genes); GUSB and GUSB promoter (controls for genomic DNA); and LTP (control for qRT-PCR).

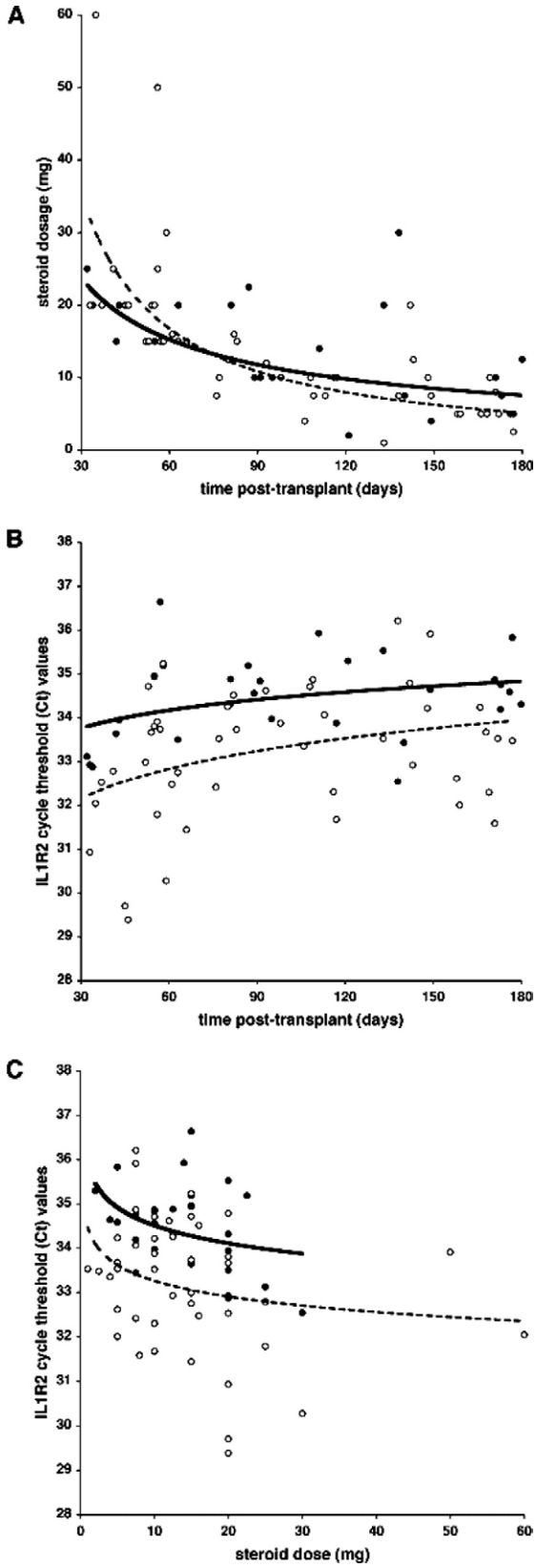


**Figure 2.** Distribution of steroid doses, time post-transplant and IL1R2 expression levels for patient samples at ≤180 days post-transplant. Filled circles represent rejection patient samples and open circles represent control group samples. Corticosteroid dosing with time post-transplant showed no differences between the rejection and control groups (A). The control group patients had, on average, 2.3-fold higher IL1R2 expression (lower Ct) relative to patients in the rejection group, regardless of time post-transplant (B) or corticosteroid dose (C). Trend lines were generated with power curve smoothing.

0 or 1A) were selected for the control group to match, as a group, the patients in the rejection case group according to their distributions of demographic characteristics, immunosuppression regimen and time post-transplant. In total, 104 patients were included in this case–control study and analyzed (Figure 1). No significant differences were observed between the rejection and control groups with regard to age, gender, immunosuppression medication regimens and mean daily steroid dose (Table 1).

Although the 39 rejection case and 65 control patients were not significantly different by race, in the sub-group of patients within 180 days post-transplant (≤180 days post-transplant), the rejection group included a higher proportion of black patients than the control group. The rejection group also had a higher percentage of baseline biopsies of Grade 1A compared with Grade 0. These imbalances between case and control patients were specifically explored to assess their potentially confounding effects. No differences were identified between the rejection and control groups with respect to the frequency of either endomyocardial biopsy procedures or clinical encounters (data not shown).

### Association With Future Moderate to Severe Rejection

To determine whether the molecular classifier that discriminates current rejection also discriminates future rejection, the gene expression scores of the baseline samples (clinically stable with biopsy Grade 0 or 1A) for the future rejection (biopsy Grade ≥3A within 80 days) and control (biopsy grades remain Grade 0 or 1A) groups were compared for the 104 patients at all times post-transplant. The mean gene expression score for the 39 patients in the rejection group was 27.4 ± 6.3, whereas that for the 65 patients in the control group was 23.9 ± 7.1 (*t*-test, $p = 0.01$; Mann–Whitney *U*-test, $p = 0.01$). The mean gene expression score for the sub-group of 28 patients in the rejection group at ≤180 days post-transplant was 28.4 ± 4.9, whereas that for the corresponding control sub-group of 46 patients was 22.4 ± 7.5 (*t*-test, $p = 0.0004$; Mann–Whitney *U*-test, $p = 0.0008$). The gene expression score for the rejection group at ≤180 days post-transplant ranged from 20.8 to 37.1 vs 4.7 to 34.0 for the control group.

Potentially confounding clinical variables (race and biopsy grade) were further explored to assess any inherent bias between the case and control groups in

**Table 5.** Time-dependent Differential Expression of 33 Non-algorithm Genes Associated With Future Grade ≥3A Rejection

| | | All times post-transplant: rejection ($n$ = 38)/ control ($n$ = 55) | | | ≤180 days post-transplant: rejection ($n$ = 27)/ control ($n$ = 40) | | |
|---|---|---|---|---|---|---|---|
| Gene | Description | Fold change | $t$-test $p$-value | FDR | Fold change | $t$-test $p$-value | FDR |
| ILIR1 | Interleukin-1 receptor, type 1 | 0.67 | 0.01 | 0.09 | 0.55 | 0.0008 | 0.012 |
| ADA | Adenosine deaminase | 1.26 | 0.002 | 0.05 | 1.35 | 0.0008 | 0.01 |
| TSC22D3 | TSC22 domain family 3 | 0.80 | 0.01 | 0.09 | 0.72 | 0.0009 | 0.007 |
| FKBP5 | FK 506–binding protein 5 | 0.85 | 0.18 | 0.38 | 0.68 | 0.007 | 0.02 |
| GZMA | Granzyme A | 1.19 | 0.15 | 0.38 | 1.40 | 0.01 | 0.08 |
| TRBC1 | T-cell receptor $\beta$ constant 1 | 1.27 | 0.08 | 0.23 | 1.50 | 0.02 | 0.07 |
| NFKB1 | Nuclear factor of kappa light chain gene enhancer in B-cells 1 | 1.09 | 0.03 | 0.18 | 1.10 | 0.02 | 0.08 |
| THBS1 | Thrombospondin 1 | 0.73 | 0.04 | 0.19 | 0.68 | 0.03 | 0.07 |
| CD163 | CD163 antigen | 0.85 | 0.20 | 0.39 | 0.72 | 0.03 | 0.08 |
| FLT3LG | fms-related tyrosine kinase 3 ligand | 1.16 | 0.12 | 0.30 | 1.31 | 0.03 | 0.08 |
| TNF | Tumor necrosis factor | 1.21 | 0.06 | 0.20 | 1.32 | 0.03 | 0.08 |
| ABCB1 | ATP-binding cassette sub-family B1 | 1.10 | 0.41 | 0.61 | 1.28 | 0.07 | 0.20 |
| CD28 | CD28 antigen, co-stimulatory molecule | 1.21 | 0.12 | 0.30 | 1.33 | 0.08 | 0.18 |
| ANXA1 | Annexin A1 | 0.89 | 0.10 | 0.29 | 0.86 | 0.10 | 0.25 |
| CD8A | CD8 antigen $\alpha$ polypeptide | 1.15 | 0.37 | 0.61 | 1.32 | 0.10 | 0.26 |
| IL1B1 | Interleukin-1$\beta$ | 1.29 | 0.19 | 0.39 | 1.45 | 0.11 | 0.28 |
| PDCD1LG2 | Programmed cell death 1 ligand 2 | 1.20 | 0.06 | 0.20 | 1.21 | 0.12 | 0.26 |
| EPOR | Erythropoietin receptor | 0.90 | 0.06 | 0.20 | 0.91 | 0.17 | 0.32 |
| CTLA4 | Cytotoxic T-lymphocyte–associated protein 4 | 1.19 | 0.17 | 0.38 | 1.23 | 0.18 | 0.34 |
| CD274 | Programmed cell death 1 ligand 1 | 1.08 | 0.38 | 0.61 | 1.15 | 0.20 | 0.37 |
| DUSP1 | Dual-specificity phosphatase 1 | 0.88 | 0.39 | 0.61 | 0.79 | 0.21 | 0.37 |
| SGK | Serum glucocorticoid-regulated kinase | 1.08 | 0.50 | 0.69 | 1.16 | 0.27 | 0.44 |
| TGFB1 | Transforming growth factor-$\beta$1 | 0.94 | 0.19 | 0.38 | 0.94 | 0.30 | 0.44 |
| IL7R | Interleukin-7 receptor | 1.08 | 0.54 | 0.70 | 1.19 | 0.30 | 0.46 |
| CD4 | CD4 antigen | 1.01 | 0.87 | 0.89 | 1.08 | 0.35 | 0.49 |
| NFKBIA | Nuclear factor of kappa light chain gene enhancer in B cells inhibitor $\alpha$ | 0.92 | 0.41 | 0.62 | 0.90 | 0.43 | 0.63 |
| NR3C1 | Nuclear receptor sub-family 3 C1 | 1.01 | 0.76 | 0.83 | 1.02 | 0.52 | 0.66 |
| IL4R | Interleukin-4 receptor | 0.98 | 0.75 | 0.83 | 0.97 | 0.56 | 0.66 |
| SELP | Selectin P | 0.88 | 0.36 | 0.57 | 0.93 | 0.62 | 0.71 |
| IL1RN | Interleukin-1 receptor antagonist | 0.97 | 0.73 | 0.83 | 0.97 | 0.78 | 0.86 |
| THBS2 | Thrombospondin 2 | 0.97 | 0.74 | 0.83 | 1.03 | 0.79 | 0.86 |
| ITGAX | Integrin-$\alpha$ X | 1.02 | 0.80 | 0.86 | 0.98 | 0.86 | 0.89 |
| TNFRSF18 | Tumor necrosis factor receptor 18 | 0.94 | 0.61 | 0.75 | 1.02 | 0.89 | 0.94 |

Transcriptional profiling of the mRNA levels of 33 non-algorithm genes was performed on PBMC samples from the study patients. Analyses were performed on the entire study population of samples for which significant RNA was available, from 93 of 104 patients at all times post-transplant and 67 of 74 patients within 180 days post-transplant. Differential expression of genes is presented as fold change, calculated as $2^{(\text{mean control Ct} - \text{mean rejection Ct})}$. Genes with mRNA levels demonstrating a fold change of >1 were up-regulated (increased) in patients with Grade ≥3A rejection relative to the control group, whereas those with a fold change of <1 were down-regulated (decreased). Genes were ordered by $t$-test $p$-value results in the group ≤180 days post-transplant, with the false discovery rate (FDR) shown. Similar data were obtained using the Mann–Whitney non-parametric test. Genes associated with steroid sensitivity are indicated in the shaded portions; ABCB1, EPOR, SELP and ITGAX demonstrated coordinated expression with known steroid-sensitive genes (data not shown).

the study. Multivariate logistic regression showed that both biopsy grade (Wald test, $p = 0.001$) and gene expression score (Wald test, $p = 0.016$) were each associated with future Grade ≥3A rejection for all patients in the study (Table 2). In the sub-group at ≤180 days post-transplant, similar results were obtained with biopsy grade (Wald test, $p = 0.025$) and gene expression score (Wald test, $p = 0.0006$). In neither set was race a significant factor, although in the sub-group at ≤180 days post-transplant, race approached significance. The independent association of classifier score with future rejection was confirmed by sub-group analysis (Table 3). The gene expression score for patients with baseline biopsy Grade 0 only was significantly greater for the rejection group than the control group ($p = 0.03$) in the period of ≤180 days post-transplant. For patients with baseline biopsy Grade 1A, the gene expression score was significantly greater in the rejection group compared with the control group for all time-points

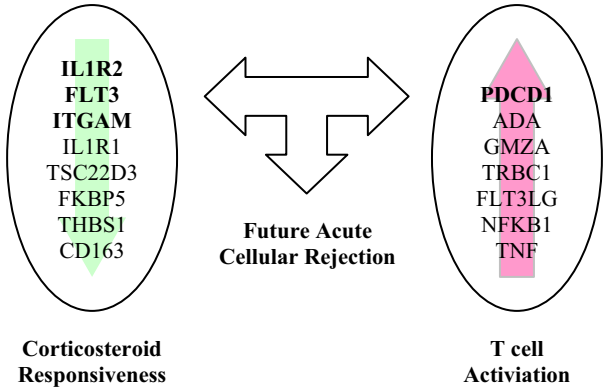


**Figure 3.** The potential relationship of decreased steroid responsiveness and increased T-cell activation as predictors of acute cellular rejection.

($p = 0.04$) and for ≤180 days post-transplant ($p = 0.008$).

To further assess the relationship between biopsy grade and gene expression score, a factorial ANOVA was performed. Study group (case or control) was found to be independently related to gene expression test score in this analysis ($F$ statistic, $p = 0.0006$); however, the ISHLT grade (0 or 1A) was not ($F$ statistic, $p = 0.89$), indicating that gene expression score is dependent on the group to which the patient belongs but is not significantly affected by ISHLT grade of the biopsy sample. So, although both gene expression score and ISHLT grade are associated with future events, gene expression score is also significant when accounting for biopsy grade.

### Individual Genes Associated With Future Rejection Events

In Table 4, the relative expression of each of the classifier genes is shown for samples in the rejection group compared with those in the control group. Expression of IL1R2, interleukin-1 decoy receptor (Type II), was lower in both the entire cohort ($p = 0.009$) and during the period of 180 days post-transplant ($p = 0.0003$). Expression levels of FLT3, fms-related tyrosine kinase 3 ($p = 0.02$), and ITGAM, integrin-α M ($p = 0.07$), the two other corticosteroid-sensitive genes, were also lower within 180 days post-transplant. By contrast, expression of PDCD1, programmed cell death 1, was higher in both the entire cohort ($p = 0.03$) and during the period of 180 days post-transplant ($p = 0.06$). Therefore, one gene (IL1R2) demonstrated significant differential expression in both time periods analyzed, whereas expression of three genes (FLT3, ITGAM and PDCD1) showed borderline significant changes in expression.

As with gene expression score, the potentially confounding clinical variables (race and biopsy grade) were further explored with respect to IL1R2 gene expression level to assess any inherent bias between the case and control groups of the study. Multivariate logistic regression showed that biopsy grade (Wald test, $p = 0.002$) and IL1R2 expression (Wald test, $p = 0.023$) were each associated with future Grade ≥3A rejection for all patients in the study. In the sub-group at ≤180 days post-transplant, only IL1R2 gene expression (Wald test, $p = 0.001$) was significant.

An analysis of corticosteroid dosing showed no differences between the rejection and control groups ($p = 0.75$), with patients in both groups managed by routine weaning protocols (Figure 2A). Irrespective of the time post-transplant or corticosteroid dose, IL1R2 gene expression showed a 2.3-fold higher level, on average, in the control group relative to patients in the rejection group (Figure 2B and C). Although steroid doses were not corrected for body weight, as this information was not systematically collected, clinicians rarely wean corticosteroids on a weight-based schedule.

To further explore the molecular pathways associated with steroid sensitivity and T-cell activation, the expression levels of 33 additional genes were measured. These included additional genes whose expression is affected by glucocorticoids, genes expressed predominantly in T cells and components of the IL-1 and PDCD1 pathways. Eleven of the 33 genes analyzed demonstrated statistically significant differential patterns of gene expression within 180 days post-transplant (Table 5). Five genes, IL1R1, TSC22D3, FKBP5, THBS1 and CD163, showed significantly reduced expression ($t$-test, $p < 0.05$, and false discovery rate <0.10) in association with future Grade ≥3A rejection. Six genes, ADA, GZMA, TRBC1, NFKB1, TNF and FLT3LG, showed significantly increased expression in the rejection case group. Five of these genes (IL1R2, ADA, TSC22D3, NFKB1 and THBS1) also demonstrated differential expression at all time-points post-transplant.

## DISCUSSION

The results of this investigation suggest that gene expression measurements of peripheral blood of heart transplant recipients conducted during the absence of histologic ACR can discriminate either continued quiescence or transition to moderate to severe rejection, weeks to months later. Only IL1R2 was significantly decreased with future rejection and three other genes (FLT3, ITGAM and PDCD1) showed borderline significant changes between the two groups. Gene expression measurements of 11 additional genes in pathways associated with PDCD1, IL1R2 and FLT3 supported the role of T-cell activation and corticosteroid-sensitive signaling pathways in future histologic rejection.

Three genes comprising the corticosteroid-sensitive gene set (IL1R2, FLT3 and ITGAM) were lower in PBMC from patients who had an episode of Grade ≥3A

rejection within 12 weeks. This is significant in the early post-transplant period as corticosteroids are the principle immunosuppressive agent subject to active dose reduction within the first 6 months after cardiac transplantation.[1] Decreased relative expression of five additional genes, IL1R1, TSC22D3, FKBP5, THBS1 and CD163, was observed in the rejection case group, each of which is known to be induced by corticosteroids.[12–15] This indicates a role for lower functional responsiveness to corticosteroids in the increased risk of cardiac rejection.

PDCD1, a key cell surface receptor regulating costimulatory pathways in T-cell activation,[16–19] was expressed at a significantly increased level in association with future rejection. Six additional genes were expressed at a significantly higher level in the rejection case group, including ADA, GZMA, TRBC1, FLT3LG, NFKB1 and TNF. These genes share the property of being transcriptionally induced by T-cell activation[20–24] or by T-cell–activating cytokines.[25] It has been shown recently that the product of the PDCD1 gene, PD-1, is found on circulating antigen-specific cells only during the course of an active immune response.[26] This suggests that increased risk of rejection may be associated with an elevated level of primed T cells in the circulation in conjunction with inadequate immunosuppression. The potential relationship of decreased steroid responsiveness and increased T-cell activation as a predictor of ACR is summarized in Figure 3.

The data from this study must be interpreted with care and in the context of the case–control study in which they were derived. The pitfalls of case–control studies include inherent spectrum bias, preventing generalization. The use of the Grade ≥3A rejection threshold in this analysis emphasized a treatment threshold outcome, but did not address milder rejection grades, such as 1B or 2. It is likely that the use of genome-wide technologies would have identified additional relevant genes and molecular pathways. The potential contribution of antibody-mediated rejection to transcriptional profiles was also not investigated.

In summary, these data show that transcriptional signals of genes regulated by corticosteroids or involved in T-cell activation in peripheral blood of heart transplant recipients are associated with the presence or absence of future clinically relevant rejection. Further confirmation of these findings may pave the way for their use in predicting ACR and in optimizing the balance of immunosuppression in heart transplantation.

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