

# Gene Expression Profiling Distinguishes a Molecular Signature for Grade 1B Mild Acute Cellular Rejection in Cardiac Allograft Recipients

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- Background:** Gene expression profiling distinguishes the absence or presence of moderate to severe grades of acute cellular rejection in cardiac allograft recipients using a 20-gene classifier. We explored the hypothesis that the rejection classifier also differentiates various forms of mild rejection and we performed sub-analyses based on time post-transplant and confirmatory pathology interpretations.
- Methods:** A post hoc analysis of 265 CARGO study patients and 714 clinical encounters focused on the correlation of rejection classifier-derived gene expression (GE) scores for blood samples accompanying endomyocardial biopsies. Biopsy grades assigned by a study center pathologist (center) were re-interpreted by three pathologists (panel) in a blinded manner.
- Results:** Mean GE scores not only differentiated Grades  $\geq 3A$  from Grade 0 ( $p < 0.00001$ , center or panel), but also from Grades 1A or 2 ( $p < 0.05$ , center or panel), based on mild rejection sub-groups defined by the ISHLT 1990 grading system. In contrast, mean GE scores for Grades 1B and  $\geq 3A$  were indistinguishable, using either center or panel interpretation. Sub-group analyses of encounters from 2 to 6 months or  $>6$  months post-transplant showed similar results for the classifier's ability to discriminate moderate to severe rejection from Grades 1A and 2 mild rejection, but indistinguishable mean GE scores for Grades  $\geq 3A$  and the Grade 1B sub-group. Of the classifier's 11 informative genes, expression of MIR and WDR40 showed statistically significant increases for both Grade 1B and Grade  $\geq 3A$  rejection, while expression of PDCD1 or SEMA7A showed similar directional patterns without achieving statistical significance.
- Conclusions:** These data demonstrate that GE scores discriminate moderate to severe rejection from Grades 1A and 2 mild rejection. However, a sub-group of mild rejection cases, defined as Grade 1B according to the 1990 grading system, share a molecular signature more consistent with moderate to severe rejection. The clinical relevance of these data remains to be defined. *J Heart Lung Transplant* 2007; 26:1270–80. Copyright © 2007 by the International Society for Heart and Lung Transplantation.

The classification of mild grades of acute cellular rejection from the International Society for Heart and Lung Transplantation (ISHLT) 1990 Working Formulation for the Standardization of Nomenclature in the Diagnosis of Heart Rejection engendered diverse efforts to establish the rele-

vance of Grades of 1A, 1B and 2 to the clinical outcomes of cardiac allograft recipients. The histologic criteria used to grade rejection severity included the distribution of inflammatory cell infiltration (e.g., focal, multifocal or diffuse) and any evidence of myocyte damage in endomyocardial biopsies.<sup>1</sup> Utilization of these criteria yielded diagnostic challenges in differentiating mild grades from one another and also from more severe grades of rejection (e.g., Grade 3A and 3B), with additional difficulties presented by nodular endocardial infiltrates (Quilty lesions).<sup>2–6</sup> Clinical investigations of the mild-grade classifications characterized their temporal occurrence, requirement for short-term therapy, and rates of progression to more severe rejection grades in both single-center and multicenter studies.<sup>6–17</sup> Three multidisciplinary reviews of the 1990 grading system, which occurred in 1994–5, 2001 and 2004, evaluated input from members of the ISHLT and the accrued clinical and scientific data to re-assess the definitions of rejection and antibody-mediated (or humoral) rejection.<sup>5,18,19</sup> These examinations

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resulted in an ISHLT-approved revision to the 1990 grading system that includes a single mild grade of rejection, defined as 1R, which subsumes the original Grades of 1A, 1B and 2, using similar histologic criteria.<sup>5</sup>

Some of the features shared by Grades 1B and 2 also overlap with those of more severe rejection grades, and encouraged studies to define the clinical utility of these mild rejection grades. Grades 1A and 1B differed in their respective focality and diffuseness of the architectural distribution of the cellular infiltrate, but they shared a requirement for an absence of myocyte damage. By contrast, the 1990 system defined Grade 2 as focal cellular infiltration, with associated myocyte damage. Moderate rejection required more extensive cellular infiltration with evidence of myocyte damage, with Grades 3A or 3B exhibiting multifocal or diffuse distributions, respectively.<sup>1</sup> Molecular sub-typing of endomyocardial biopsies has demonstrated evidence of myocyte apoptosis in association with Grade 1B, which is a feature of myocyte damage typical of Grade 3A, but not of less severe rejection.<sup>20</sup> Such data suggest that Grade 1B may share molecular similarities with Grades  $\geq 3A$ , and that adjunctive molecular approaches, such as transcriptional profiling,<sup>21</sup> may provide novel insights into tissue injury processes that may complement the light-microscopic criteria used to differentiate mild grades of rejection.

Gene expression profiling of peripheral blood mononuclear cells (PBMCs) provides molecular insights into host responses to cardiac allografts,<sup>22,23</sup> permitting clinical discrimination between the absence and presence of moderate to severe acute cellular rejection.<sup>24</sup> A 20-gene classifier, comprised of 11 informative and 9 control genes, yields a gene expression (GE) score on a scale of 0 to 40 that enables clinicians to rule out rejection with a value below a specified threshold.<sup>24</sup> To explore the hypothesis that the multi-gene rejection classifier also distinguishes different forms of mild rejection, we performed a post hoc gene expression analysis of blood samples associated with biopsies assigned Grades 1A, 1B and 2 (1990 system) in the CARGO study.<sup>6,24</sup> In this work we showed that the mean GE scores for Grade 1B are indistinguishable from those associated with Grades  $\geq 3A$ , and defined the genes and pathways shared by these rejection grades. The data also demonstrate that gene expression profiling differentiates Grades  $\geq 3A$  and 1B from Grades 1A and 2, thus providing novel molecular insights into the spectrum of rejection severity.

## METHODS

### Study Design

We performed a post hoc analysis of the relationship between gene expression scores, calculated using a 20-gene classifier that discriminates between the absence and presence of moderate to severe acute cellular rejection,

and histopathologic grades of tissue specimens obtained by endomyocardial biopsies. This analysis was performed using clinical information, endomyocardial biopsy specimens and peripheral blood samples obtained during the CARGO study from September 2001 to June 2003 in protocols approved by the institutional review boards of the participating study centers. Analysis of patient data, determination and derivation of gene expression profiles, grading of the biopsy specimens using the 1990 system adopted by the ISHLT, and re-classification of tissues by a three-member pathology panel were performed as previously described.<sup>24</sup> To avoid introducing any bias, no additional pathology interpretations were performed during the post hoc analysis. We analyzed pathologic grades of endomyocardial tissues and GE scores (scale of 0 to 40) derived from the rejection classifier for all time-points after transplantation and for two distinct periods, including 2 to 6 months and beyond 6 months in the post-operative period to differentiate early and late rejection and dynamic steroid dosing.

### Study Population

We analyzed data from 265 adult and pediatric patients from 8 participating centers in the CARGO study, which enrolled a total of 737 patients. All study subjects had previously provided written informed consent. Inclusion criteria for the study included: the availability of clinical data; an endomyocardial biopsy with a concurrent blood sample collected in a CPT tube (Becton Dickinson Co., Franklin Lakes, NJ); blood sampling beyond 55 days after transplantation; a blood sample obtained at a clinical encounter  $>21$  days since treatment for rejection; and availability of a gene expression score that passed appropriate quality analyses in the XDx Reference Laboratory. Exclusion criteria included: lack of sufficient blood or collection in the appropriate CPT tube; missing medication data; and lack of clinical encounter data. Blood samples obtained earlier than 55 days post-transplantation were excluded due to confounding issues arising from clinical instability.

### Study End-points

The primary study end-points were the center and panel-derived ISHLT grades for each biopsy specimen and the GE scores derived from transcriptional profiling of the CPT blood sample. The secondary study end-point included the time elapsed after transplantation (time post-transplant), which was further differentiated into two periods of 2 to 6 months and beyond 6 months after transplantation surgery, to differentiate early and late rejection and dynamic steroid dosing.

### Panel Pathology Interpretation

To refine the stringency of pathologic grading of endomyocardial biopsies, histologic sections used for assign-

ment of ISHLT grades based on the 1990 system at the participating CARGO study center (center) were requested and assigned a unique identifier number before being re-interpreted by a three-member CARGO study panel of pathologists (M.B., G.B., C.M.) in a blinded fashion (panel). Confirmation of each of the grades required satisfaction of the following criteria: Grade 0, assignment of Grade 0 by two or more panel members, with no dissenting Grade >1A; Grade 1A, assignment of Grade 1A by two or more panel members, with no dissenting Grade  $\geq$ 3A; Grade 1B, assignment of Grade 1B by at least one panel member, with no dissenting Grade  $\geq$ 3A or Grade 0; Grade 2, assignment of Grade 2 by at least one panel member, with no dissenting Grade  $\geq$ 3A or Grade 0; and Grades  $\geq$ 3A, assignment of Grades  $\geq$ 3A by at least one panel member, with no dissenting Grade 0. A more conservative stance was used to confirm Grades  $\geq$ 3A to avoid missing any episodes of moderate to severe rejection.

### Determination of Gene Expression Scores

Gene expression profiling was performed as previously described.<sup>24</sup> Briefly, total RNA was isolated from PBMCs, using the RNeasy kit (Qiagen, Inc., Valencia, CA), isolated from 8 ml of venous blood using density gradient centrifugation (CPT tubes). For each of the 20 genes in the rejection classifier, triplicate 10- $\mu$ l quantitative real-time polymerase chain reactions (qRT-PCRs) were performed using FAM-TAMRA probes and standard Taq-Man protocols on cDNA reverse transcribed from 5 ng of total RNA, and then analyzed (ABI 7900HT; Applied Biosystems, Foster City, CA). Gene expression levels assessed as cycle threshold ( $C_T$ ) values were used to calculate the GE score. Individual  $C_T$  values were reported for the 11 informative genes in the algorithm, including ITGA4, PDCD1, MIR, WDR40, G6b, PF4, FLT3, IL1R2, ITGAM, RHOU and SEMA7A (see Table 1).

Differential gene expression was determined by calculating the difference in cycle threshold ( $C_T$ ) values ( $C_T$  difference) for individual genes and converting to fold-difference using the following relationship: fold difference =  $2^{(C_T \text{ difference})}$ . A lower  $C_T$  value represents a higher level of gene expression. Therefore, a decrease in  $C_T$  value indicates an increase in gene expression, which is expressed as a positive fold-difference, whereas an increase in  $C_T$  value indicates a decrease in gene expression, which is expressed as a negative fold-difference.

### Assignment of Revised Grades for Endomyocardial Biopsies

The 2004 revision of the 1990 grading system adopted by the ISHLT subsumes Grade 1B into revised Grade 1R.<sup>1,5</sup> For the purposes of sub-analysis, the 1990 grades were re-classified using the revised criteria (in paren-

**Table 1.** List of Informative Genes in AlloMap Molecular Expression Testing for Calculation of Gene Expression Scores

Gene	Description	Cell/function/metagene
FLT3	Fms-related tyrosine kinase 3	Corticosteroid
G6b	G6b protein (C6orf25)	Platelet
IL1R2	Interleukin-1 receptor, Type II	Corticosteroid
ITGA4	Integrin alpha 4	T cell
ITGAM	Integrin alpha M	Corticosteroid
MIR	Membrane-associated ring finger 8	Hematopoiesis
PDCD1	Programmed cell death 1	T cell
PF4	Platelet factor 4	Platelets
RHOU	Ras homolog gene family U	Morphology
SEMA7A	Semaphorin 7A	Macrophage
WDR40A	WD repeat domain 40A	Hematopoiesis

Genes are listed alphabetically by symbol as designated by the National Center for Biotechnology Information, with accompanying description and annotated relevance by cell, function or CARGO study metagene.<sup>23</sup>

theses) as follows: Grade 0 (Grade 0R); Grades 1A, 1B and 2 (Grade 1R); Grade 3A (Grade 2R); and Grades 3B and 4 (Grade 3R). Grades 2R and 3R were analyzed collectively and designated as Grades  $\geq$ 2R for purposes of the study.

### Statistical Analyses

Differential gene expression was assessed with fold-difference. Because a unit  $C_T$  value represents a doubling of DNA material in a polymerase chain reaction, fold-difference is calculated from the difference in  $C_T$  values between two sample sets for individual genes ( $DC_T$ ) as follows:

$$FD = \begin{cases} 2^{-\Delta C_T} & \text{if } \Delta C_T < 0 \text{ (positive for increased expression)} \\ -2^{\Delta C_T} & \text{if } \Delta C_T > 0 \text{ (negative for decreased expression)} \end{cases}$$

Statistically significant ( $p < 0.05$ ) differences in the distribution of GE scores between two sample sets were determined by 2-tailed, 2-sample  $t$ -test using STATISTICA, version 7.1 (StatSoft, Inc., Tulsa, OK). All indicated errors on bar graphs are standard deviations. Significant fold-differences were determined using the same statistical test calculated for the distributions of gene expression levels in two sample sets.

## RESULTS

### Clinical Characteristics of Study Cohort

The sub-group of 265 CARGO study patients who met the inclusion criteria for this analysis demonstrated overall similarity to the entire group of 737 patients enrolled and also to the patient population assessed by the United Network for Organ Sharing (UNOS) (Table 2). Fewer patients <18 years old ( $n = 10$ ) satisfied the inclusion criteria, whereas the presence of patients >50 years of age exceeded that of the populations of both the CARGO study and the UNOS

**Table 2.** Comparison of Study Cohort to CARGO Study and UNOS Databases

	Study cohort		CARGO		UNOS	
	N	Percent	N	Percent	N	Percent
<b>Patient demographics</b>						
Total patients	265		737		2,127	
<b>Age</b>						
<18 y	10	3.8%	159	21.6%	313	14.7%
18–34 y	29	10.9%	66	9.0%	240	11.3%
35–49 y	40	15.1%	132	17.9%	406	19.1%
50–64 y	136	51.3%	316	42.9%	958	45.0%
≥65 y	50	18.9%	63	8.5%	209	9.8%
Unknown	0	0.0%	1	0.1%	1	0.0%
<b>Race</b>						
White	188	70.9%	534	72.5%	1,489	70.0%
Black	44	16.6%	113	15.3%	371	17.4%
Hispanic	19	7.2%	49	6.6%	169	7.9%
Asian	5	1.9%	17	2.3%	65	3.1%
Other	9	3.4%	24	3.3%	33	1.6%
<b>Gender</b>						
Male	200	75.5%	530	71.9%	1,541	72.4%
Female	65	24.5%	207	28.1%	586	27.6%
<b>Treatment characteristics</b>						
Total encounters	714		5,713			
<b>Time post-transplant</b>						
Average	277		241			
≤2 mo	0	0.0%	903	15.8%		
>2 mo to ≤6 mo	357	50.0%	2,502	43.8%		
>6 mo to ≤1 y	246	34.5%	1,320	23.1%		
>1 y	111	15.5%	988	17.3%		
<b>Local 1990 ISHLT grade</b>						
Grade 0	343	48.0%	2,851	49.9%		
Grade 1A	235	32.9%	2,137	37.4%		
Grade 1B	34	4.8%	217	3.8%		
Grade 2	64	9.0%	303	5.3%		
Grade ≥3A	38	5.3%	205	3.6%		
<b>Steroid dose</b>						
<10 mg/d	353	54.3%	2,197	38.5%		
≥10 to <20 mg/d	239	36.8%	1,684	29.5%		
≥20 to <40 mg/d	53	8.2%	1,410	24.7%		
≥40 mg/day	5	0.8%	422	7.4%		
<b>Immunosuppression</b>						
Corticosteroids	642	89.9%	4,696	82.2%		
Cyclosporine	360	50.4%	2,879	50.4%		
Tacrolimus	343	48.0%	2,091	36.6%		
Mycophenolate mofetil	584	81.8%	4,113	72.0%		
Sirolimus	97	13.6%	560	9.8%		

UNOS study data from 2005 used for comparative analysis upon CARGO study completion.

database. No clinical encounters that occurred at <2 months after transplantation were included in the analysis, although they comprised 15.8% of the CARGO study encounters. In addition, patients receiving steroid doses of <10 mg/day or >20 mg/day were over- or under-represented, respectively, in the study population. An analysis of immunosuppressive regimens by class revealed an over-representation of

patients receiving mycophenolate compared with the entire CARGO study group.

#### Gene Expression Scores Demonstrate Genomic Similarity of Grade 1B to Grades ≥3A

The mean GE scores for all endomyocardial biopsies performed on cardiac allograft recipients >2 months after transplantation were determined for individual

acute cellular rejection grades. Grades assigned by each participating center pathologist (center) were re-interpreted by the three-member CARGO study pathology panel (panel)<sup>24</sup> to yield the following distribution of biopsy grades: 0,  $n = 176$ ; 1A,  $n = 17$ ; 1B,  $n = 12$ ; 2,  $n = 21$ ; and  $\geq 3A$ ,  $n = 24$ . The mean GE scores differentiated moderate to severe rejection (Grades  $\geq 3A$ ) ( $32 \pm 0.9$ ) from Grades 0 ( $25.3 \pm 0.5$ ), 1A ( $23.8 \pm 2.1$ ) and 2 ( $26.9 \pm 1.5$ ) ( $t$ -test:  $p < 0.00001$ ,  $p < 0.001$  and  $p < 0.01$ , respectively) as interpreted by the panel pathologists (Figure 1); similar results were obtained based on interpretations by the center pathologist (data not shown). By contrast, the mean GE score for Grade 1B was indistinguishable from that for Grades  $\geq 3A$ , as interpreted by either the panel ( $29.8 \pm 2.0$  vs  $32.0 \pm 0.9$ ;  $t$ -test:  $p = 0.25$ ) (Figure 1) or center ( $29.7 \pm 1.1$  vs  $30.7 \pm 0.9$ ;  $t$ -test:  $p = 0.49$ ) (data not shown) pathologist(s).

#### Differential Expression of Individual Rejection Algorithm Genes

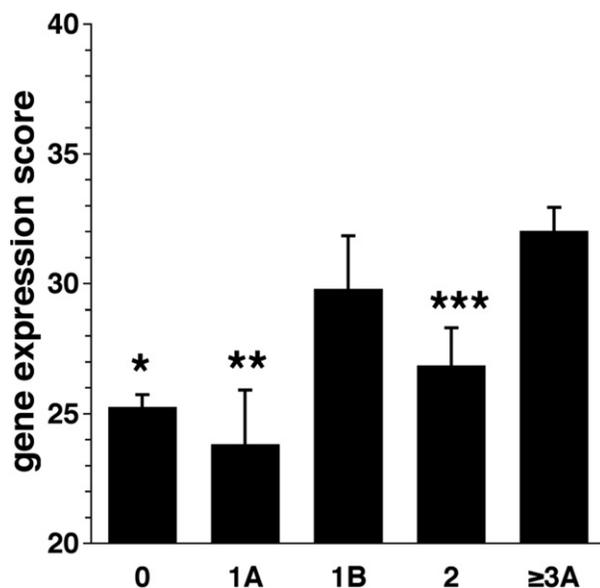
To determine which of the classifier's 11 informative genes contributed to the molecular similarity between Grade 1B and Grades  $\geq 3A$ , we calculated the fold-

difference in expression for each gene based on the measured change in cycle threshold (Ct) values between Grade 0 and Grades 1A, 1B, 2 and  $\geq 3A$ , respectively, for each gene. The fold-difference for PDCD1, MIR, WDR40 and SEMA7A increased for blood samples associated with biopsies assigned Grades  $\geq 3A$ , as interpreted by panel pathologists (Figure 2). Similar results were obtained based on grades assigned by the individual center pathologists (data not shown). The expression profile for Grade 1B showed similar increased expression of MIR and WDR40; the expression of PDCD1 or SEMA7A demonstrated the same directional change as that observed for Grades  $\geq 3A$ , but did not achieve statistical significance. Differences in directional changes of expression occurred for PF4 and IL1R2 for Grade 1B relative to Grades  $\geq 3A$ . By contrast, the patterns of gene expression for Grades 1B and  $\geq 3A$  differed from those observed from Grades 1A and 2. These data distinguish Grade 1B as a sub-group of rejection with a peripheral gene expression profile more closely resembling that for moderate to severe rejection than for other grades of mild rejection.

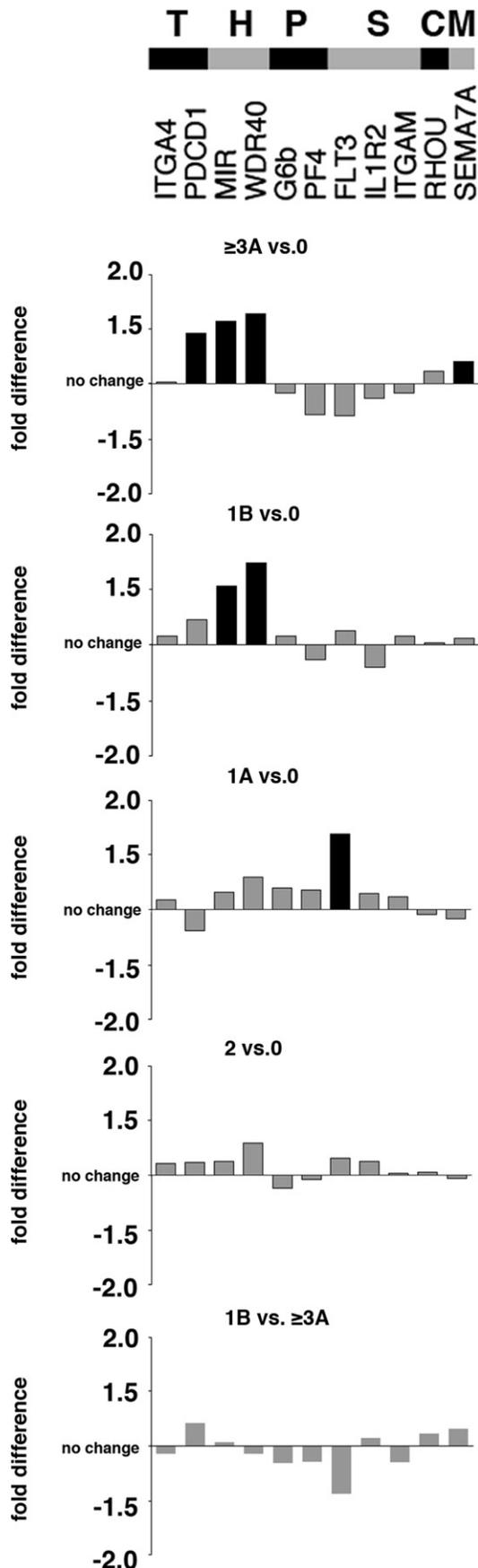
#### Gene Expression Scores Discriminate Rejection in a Manner Independent of Time Post-transplant

To determine whether time post-transplant influences the relationship between GE scores and rejection grades, we performed a sub-analysis using the periods of 2 to 6 months and beyond 6 months post-transplant. Based on the biopsy grades re-assigned by the study pathology panel (0,  $n = 176$ ; 1A,  $n = 17$ ; 1B,  $n = 12$ ; 2,  $n = 21$ ; and  $\geq 3A$ ,  $n = 24$ ) for the period of 2 to 6 months, the classifier yielded a mean GE score for Grades  $\geq 3A$  of  $30.8 \pm 1.4$ , which was also not significantly different from that for Grade 1B of  $28.5 \pm 3.9$  ( $t$ -test:  $p = 0.49$ ; Figure 3A). Similar results were obtained based on interpretations by individual center pathologists for Grades  $\geq 3A$  ( $30.0 \pm 1.2$ ) and Grade 1B ( $27.2 \pm 1.7$ ) ( $t$ -test:  $p = 0.21$ ). The mean GE scores for Grades  $\geq 3A$  not only differentiated Grade 0, but also Grades 1A and 2 (Figure 3A). For endomyocardial biopsies obtained beyond 6 months (0,  $n = 88$ ; 1A,  $n = 77$ ; 1B,  $n = 6$ ; 2,  $n = 13$ ; and  $\geq 3A$ ,  $n = 11$ ), those assigned grades of moderate to severe rejection ( $\geq 3A$ ) showed mean GE scores of  $33.5 \pm 1.0$ , which were similar to those for Grade 1B of  $31.1 \pm 1.5$  ( $t$ -test:  $p = 0.19$ ; Figure 3B). The results for Grades  $\geq 3A$  ( $31.3 \pm 1.2$ ) and Grade 1B ( $33.0 \pm 0.9$ ) based on individual center pathologist interpretation were also similar ( $t$ -test:  $p = 0.30$ ; data not shown). In addition, the mean GE scores for Grades 0, 1A and 2 were significantly lower than for Grades  $\geq 3A$ , as interpreted by the pathology panel (Figure 3B).

Further analyses of the individual expression levels of the classifier's informative genes revealed time-depen-



**Figure 1.** Gene expression scores demonstrate molecular similarity of Grade 1B and moderate to severe acute cellular rejection (Grades  $\geq 3A$ ). Mean GE scores were determined for rejection grades after re-interpretation of available endomyocardial biopsy slides by the CARGO study pathology panel. Rejection grades were re-classified based on adherence to the 1990 grading system and the satisfaction of criteria as specified in Methods. Panel interpretation decreased and re-distributed the rejection grades assigned by the individual center pathologists (0,  $n = 343$ ; 1A,  $n = 235$ ; 1B,  $n = 34$ ; 2,  $n = 64$ ;  $\geq 3A$ ,  $n = 38$ ) resulting in the following distribution: 0,  $n = 176$ ; 1A,  $n = 17$ ; 1B,  $n = 12$ ; 2,  $n = 21$ ;  $\geq 3A$ ,  $n = 24$ . Mean GE scores differentiated Grades  $\geq 3A$  from Grades 0, 1A and 2 ( $t$ -test:  $*p < 0.00001$ ;  $**p < 0.001$ ; and  $***p < 0.01$ , respectively).

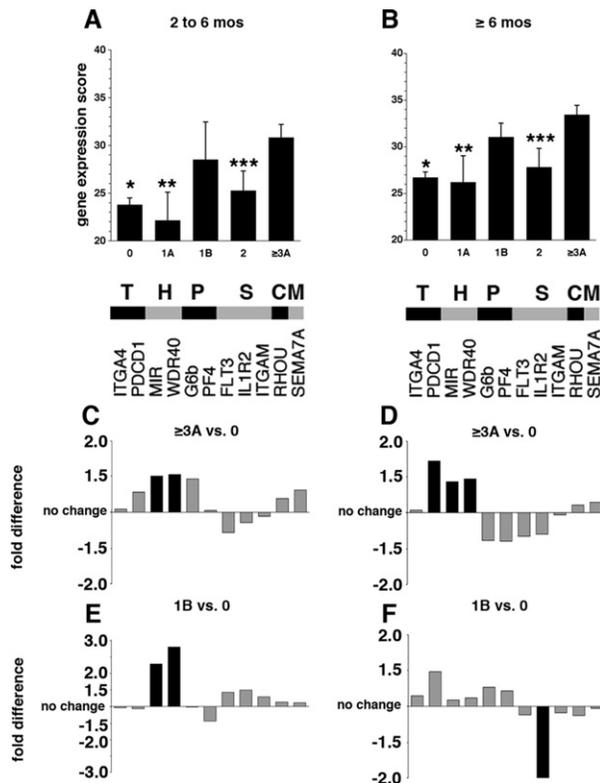


dent contributions to the transcriptional profiles of Grade 1B and Grades  $\geq 3A$ . During the period of 2 to 6 months, both MIR and WDR40 showed a positive fold-difference for Grades  $\geq 3A$  and Grade 1B (Figure 3C and E). For Grades  $\geq 3A$ , PDCD1 and SEMA7A showed small increases in fold-difference that were not statistically significant, whereas for Grade 1B the molecular profile remained similar to that shown in Figure 2, with additional changes that were not significant. For biopsies obtained after 6 months post-transplant, the gene expression profile for Grades  $\geq 3A$  showed increased expression of PDCD1, MIR and WDR40, with an overall pattern similar to that shown in Figure 2 for all periods beyond 2 months post-transplant. The most significant change in gene expression for Grade 1B after 6 months was a decrease in IL1R2 (Figure 3F). Expression of PDCD1 increased for both Grades  $\geq 3A$  and Grade 1B beyond 6 months; whereas analyses based on the individual center pathologist interpretations demonstrated this increase to be statistically significant (data not shown), the re-interpretation of Grades  $\geq 3A$  by the study panel pathologists resulted in the additional significance of MIR and WDR40, but resulted in a loss of significance for the differential expression of PDCD1 for Grade 1B.

#### Gene Expression Scores Correlate With Re-classified Biopsy Grades

The re-interpretation of grades assigned to endomyocardial biopsy specimens by a panel of pathologists in an investigative setting may result either in their confirmation or their re-classification to rejection grades of lesser or greater severity.<sup>6,13,14</sup> To define the relationship between the gene expression profiles of biopsies with confirmed grades to those with re-assigned grades, we examined their mean GE scores and the individual expression levels of the classifier's informative genes. The mean GE score for 176 Grade 0 biopsies ( $25.3 \pm 0.5$ ) confirmed by panel re-analysis differed from that ( $28.3 \pm 1.1$ ) for 25 biopsies re-assigned to more ad-

**Figure 2.** Differential expression of individual algorithm genes. The expression of each gene is shown as the fold-difference between Grade 0 and Grades 1A, 1B, 2 and  $\geq 3A$  (panel interpretation) or Grades 1B and  $\geq 3A$ , based on absolute cycle threshold ( $C_T$ ) value differences (see Methods) for individual genes in diverse pathways: T-cell activation (T); hematopoiesis (H); platelet activation (P); steroid-susceptibility (S); cell morphology (C); and macrophage activation (M). A fold-difference of  $>1.0$  indicates an increase in relative gene expression, whereas a value of  $<1.0$  indicates a decrease. Values with  $t$ -tests associated with  $p < 0.05$  are indicated in black, whereas those not achieving statistical significance are indicated in gray. Comparison of the individual expression profiles for Grades 1B and  $\geq 3A$  revealed no statistically significant differences.



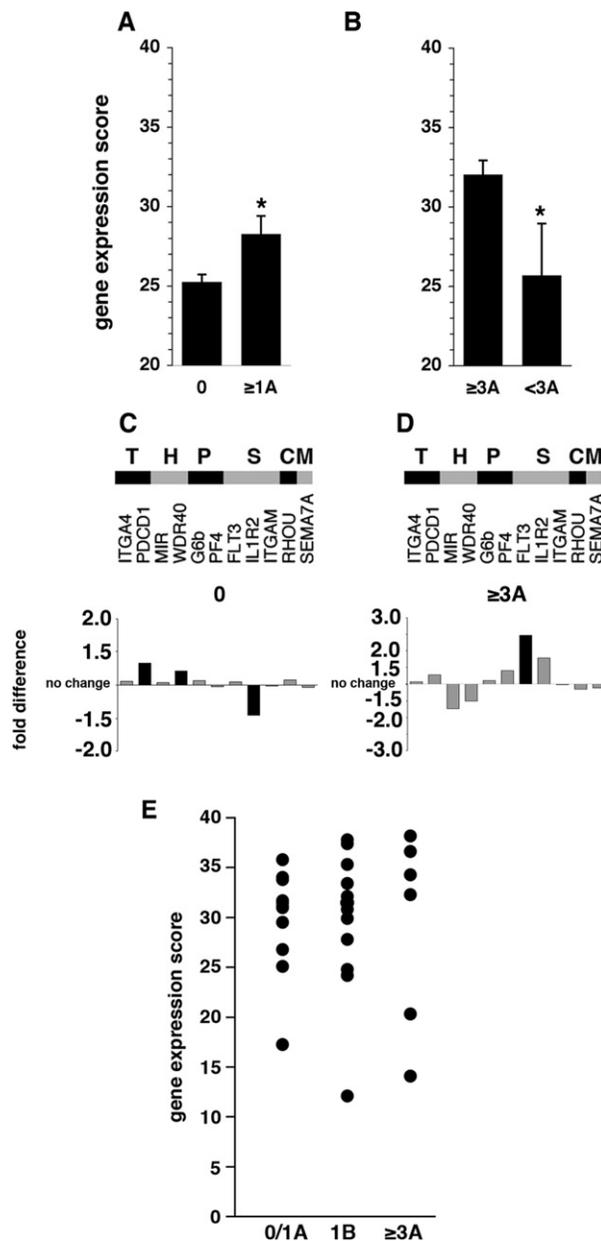
**Figure 3.** Gene expression scores differentiate mild from moderate to severe grades of acute cellular rejection in a manner dependent on time post-transplant. Sub-group analyses using mean GE scores were performed on rejection grades for endomyocardial biopsies performed on patients within 2 to 6 months (A) or beyond 6 months after transplantation (B). (A) Mean GE scores were determined for all rejection grades (0,  $n = 174$ ; 1A,  $n = 126$ ; 1B,  $n = 19$ ; 2,  $n = 22$ ;  $\geq 3A$ ,  $n = 16$ ) as interpreted by the center pathologist (center) or by the study pathology panel (panel) (0,  $n = 176$ ; 1A,  $n = 17$ ; 1B,  $n = 12$ ; 2,  $n = 21$ ;  $\geq 3A$ ,  $n = 24$ ). Mean GE scores for panel rejection grades discriminated Grades  $\geq 3A$  from Grades 0, 1A and 2 ( $*p < 0.000001$ ;  $**p < 0.001$ ;  $***p < 0.001$ , respectively). Mean GE scores were similar for Grade 1B ( $28.5 \pm 3.9$ ) and Grades  $\geq 3A$  ( $30.8 \pm 1.4$ ;  $t$ -test:  $p = 0.49$ ) based on interpretations by the study panel, as well as for Grade 1B ( $27.2 \pm 1.7$ ) and Grades  $\geq 3A$  ( $30.0 \pm 1.2$ ), based on assignments by the center pathologists ( $t$ -test:  $p = 0.21$ ; data not shown). (B) The mean GE score for Grade 1B ( $31.1 \pm 1.5$ ) was similar to that for Grades  $\geq 3A$  ( $33.5 \pm 1.0$ ;  $p = 0.19$ ) based on panel pathology interpretation, whereas those using individual center interpretations were similar (Grades  $\geq 3A$  [ $31.3 \pm 1.2$ ] and Grade 1B [ $33.0 \pm 0.9$ ];  $t$ -test:  $p = 0.30$ ) (data not shown). The use of panel re-interpretations of rejection Grades (0,  $n = 88$ ; 1A,  $n = 77$ ; 1B,  $n = 6$ ; 2,  $n = 13$ ;  $\geq 3A$ ,  $n = 11$ ) demonstrated differentiation of Grades  $\geq 3A$  from Grades 0, 1A and 2 ( $p < 0.0001$ ,  $p = 0.01$ ,  $p = 0.027$ , respectively). By contrast, mean GE scores for center rejection grades (0,  $n = 169$ ; 1A,  $n = 109$ ; 1B,  $n = 15$ ; 2,  $n = 42$ ;  $\geq 3A$ ,  $n = 22$ ) determined for biopsies obtained after 6 months revealed discrimination of Grades  $\geq 3A$  from Grade 0 ( $p < 0.01$ ), but not from Grades 1A, 1B or 2 ( $p = 0.17$ ,  $p = 0.30$ ,  $p = 0.07$ , respectively). (C–F) Differential expression of classifier genes expressed as fold-difference. Values with  $t$ -tests associated with  $p < 0.05$  are indicated in black, whereas those not achieving statistical significance are indicated in gray.

vanced rejection grades ( $\geq 1A$ ) ( $p = 0.023$ ; Figure 3A). A similar analysis performed for biopsies assigned Grades  $\geq 3A$  showed a mean GE score of  $32.0 \pm 0.9$  for 24 biopsies with confirmed grades, whereas that for the 5 biopsies re-assigned to less advanced rejection grades ( $< 3A$ ) was  $25.7 \pm 3.3$  ( $p = 0.014$ ; Figure 3B). Biopsies assigned Grade 1B showed a range of GE scores, and no differences in mean GE scores were observed among biopsies with either confirmed grades (1B) or those re-assigned to Grades 0/1A or Grades  $\geq 3A$ . However, the scatterplot of GE scores (Figure 4E) for Grade 1B biopsies re-assigned to Grades  $\geq 3A$  by the study panel revealed four scores (32, 34, 37 and 38) in the range associated with Grade  $\geq 3A$  rejection, as shown in Figures 1 and 3. Re-interpretation of biopsy specimens originally classified as mild rejection (Grades 1A or 2) also yielded no significant differences in mean GE scores between the confirmed and re-classified biopsy specimens (data not shown).

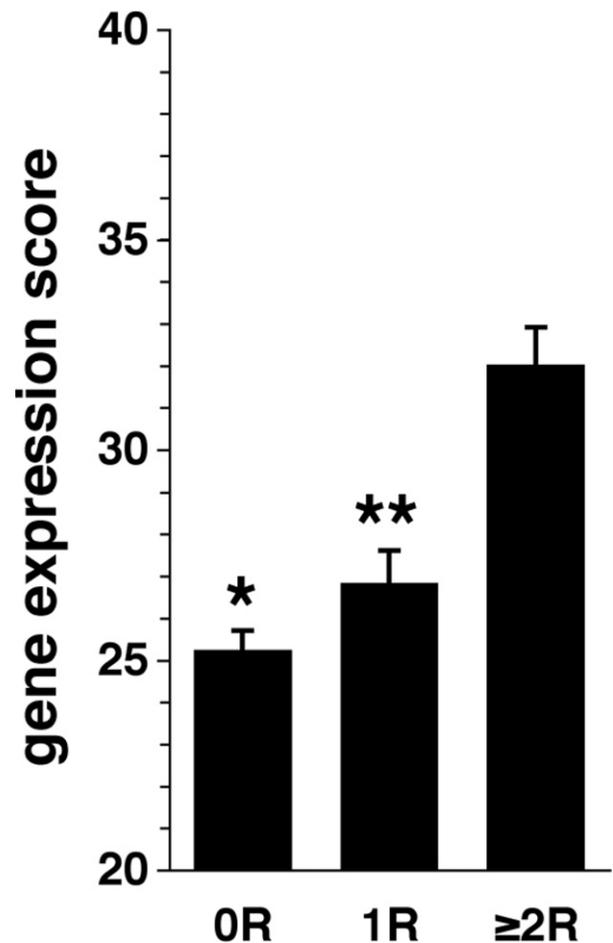
Analysis of the classifier's informative genes in blood samples associated with either confirmed or re-assigned rejection grades demonstrated differential patterns of expression. Blood samples associated with Grade 0 biopsies re-assigned to more severe rejection grades showed increased expression of PDCD1 and WDR40, whereas that for IL1R2 decreased (Figure 3C). For biopsies originally graded as moderate to severe rejection ( $\geq 3A$ ), but then re-assigned less severe grades by the study panel, expression of FLT3 increased, whereas the expression levels of a number of genes decreased but did not achieve statistical significance. These data demonstrate a correlation between the mean GE scores yielded by the classifier with rejection grades either confirmed or re-assigned by the pathology panel.

### Gene Expression Scores Discriminate the Revised Rejection Grades

The revised 2004 grading system subsumes the Grade 1B classification into Grade 1R, due to the lack of differentiating outcomes data for Grades 1A, 1B or 2.<sup>1,5</sup> Irrespective of the use of either panel or center interpretations of endomyocardial biopsy specimens, gene expression scores for Grade 1R were intermediate between those for Grades 0R and  $\geq 2R$  for all biopsy samples beyond 2 months post-transplantation (Figure 5; data for center interpretations not shown). Mean GE scores for Grades  $\geq 2R$  ( $32.0 \pm 0.9$ ) were greater than those for either Grades 0R ( $25.3 \pm 0.5$ ,  $t$ -test:  $p < 0.0000007$ ) or 1R ( $26.9 \pm 0.8$ ;  $t$ -test:  $p = 0.00157$ ) by panel interpretation (0R,  $n = 178$ ; 1R,  $n = 95$ ;  $\geq 2R$ ,  $n = 24$ ;  $t$ -test:  $p < 0.002$ ). Similar results (0R,  $25.9 \pm 0.30$ ; 1R,  $27.7 \pm 0.4$ ;  $\geq 2R$ ,  $30.7 \pm 0.9$ ;  $t$ -test:  $p < 0.002$ ) were obtained using individual center pathologist interpretations (data not shown). Grade 1R demonstrated a mean GE score that was indistinguishable from that of



**Figure 4.** Gene expression scores substantiate biopsy grade re-classification by the pathology panel. Re-interpretation of rejection grades for endomyocardial biopsies obtained from patients beyond 2 months after transplantation by the panel resulted in revised assignments for grades originally reported by the center pathologist. (A) The mean GE score for 176 Grade 0 biopsies ( $25.3 \pm 0.5$ ) confirmed by panel re-analysis differed from that ( $28.3 \pm 1.1$ ) for 25 biopsies re-assigned to more advanced rejection Grades ( $\geq 1A$ ) ( $p = 0.023$ ). (B) The mean GE score for 24 biopsies with confirmation of Grades  $\geq 3A$  by the panel ( $32.0 \pm 0.9$ ) differed from 5 biopsies, which were re-assigned to less advanced rejection grades ( $< 3A$ ) ( $25.7 \pm 3.3$ ;  $p = 0.014$ ). Mean GE scores ( $\pm$ SE) were not statistically different for re-classified rejection scores upon panel re-interpretation of Grades 1A, 1B and 2 (data not shown). (C, D) Fold-differences for individual algorithm genes demonstrated differential gene expression in PBMCs associated with confirmed and unconfirmed Grade 0 and Grades  $\geq 3A$ . Values with  $t$ -tests associated with  $p < 0.05$  are indicated in black,



**Figure 5.** Gene expression scores discriminate Grade 1R from Grades 0 or  $\geq 2R$ . Mean gene expression (GE) scores were determined for blood samples associated with endomyocardial biopsies performed on allograft recipients beyond 2 months following transplantation (e.g., entire study cohort). Mean GE scores based on study panel interpretations (OR,  $n = 178$ ; 1R,  $n = 95$ ;  $\geq 2R$ ,  $n = 24$ ) were  $25.3 \pm 0.5$ ,  $26.9 \pm 0.8$  and  $32.0 \pm 0.9$  for Grades OR, 1R and 2R, respectively ( $t$ -test:  $*p < 0.0000007$  and  $**p = 0.00157$  for OR and 1R with respect to  $\geq 2R$ ). Similar results (OR,  $25.9 \pm 0.30$ ; 1R,  $27.7 \pm 0.4$ , and  $\geq 2R$ ,  $30.7 \pm 0.9$ ;  $t$ -test:  $p < 0.002$ ) were obtained using individual center pathologist interpretations (OR,  $n = 343$ ; 1R,  $n = 333$ ;  $\geq 2R$ ,  $n = 38$ ) (data not shown). Grade 1R demonstrated a mean GE score that was indistinguishable from that of Grade OR ( $t$ -test:  $p = 0.0624$ ) based upon interpretations by the study panel.

Grade OR ( $t$ -test:  $p = 0.0624$ ) based on interpretations by the study panel. Similarly, sub-analyses using the two time periods of 2 to 6 months and beyond 6 months after transplantation also showed discrimination of

while those not achieving statistical significance are indicated in gray [Note change in y-axis scale for panel (D)]. (E) Scatterplot of Grade 1B biopsies re-interpreted by the study panel pathology. Of 31 biopsies, 13 were confirmed as Grade 1B (1B), while the remained were re-assigned as Grade 0/1A ( $n = 12$ ) or Grades  $\geq 3A$  ( $n = 6$ ).

Grade  $\geq 2R$  rejection from Grades 0R and 1R by GE scores (data not shown).

## DISCUSSION

Unique transcriptional signatures of circulating white blood cells provide molecular information that can serve as a clinical adjunct in the management of acute cellular rejection in cardiac allograft recipients. The development of the multigene classifier for cardiac allograft rejection optimized genomic profiling of PBMCs associated with pathologically confirmed biopsies correlating either with the absence of any endomyocardial injury or the rejection treatment threshold. Molecular expression testing using this classifier distinguishes between the absence (Grade 0/0R) or presence of moderate to severe rejection (Grades  $\geq 3A/2R$ ), with a statistical significance ( $p < 0.0001$ ) and high negative predictive value that confer diagnostic utility.<sup>24</sup> In this work we have shown that the classifier yields mean GE scores for Grades 1A and 2 that differ from those for Grades  $\geq 3A$ , demonstrating its ability to differentiate moderate to severe rejection from two classes of mild rejection, in addition to Grade 0. We have also provided novel data showing evidence of molecular similarity between Grade 1B and Grades  $\geq 3A$ , suggesting their potential overlap along a molecular spectrum of rejection severity. In a manner similar for Grades  $\geq 3A$ , the mean GE score for Grade 1B also suggests its molecular distinction from other Grades (1A and 2) classified as mild rejection.

Both the pathologic interpretive stringency and the time post-transplant influence the assignment and GE scores of cardiac allograft rejection grades. Re-interpretation of endomyocardial biopsy grades by a panel of pathologists in a study setting may result in the re-assignment of rejection grades, yielding a re-distribution that includes confirmed grades, and those with less or more severe rejection grades.<sup>6,24</sup> In this study, panel re-interpretation resulted in a broader distribution of mean GE scores for all rejection grades, but continued to highlight the similarity of the mean GE scores for Grade 1B with those for Grades  $\geq 3A$ . Biopsies with an original assignment of Grade 0 that changed to Grades  $\geq 1A$  by the panel demonstrated higher mean GE scores, whereas those with center-assigned Grades of  $\geq 3A$ , which were re-interpreted as less severe rejection, showed lower mean GE scores compared with those biopsies with confirmed grades. These data suggest that the classifier provides objective GE scores that correlate with panel re-interpretations of biopsy rejection grades. Further sub-analyses based on early and late periods post-transplantation, defined as 2 to 6 months or beyond 6 months, revealed a time-dependent increase in the mean GE scores, not only for Grades  $\geq 3A$  but also

for those associated with Grade 1B, which were of similar magnitude.

Host immune responses to cardiac allografts include perturbations in diverse pathways regulating cellular homeostasis and trafficking that can be assessed by profiling dynamic changes in the intracellular expression of genes in PBMCs.<sup>22-24</sup> Four of the rejection classifier's 11 informative genes, including PDCD1, SEMA7A, MIR and WDR40, demonstrated patterns of expression that were similar for Grade 1B and Grades  $\geq 3A$ , but varied in a manner dependent on time post-transplant. Expression of two of these genes, PDCD1 and WDR40, also increased in Grade 0 biopsies re-assigned to higher grades, substantiating their association with more extensive endomyocardial injury.

Expression of PDCD1 (programmed cell death 1, PD1) encodes a 50- to 55-kd glycosylated surface receptor, which is a CD28 homolog, on circulating T cells by activation through the T-cell receptor.<sup>25,26</sup> Semaphorin 7A (SEMA7A) is a GPI-linked member of the semaphorin family with putative immune functions due to its expression by human T lymphocytes, natural killer cells and monocytes, and additional data identifying it as a negative regulator of murine T-cell activation and function.<sup>27</sup> MIR, the gene for an E3 ubiquitin ligase originally designated as a cellular modulator of immune recognition (c-MIR),<sup>28,29</sup> and WDR40, a WD repeat domain protein awaiting functional definition,<sup>30</sup> have been associated with hematopoiesis, which may be influenced by systemic activation as part of alloimmune responses in cardiac allograft recipients.<sup>31</sup> The observed patterns of differential and directional gene expression for these four classifier genes suggest that they contribute to the genomic similarity between Grade 1B and Grades  $\geq 3A$  via mechanisms that remain to be clarified using both molecular and clinical approaches. The similar directional changes observed for these differentially expressed genes is important because the rejection classifier includes a positive or negative coefficient for each gene<sup>24</sup> that reflects its net change in expression relative to the absence of rejection.

The similarity of GE scores and the associated gene pathways shared by Grade 1B and Grades  $\geq 3A$  provides new molecular insights that may influence the clinical interpretation and management of rejection in cardiac allograft recipients. The 1990 grading system used a classification that defined Grade 1B and Grades  $\geq 3A$  as sharing a requirement for either diffuse or multifocal mononuclear cell infiltration. Both grades share a requirement for a distribution of inflammation beyond localized involvement, but they differ in the requirement for evidence of myocyte damage, which was absent in Grade 1B and present in Grades  $\geq 3A$ . However, in a study by Laguens et al, in situ labeling of

nuclear DNA breaks using the TUNEL assay in endomyocardial biopsy tissues demonstrated apoptotic nuclei in 3 of 8 Grade 1B specimens and 8 of 8 biopsies assigned Grades  $\geq 3A$ , whereas none were detected in 33 biopsy specimens assigned Grade 0 or 1A.<sup>20</sup> These data suggest that a sub-group of biopsy specimens assigned Grade 1B may already have evidence of alloimmune-mediated myocyte apoptosis (or programmed cell death) and damage not detected by light microscopic techniques. Histologic evidence of myocyte damage may be difficult to define<sup>19</sup> and requires cell death or myocytolysis in the absence of a contraction band or coagulation necrosis.<sup>5</sup> Such data showing myocyte apoptosis in Grade 1B biopsies at the DNA level adds to the evidence of molecular similarity between Grade 1B and Grades  $\geq 3A$  provided by GE scores, thereby blurring the histologic distinction between these two forms of diffuse rejection.

Our data suggest that Grade 1B biopsies, or a sub-group thereof, may not only be molecularly similar to Grades  $\geq 3A$  biopsies, but also molecularly distinct from Grade 1A and 2 biopsies with which they comprise the group classified as mild (1R) rejection. Despite its sharing of the histologic characteristic of diffuseness with Grades  $\geq 3A$ , Grade 1B is defined as mild rather than moderate to severe. The reliance on light-microscopic evidence for myocyte injury suggests that some Grade 1B assignments may represent false-negative assessments of potential moderate to severe rejection that might be precluded by the introduction of molecular adjunctive techniques for tissue analysis. The use of GE scores may also provide such supplementary information in the setting of high clinical suspicion for moderate to severe rejection with a Grade 1B biopsy result, if the score substantiates the pre-test impression by suggesting an increased risk of heightened alloimmune response.

A biopsy result of Grade 1B occurred in 3.4% of 3,968 biopsies from 562 patients in the CARGO study, similar to the 3.78% graded as 3A and 3B, but less prevalent than Grades 1A (39.4%) or 2 (6.2%).<sup>6</sup> Therefore, Grade 1B is as clinically frequent as moderate to severe rejection. The grouping of Grade 1B together with the more frequent Grades 1A/2 into Grade 1R and the GE score data for the 1R mild rejection grade in Figure 5 suggest the potential loss of the molecularly distinct Grade 1B rejection with the adoption of the revised grading system. Although Grade 2 shares the requirement for myocyte damage with Grades  $\geq 3A$ , its lack of molecular similarity may be due to its focality and the confounding presence of Quilty lesions.<sup>6,9</sup> Further clarification of Grade 1B rejection at the molecular level may guide future clinical practice by refining both the definitions for acute cellular rejection and the threshold for rejection therapy.

The use of a post hoc analysis of the CARGO study clinical database, sample archive and gene expression data introduced potential limitations to the interpretation of these data. To avoid introducing any bias into the interpretative stringency of endomyocardial biopsies due to differences in intra- or inter-reader variability of the panel pathologists since the conclusion of the original analysis, no additional biopsy interpretations were performed for the current work. However, this decreased the number of biopsy samples with both center and panel pathology interpretations available, yielding smaller numbers of panel-confirmed grades for the sub-analyses. Although fewer in number, the confirmed biopsies provided conservatively defined and clinically relevant rejection grades necessary for defining gene expression profiles. Overall, the panel interpretation enhanced the diagnostic certainty of the assigned rejection grade, and the number of Grade 1B and Grades  $\geq 3A$  samples analyzed in this study compares favorably with other investigations. The time dependency of the relationship between mean GE scores and rejection grades suggest that intrinsic or extrinsic factors that vary by time post-transplant may alter the peripheral molecular signatures of PBMCs in heart transplant recipients.

In conclusion, mild grades of rejection defined by ISHLT grading systems for cardiac allograft pathology demonstrate gene expression scores intermediate between those for the absence and presence of moderate to severe rejection. Mean gene expression scores for Grade 1B of the 1990 grading system are more similar to those for Grades  $\geq 3A$  than to those for Grades 0, 1A or 2, suggesting a genomic similarity that substantiates prior evidence that a sub-group of Grade 1B rejections may share characteristics typical of more severe grades of rejection. Defining the clinical outcomes of mild grades of rejection and the classifier-derived GE score may refine its clinical utility as a prognosticator for moderate to severe acute cellular rejection in the management of heart transplant patients.

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