Gene expression profiling from endomyocardial biopsy tissue allows distinction between subentities of dilated cardiomyopathy

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Supplemental material is available online.

Objective: Expression profile analysis using endomyocardial biopsy specimens from patients with cardiomyopathies promises to improve the differential diagnosis of heart failure.

Methods: In this study, left ventricular endomyocardial biopsy specimens were obtained from 50 patients and histopathologically classified according to the World Heart Federation Task Force criteria as having dilated cardiomyopathy (n = 17), inflammatory cardiomyopathy (n = 11), myocarditis (n = 15), or pericarditis (n = 7). Microarrays were performed by hybridization of synthesized complementary DNA against a Lab-Arraytor60-combi microarray (SIRS-Lab, Jena, Switzerland). Differentially expressed genes were clustered hierarchically according to their variation in hybridization signals.

Results: In samples from patients with dilated cardiomyopathy, two different types of gene expression profiles were distinguishable. One pattern was unique for dilated cardiomyopathy and inflammatory cardiomyopathy, respectively, and the other more closely resembled that seen in samples from inflammatory heart disease. Additionally, we confirmed the microarray data by showing that dilated cardiomyopathy is associated with a reduced myocardial toll-like receptor 9 expression that resulted from progressive loss of functional cardiomyocytes. Taken together, our data demonstrate the utility and validity of microarrays from endomyocardial biopsy specimens in detecting subentities of dilated cardiomyopathy that do not differ histopathologically, but transcriptionally, from each other. The gene expression profile observed in one subgroup of patients with dilated cardiomyopathy is indicative of ongoing immune activation, albeit infiltrating immunocompetent cells were not detected histopathologically.

Conclusion: Thus, our transcriptional data indicate that dilated cardiomyopathy constitutes a heterogeneous disease with an broad overlap to inflammatory heart disease.

Heart transplantation is the sole surgical option for selected patients with severe cardiomyopathy who do not respond to drug therapy. Accurate clinical assessment is obligatory to decide which patients will benefit from heart transplantation. Several studies have been carried out to determine unfavorable prognoses and to provide an early indication for cardiac transplantation. Despite recent advances in our pathophysiologic knowledge of end-stage heart disease, the differential diagnosis of nonischemic cardiomyopathies still remains a challenge for the clinician, particularly in early stages of the disease.^{1,2}

Dilated cardiomyopathy (DCM), a leading cause of heart failure and heart transplantation, is characterized by dilatation and impaired contraction of the left or both ventricles; it may be idiopathic, familial/genetic, viral, and/or immune. Various pathogenic human viruses have been identified as causative agents for myocardial damage, in particular parvovirus B19, enteroviruses (especially Coxsackie B virus), adenoviruses, and herpesviruses including cytomegalovirus.³⁻⁶ Replication of viral genomes resulting in

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Abbreviations and Acronyms

cDNA	= complementary DNA
DCM	= dilated cardiomyopathy
DCMi	= dilated cardiomyopathy with inflammation
LV	= left ventricular
mRNA	= messenger RNA
RT-PCR	= reverse-transcriptase polymerase chain reaction
TLR9	= toll-like receptor 9

a transient phase of myocytolysis is usually controlled by an appropriate immune reaction directed at viral clearance. However, a complete elimination of the replicating virus is not achieved in all cases, whereas in others the viral genome persists without productive replication, resulting in a period of disease inactivity in which the patient often improves or completely recovers. Most patients experience exclusively this transient phase of viral infection, as the virus is being cleared with little or no sequelae. Patients usually seek medical care at this stage because of congestive heart failure and the occurrence of ventricular arrhythmias. However, even in these patients the harmful immune activation usually dissipates. Tissue remodeling continues in only a minority of affected individuals, resulting in the ongoing loss of contractile myocardium.² In the latter case, the disease progresses into DCM and clinical signs of chronic heart failure develop owing to impaired systolic and diastolic function of the left ventricle. According to this postulated model of disease progression, the acute inflammation with infiltrating immunocompetent cells finally leads to a remodeled heart organ with predominant reparative fibrosis and, if any, only sparse and focally distributed persisting cellular infiltrates.

Given that the prognosis of patients varies substantially depending on the underlying etiology, early assessment of an accurate diagnosis for patients with new-onset heart failure is of considerable therapeutic importance. The use of cardiac biomarkers or modern noninvasive imaging techniques including magnetic resonance imaging is limited for diagnostic evaluation because of their relatively low sensitivity and specificity as compared with biopsy-proven methods.^{2,3,7} Endomyocardial biopsy has been shown to refine the diagnostic procedure, especially in patients with rapidly progressive heart failure that is refractory to conventional therapeutic strategies or if progressive conduction abnormalities or life-threatening ventricular arrhythmias occur.⁷ Despite the often patchy nature of acute myocardial inflammation, which contributes to the underdiagnosis owing to sampling error, endomyocardial biopsy is still regarded as the gold standard for the antemortem diagnosis of myocarditis.^{3,7,8} The immunohistochemical assessment of endomyocardial biopsy samples has ameliorated the early detection of inflammatory tissue reactions in the heart.^{4,6,9-11} However, the number of cardiac-

specific epitopes to be tested is restricted depending on the avidity and availability of the corresponding antibodies. Recent advances in microarray technology allow measuring the expression of thousands of genes simultaneously in a defined small tissue sample.¹²⁻¹⁴ Thus, we asked whether microchip technology is principally suitable to distinguish myocarditis from other heart diseases in a diagnostic setting. In particular, we were interested to determine whether gene expression profiling from endomyocardial biopsy tissue adds further information for the identification of patients with cardiomyopathies.

Patients and Methods**Patient Selection and Diagnosis of Heart Disease**

Patients with signs of heart failure, who had been referred to our hospital for further diagnostic investigation because of suspected inflammatory heart disease, were enrolled in our study. The patients usually had a history of recent onset of cardiac arrhythmias, undefined electrocardiographic abnormalities, and atypical chest pain suggesting nonischemic cardiomyopathy and/or myocardial inflammation. Inclusion criteria for participating in the study were a histologically proven diagnosis of one of the following four cardiac diagnoses: pericarditis, myocarditis, and dilated cardiomyopathy with or without inflammation (DCM and DCMi, respectively). For all study patients the accurate cardiologic diagnosis was obtained before the microchip assays were performed. Patients with pericarditis usually had a hemodynamically relevant pericardial effusion and/or typical electrocardiographic alterations. Myocarditis was defined as an inflammation of the heart according to the World Heart Federation criteria (≥ 14 lymphocytes and macrophages per square millimeter) independent of the clinical phenotype or the presence of heart failure or ventricular dilatation.^{15,16} DCMi was diagnosed if, in addition to histologic signs of inflammation, impaired left ventricular (LV) function was proven by echocardiography (LV end-diastolic diameter > 55 mm, LV end-diastolic volume index > 100 mL/m², or LV ejection fraction $< 50\%$).¹⁶ If no inflammation was detectable, patients with impaired LV function were classified as having DCM.

In each patient, coronary angiography was performed to exclude significant coronary artery disease with a luminal narrowing of major arteries of 50% or more. For all patients, the prevalence of the parvovirus B19 genome was tested as described before.⁴ Patients with suspected inflammatory heart disease who underwent endomyocardial biopsy for therapeutic and/or diagnostic reasons were asked to participate in our study. If they agreed, written informed consent was obtained. The investigation conforms with the principles outlined in the Declaration of Helsinki. This study protocol was approved by the local ethical review board.

Tissue Collection

From each patient undergoing cardiac catheterization, multiple LV endomyocardial specimens were obtained with a flexible biptome. Each tissue specimen was then divided into two parts: one for histopathologic examination and the other for RNA analyses. The biopsy specimens were immediately snap-frozen in liquid nitrogen and banked at -80°C for storage until further use.

Immunohistochemical Stainings

Frozen sections of the banked endomyocardial tissue samples were cut (5 μm) and stained immunohistochemically for the detection of

infiltrating cells. Monoclonal antibodies against CD3, CD4, and CD11c, all obtained from Dako Diagnostics Ltd (Copenhagen, Denmark), were used in this study. Avidin–biotin double sandwich technique (Vectastain Elite ABC Kit; Vector Laboratories, Inc, Burlingame, Calif) was used in combination with a monoclonal antibody against the endothelial antigen EN 4 (Sanbio BV, AmUden, The Netherlands) to distinguish infiltrating lymphocytes (red staining) adjacent to myocytes from those located nearby or inside intramyocardial vessels and capillaries (blue staining). Toll-like receptor 9 (TLR9) was stained with a rabbit polyclonal antibody (H-100) purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Detection of bound immunoglobulins was achieved with the ABC method employing biotinylated secondary antibodies and avidin–horseradish peroxidase complexes (Vectastain Elite ABC Kit from Vector Laboratories). Diaminobenzidine producing a brown reaction product was used as a substrate for visualization of the enzyme reaction. Finally, the sections were counterstained with Mayer's hematoxylin.

RNA Isolation, Complementary DNA Synthesis and Microarray Hybridization

Total RNA was extracted from endomyocardial biopsy specimens or autoptic heart tissue using the Qiagen RNeasy Kit and eluted in a volume of 30 μ L of sterile water as recommended by the manufacturer. RNA concentrations were determined spectrometrically at a wavelength of 260 nm. Because of minimal total RNA in the samples, complementary DNA (cDNA) synthesis was performed by the BD Super SMART cDNA synthesis technology (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. Reverse transcription was in the presence of aminoallyl-dUTP and cDNA was labeled by the use of the Alexa Fluor 647 system (Invitrogen, Carlsbad, Calif). Alexa Fluor 647–labeled cDNA was cohybridized with pooled Alexa Fluor 555–labeled cDNA obtained from 6 autoptic hearts from patients who died of noncardiac diseases and served as control samples. Synthesis of cDNA included a DNase I treatment (Promega Corporation, Madison, Wis) to prevent cellular DNA contamination in the polymerase chain reaction (PCR). Each RNA pair (sample vs reference) was hybridized against a Lab-Arraytor60-combi microarray, comprising 578 probes for 556 human genes involved in inflammatory processes and 22 positive/negative controls (SIRS-Lab, Jena, Switzerland). After incubation in a formamide-based hybridization buffer for 10 hours at 42°C, microarrays were washed and dried. Hybridization signal intensities were measured with a GenePix scanner (Axon Instruments, division of Molecular Dynamics, Sunnyvale, Calif).

Microarray Data Pre-processing

Data pre-processing included the following 4 steps: (1) spot detection and quantification, (2) spot flagging according to the defined signal-to-noise threshold value, (3) correction of systematic bias including the normalization and the variance-stabilized transformation of raw signals, and (4) averaging of printing replicates. For the first 2 steps, the GenePix Analysis Software was used. The raw expression signals for each spot were quantified as the median spot intensity in the red and green channel, respectively. The spots were flagged corresponding to the settings of the GenePix Software. For the third step the approach of Huber and colleagues¹⁷ was used and the additive and multiplicative errors were estimated. The normalized signals were transformed by the arcsinh function. Per gene, replicates

with the highest flag value were selected and the corresponding signal intensities were averaged.

Statistical Data Analysis

In the analysis, the gene expression profiles of 50 patients with sufficient hybridization quality were included. The statistical analysis was performed for 481 reliable genes, that is, genes with detectable signal intensity and sufficient variation throughout the experiment. Missing values were imputed employing the k-nearest neighbor algorithm with $k = 15$.¹⁸ A vector of p values was obtained from a multivariate permutation test based on 1-way analysis of variance statistics, where the effect of the disease type (DCM, DCMi, myocarditis, and pericarditis) was investigated for each gene. Genes with statistically significant differences were identified by tuning the q value, thus controlling the positive false discovery rate.¹⁹ The corresponding approach also allowed for estimation of the proportion of differentially expressed genes. The gene expression patterns of selected genes were ordered by hierarchical cluster algorithm with Euclidean distance and averaged linkage method.

Reverse-transcriptase PCR (RT-PCR)

Verification of the microarray results was confirmed by real-time PCR using the SYBR-Green method (Invitrogen). Gene-specific primers were designed using Primer 3 software (Applied Biosystems, Foster City, Calif) to amplify fragments of about 200 base pairs in length. The reverse-transcriptase (RT) PCR reactions were carried out in a total volume of 25 μ L, containing 25 ng messenger RNA (mRNA), 7.5 μ mol/L of each specific primer pair, and 2.5 μ L of SYBR Green. The following protocol was applied: reverse transcription at 50°C for 30 minutes followed by a denaturation step at 95°C 15 minutes, and 40 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 45 seconds. A melting curve analysis was run after final amplification via a temperature gradient from 55°C to 94°C in 0.5°C increment steps measuring fluorescence at each temperature for a period of 10 seconds. All reactions were carried out in at least duplicate for each sample. The relative expression of a transcript was calculated as the ratio between the specific transcript level and the level of *gapdh* as determined for each sample. Using the Bio-Rad iQ-iCycler system software (Bio-Rad Laboratories, Hercules, Calif), the threshold (Ct) at which the cycle numbers were measured was adjusted to areas of exponential amplification of the traces. The $\Delta\Delta$ -method was used to determine comparative relative expression levels by applying the formula $2^{-(\Delta C_t \text{ target} - \Delta C_t \text{ reference sample})}$, as described previously.²⁰

Results

Characterization of the Study Population

The total study population included 50 patients. Among them, DCM was diagnosed in 17 study participants and DCMi in 11 subjects. The diagnosis of myocarditis was confirmed in 15 patients. A fourth group consisting of 7 subjects with pericardial effusion was included in the study, in which endomyocardial biopsy had revealed no pathologic signs on histologic examination. This group of patients was therefore diagnosed to have pericarditis. With the exception of the latter group, the majority of all study participants in each group were men (DCM, 89.9%; DCMi, 90.0%; and myocarditis,

TABLE 1. Patient characteristics

	DCM	DCMi	Myocarditis	Pericarditis
Age (y)	48.8 ± 14.6	45.8 ± 9.6	41.7 ± 19.7	50.6 ± 21.7
Sex (F/M)	2/15	1/8	3/12	4/3
EF (%)	27.2 ± 10.6*	27.8 ± 10.0*	69.1 ± 14.8	65.1 ± 22.5
LVEDD (mm)	67.3 ± 6.9*	64.5 ± 7.0*	50.2 ± 5.3	45.1 ± 4.2
Parvovirus B19 (pos./neg.)	4/13	4/5	7/8	3/4

EF, Ejection fraction; LVEDD, left ventricular end-diastolic diameter; pos./neg., positive/negative. **P* < .05; difference between patient groups having myocarditis or pericarditis.

82.3%). As expected, the echocardiographically measured LV end-diastolic diameter was significantly elevated in both DCM and DCMi patients (67.3 ± 6.9 mm and 64.5 ± 7.0 mm, respectively) as compared with patients with myocarditis or pericarditis (50.2 ± 5.3 mm and 45.1 ± 4.2 mm; *P* < .05). Similarly, LV ejection fraction differed significantly between DCM and DCMi patients on the one hand (27.2% ± 10.6% and 27.8% ± 9.6%, respectively) and patients with myocarditis or pericarditis on the other (69.1% ± 14.8% and 65.1% ± 22.5%; *P* < .05). Further details on the study population are presented in Table 1.

Immunohistochemical Detection of Immunocompetent Cells in Biopsy Tissue

Immunohistochemistry was performed in biopsy samples to detect immunocompetent cells in the myocardium, thereby distinguishing between different entities of heart diseases. Figure 1 demonstrates the presence of CD3-positive T cells in mononuclear infiltrates in patients with myocarditis, whereas in patients with DCM and pericarditis staining

with anti-CD3 antibody was usually negative (Figure 1, A). In endomyocardial biopsy tissue from patients with DCMi, CD3-positive T cells only occasionally were detected scattered throughout the section. Similarly, CD4-positive cells were detected in specimens from inflammatory heart disease and infrequently in patients with DCMi but were generally absent in DCM and pericarditis (Figure 1, B and C). Dendritic cells expressing CD11c were most often detected in inflammatory infiltrates from patients with myocarditis (Figure 1, D). However, dendritic cells were also detected but less frequently in tissue specimens from the remaining patient groups.

Differential Gene Expression in Different Heart Diseases

Next, we performed microarrays to explore putative differences in the expression profile of genes involved in inflammatory reactions among the four entities of heart disease. A list of genes that showed differential expression between the patient groups was generated by ranking the genes according to the

Figure 1. Immunohistochemical detection of infiltrating immune cells in endomyocardial biopsy samples. Shown are the localization of CD3- (A) and CD4-positive T cells (B and C) in patients with myocarditis (A and B) and DCM (C), respectively, as well as CD11c-positive dendritic cells in a patient with myocarditis (D). Double staining using a monoclonal antibody against endothelial antigen EN 4 was applied to distinguish infiltrating lymphocytes (red staining) from intramyocardial vessels and capillaries (blue staining). Note the absence of a positive CD4 immunostaining in heart tissue obtained from a DCM patient (C).

relative median expression. We found differences in the gene expression between the patient groups for approximately 45% of genes tested. A set of 42 genes with a q value of less than 17 (corresponding to a p value of less than .026) was considered as being significantly changed (thus 7 false positive decisions were accepted). Data corresponding to the selected genes are summarized in Supplemental Table E1 and Figures 2 and 5.

The mean expression of the 6 genes with least q values (*cflar*, *c3*, *serca2*, *serping*, *cis*, and *galc*) was higher for the patient groups with myocarditis and pericarditis, respectively, as compared with those with DCMi or DCM. For the majority of the selected genes the expression level was elevated in the patients with myocarditis and pericarditis as compared with study participants with DCM or DCMi. The expression of some genes, for example, *fos*, *nppb*, *map3k*, *tnfrsf6*, or *ppbp*, was reduced in the group with pericarditis when compared with the remaining patient groups. To better visualize the obtained expression patterns, variations in hybridization signals were ordered by hierarchical clustering. In Figure 2, the transcription patterns are presented by a colored image and the corresponding dendrogram. It can be seen in this figure that with respect to the gene expression pattern, the patient group with DCM encompasses two distinct subgroups. Whereas the gene expression in 7 patients of the DCM group was similar to that observed in myocarditis, the gene expression in the remaining 10 patients corresponded well to that seen in patients with DCM. Interestingly, the former subgroup of patients with DCM, who more likely resembled the expression profile of myocarditis patients, had significantly higher LV shortening fractions ($18.9\% \pm 6.3\%$ vs $11.3\% \pm 3.0\%$; $P = .003$; see Figure 3).

Next, we compared the two DCM subgroups separately with the DCMi patients for their echocardiographically assessed shortening fraction (for DCMi patients; $16.0\% \pm 3.2\%$). Statistical significance was achieved only for the subgroup of DCM patients with a unique gene expression ($P = .003$), but not for those DCM patients with a gene expression pattern resembling that of myocarditis ($P > .05$). No association was seen between parvovirus B19 positivity and gene expression profiling.

Validation of Differentially Expressed Genes Using RT-PCR

The differential gene expression observed in the microarrays was verified by two independent measurement methods. First, we performed real-time RT-PCR from the same set of tissue samples and compared these results with the findings from the microarrays. On the basis of biologic considerations and statistical significance, two genes were selected for RT-PCR measurements: *serca* and *tlr9*. The sarcoplasmic reticulum Ca^{2+} -ATPase *Serca* was included in the microarray and chosen for RT-PCR validation, because it is widely known that *Serca* expression is down-regulated in the hearts of patients with DCM.²¹ The second gene included for validation

of the microarray results by quantitative RT-PCR was *tlr9*. Expression of *gapdh* was not significantly changed between the samples and therefore *gapdh* served as a control for normalization of the RT-PCR measurements.

Our RT-PCR results confirmed that *serca* expression in DCM and DCMi patients was down-regulated as compared with that in patients with pericarditis or myocarditis (Figure 4, B). The decreased *serca* expression in the former patient groups as measured by RT-PCR correlated well with the results from the microchip experiment. Similarly, expression of *tlr9* coding for TLR9 was significantly reduced in DCM patients as judged by both RT-PCR and microarrays (Figure 4, A). The expression level of *tlr9* was intermediate in DCMi patients and high in patients with acute inflammatory heart disease. Thus, our data showed that the RT-PCR results generally paralleled those seen by microarray analysis.

Expression of TLR9 in Endomyocardial Biopsy Tissue

Next, we tested whether immunohistochemical stainings confirmed the expression data obtained from the microarrays (Figure 5). Since microarray and RT-PCR techniques both revealed down-regulation of *tlr9* in DCM and DCMi patients, we asked whether the reduced TLR9 expression was also detectable at the level of protein expression. In immunohistochemical stainings using a specific anti-TLR9 antibody, we confirmed the expression patterns described above at the mRNA level. TLR9 was localized predominantly in the cytosol of cardiomyocytes but was generally absent in noncardiomyocytes. It was found that the staining intensity of TLR9 immunopositivity in biopsy specimens from patients with DCM was significantly lower as compared with the other three disease entities (Figure 5, D). There was a significantly higher expression of TLR9 in tissue samples from myocarditis and pericarditis patients (Figure 5, A and B). Also here TLR9-positive cells were exclusively cardiomyocytes as judged by their characteristic shape and the presence of a contractile apparatus, whereas lymphocytes or antigen-presenting cells comprising inflammatory infiltrates were typically negative. Biopsy samples from patients with DCMi often showed an intermediate staining pattern (Figure 5, C). Taken together, immunohistochemical detection of TLR9 used here as an independent method for assessing gene expression confirmed the differential gene expression pattern between the distinct disease conditions, as had already been revealed before by microarray and RT-PCR techniques.

Discussion

In this study, we compared the global gene expression profiles from four clinically and histopathologically defined entities of heart disease using a custom-designed DNA microarray technology in an attempt to identify potential molecular markers that facilitate the differential diagnosis of cardiomyopathies. The set of genes tested here for cardiac

Figure 2. Gene expression characteristics in patients with different clinical diagnoses. From statistical comparison, 42 genes were identified that significantly differed between the patient groups. The gene expression patterns of these genes were ordered by hierarchical cluster algorithm with Euclidian distance and averaged linkage method. Each gene is represented by a single row of colored boxes and each sample by a single column. The associated colors represent variance-normalized expression for individual genes. While the intensity of red color indicates a relative expression greater than the mean, green indicates a decreased expression level compared with the mean. (B) Distribution of the gene expression throughout the experiment. To minimize the number of false positives, we excluded such genes from the statistical analysis whose mean expression intensity were less than those of any the negative control or whose expression variation throughout the experiment was less than those of the most positive controls. (C) Statistical comparison of the gene expression patterns. *Left panel*, Histogram of *p* values obtained from the statistical test. While the line at the height of *h*₀=12 is the histogram expected portion of null *p* values if none gene was differentially expressed, the line at the height of *h*₁=6.9 is the histogram of the estimated portion of null *p* values. *Middle panel*, The *q* values versus their respective *p* values. *Right panel*, The number of genes considered as being differentially expressed versus the respective *q* values. For a *q* value closed to 0.17 a particular increase in the curve can be observed.

Figure 3. The gene expression profiles between two subgroups of DCM patients differ significantly depending on the severity of impaired LV systolic function. Echocardiographically measured shortening fractions (A) and ejection fractions (EF) (B) are presented separately for both subgroups of DCM patients with distinct gene expression patterns.

expression was selected on the basis of the involvement of the corresponding gene products in executing inflammatory reactions. The data presented demonstrate that hierarchical gene clustering in microarray analysis allows for the identification of distinct gene expression patterns that distinguish different heart diseases from each other. Despite considerable interindividual variability, our microarray results show that in the myocardium of patients with DCM and DCMi, a panel of genes involved in immune regulation is differentially expressed as compared with that in patients with myocarditis or pericarditis. Within the group of DCM patients we observed two different expression patterns for inflammatory markers, which were clearly distinguishable from each other. One gene expression profile resembled more closely that seen also in patients with myocarditis, whereas the other pattern was unique for DCM and DCMi patients. Individuals exhibiting the latter type of gene expression had a significantly reduced LV shortening fraction as compared with those from

the former subgroup, indicating that their LV systolic function was more profoundly impaired. These data suggest that DCM patients with a gene expression pattern more closely resembling that of myocarditis may be classified as belonging to an intermediate subgroup that is transcriptionally, but not necessarily histopathologically, distinguishable from subjects with a DCM-specific gene expression profile.

Additionally, our microarray results indicate that there is a substantial overlap in gene expression profiles between the subgroup of DCM patients with only slightly impaired LV systolic function and those diagnosed as DCMi. In the subgroup of DCM patients exhibiting a unique gene expression pattern, the shortening fraction was significantly more reduced as compared with that of patients with a transcription pattern resembling that of myocarditis. Thus, belonging to a transcriptionally defined DCM subgroup seems to be associated with a reduced systolic function of the left ventricle. The different myocardial gene expression profiles seen in both DCM subgroups appear to more likely reflect the severity of LV impairment than the presence of a still ongoing immune reaction, which is undetectable in the diverse immunohistochemical techniques applied here for the detection of infiltrating cells.

The expression levels of the selected genes were generally more decreased in DCM and DCMi patients as compared with those diagnosed as either myocarditis or pericarditis. This finding was not unexpected inasmuch as the genes selected for the microarrays were chosen with respect to their engagement in inflammatory processes. However, it is unclear whether the reduced activation level found here for numerous genes simply reflects the nosologic heterogeneity and clinical severity of the disease or whether it indicates a more profound alteration in transcriptional activity that accompanies the phenotypic dedifferentiation process of cardiomyocytes. Nevertheless, our microarray results allow DCM patients to be divided into two distinct subgroups that differ considerably with respect to the transcriptional activation of inflammatory genes. Thus, microarrays obtained from endomyocardial biopsy tissue add further information for the subclassification of DCM patients that are not covered by conventional methods of gene expression measurements.

Additionally, we tested the microarray technique for screening novel markers whose expression is specifically changed in DCM. Recently, this approach has been successfully applied in numerous studies.^{14,22-31} Here we focused on a gene product that has not been related to the pathogenesis of DCM so far, but whose expression was gradually reduced as determined in our microarrays. The gene coding for TLR9 was identified as such a marker that is significantly down-regulated in DCM patients. Interestingly, our RT-PCR results confirmed that the induction of the *tlr9* gene is critically impaired in DCM and DCMi patients, whereas significantly higher expression levels were measured in myocarditis and pericarditis patients. Expression of TLR9 was restricted to

Figure 4. Comparison of expression data between microarrays and RT-PCR measurements. Shown are the microarray (left) and RT-PCR results (right) for the expression of *tlr9* coding for toll-like receptor 9 (A) and *serca* (B) in patients grouped with respect to the underlying heart disease. Note that *serca* and *tlr9* mRNA are both down-regulated in DCM and DCMi as compared with patients with myocarditis or pericarditis and that these findings were independent of the particular technique used.

differentiated cardiomyocytes as observed immunohistochemically, whereas nonmyocytes usually expressed much lower, if any, immunodetectable TLR9. With increasing amounts of fibrotic components, the signal intensity in the anti-TLR9 stained tissue specimens was critically decreased, as was observed in DCM patients. Thus, three independent techniques demonstrated down-regulation of the *tlr9* gene as was first revealed in the microarray analyses and later confirmed both at the mRNA and protein level. Members of the toll-like receptor family such as TLR9 have been implicated in the recognition of exogenous and endogenous ligands and activate the nuclear factor κ B pathway.³² The high expression level of TLR9 in morphologically intact cardiomyocytes suggests a functional role of these cells in the recognition of the cognate ligand, which is unmethylated CpG DNA.

Our data suggest that implementing microarray-based information on gene expression broadens our understanding for the pathogenesis and subclassification of DCM and related diseases. RNA amplification protocols combined with gene expression profiling appear to refine the clinical diagnosis of cardiomyopathies. Novel disease-associated markers may be identified that are suitable for the screening of patients with otherwise unexplained heart disease. Advanced DNA microchip technology promises to identify target genes of potential therapeutic interest that are involved in diverse pathologic pathways such as remodeling and apoptosis. The expression of these genes may then be specifically modulated by gene-targeting approaches that are currently under development.

Taken together, our microarray analysis based on genes involved in immune regulation allows for the distinction

Figure 5. Decreased expression of toll-like receptor 9 (TLR9) in DCM and DCMi patients as shown by immunohistochemistry. Endomyocardial biopsy specimens were stained for the presence of TLR9 in patients diagnosed as myocarditis (A), pericarditis (B), DCMi (C), and DCM (D), respectively.

between different entities of DCM. In a subgroup of DCM patients with impaired LV function, we detected evidence of continuous immune activation, as judged by the elevated expression of inflammatory genes. Thus, despite the absence of infiltrating immunocompetent cells, our transcriptional data demonstrate ongoing activation of the immune system in this DCM subgroup. The robustness of the gene expression signatures shown here can be successfully applied to identify novel disease-associated markers, such as TLR9, that are dysregulated in specific cardiac entities.

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TABLE E1. For genes with $q < .17$ (in ascending) the data are presented as the means $\pm$ standard deviations of normalized and transformed expression signals

Acc.-No	Gene symbol	Mean $\pm$ SD				<i>p</i> value	<i>q</i> value
		DCM	DCMi	Myocarditis	Pericarditis		
NM_003879	CFLAR	0.33 $\pm$ 0.24	0.16 $\pm$ 0.28	0.63 $\pm$ 0.21	0.7 $\pm$ 0.42	.000	.029
NM_000064	C3	0.06 $\pm$ 0.1	0.05 $\pm$ 0.2	0.27 $\pm$ 0.16	0.24 $\pm$ 0.17	.000	.029
NM_001681.2	SERCA2	0.88 $\pm$ 0.41	0.69 $\pm$ 0.42	1.24 $\pm$ 0.22	1.37 $\pm$ 0.36	.000	.029
NM_000062	SERPING1	0.03 $\pm$ 0.18	0.12 $\pm$ 0.21	0.35 $\pm$ 0.22	0.24 $\pm$ 0.22	.001	.075
NM_001734	C1S	0.18 $\pm$ 0.17	0.17 $\pm$ 0.17	0.43 $\pm$ 0.22	0.3 $\pm$ 0.04	.002	.113
NM_000153	GALC	0.18 $\pm$ 0.21	0.02 $\pm$ 0.21	0.3 $\pm$ 0.17	0.32 $\pm$ 0.22	.004	.152
NM_016562	TLR7	0.01 $\pm$ 0.1	0.0 $\pm$ 0.09	-0.12 $\pm$ 0.09	-0.08 $\pm$ 0.14	.005	.152
NM_013261.1	PGC-1	0.12 $\pm$ 0.09	0.07 $\pm$ 0.1	0.14 $\pm$ 0.11	0.22 $\pm$ 0.09	.005	.152
NM_006573	TNFSF13B	-0.23 $\pm$ 0.12	-0.08 $\pm$ 0.09	-0.24 $\pm$ 0.16	-0.22 $\pm$ 0.09	.005	.152
NM_004633	IL1R2	0.03 $\pm$ 0.05	0.1 $\pm$ 0.13	-0.01 $\pm$ 0.06	-0.03 $\pm$ 0.11	.006	.152
NM_005252	FOS	0.08 $\pm$ 0.11	0.09 $\pm$ 0.09	0.02 $\pm$ 0.06	-0.07 $\pm$ 0.16	.006	.152
NM_000657	BCL2.2	-0.21 $\pm$ 0.09	-0.13 $\pm$ 0.07	-0.27 $\pm$ 0.09	-0.2 $\pm$ 0.07	.007	.154
NM_080738	EDARADD	0.27 $\pm$ 0.19	0.22 $\pm$ 0.19	0.46 $\pm$ 0.14	0.37 $\pm$ 0.17	.008	.154
NM_002521.1	NPPB	0.11 $\pm$ 0.6	-0.07 $\pm$ 0.62	-0.26 $\pm$ 0.57	-0.82 $\pm$ 0.65	.009	.154
NM_006301	MAP3K12	-0.07 $\pm$ 0.07	-0.01 $\pm$ 0.07	-0.03 $\pm$ 0.06	-0.18 $\pm$ 0.22	.010	.154
NM_000201.1	ICAM1	-0.08 $\pm$ 0.09	0.02 $\pm$ 0.08	-0.08 $\pm$ 0.1	0.02 $\pm$ 0.09	.010	.154
NM_000874	IFNAR2	0.59 $\pm$ 0.43	0.36 $\pm$ 0.28	0.82 $\pm$ 0.29	0.82 $\pm$ 0.33	.010	.154
NM_003805	CRADD	0.02 $\pm$ 0.12	-0.04 $\pm$ 0.12	0.11 $\pm$ 0.09	0.1 $\pm$ 0.13	.011	.154
NM_002745	MAPK1.1	0.22 $\pm$ 0.18	0.15 $\pm$ 0.13	0.32 $\pm$ 0.11	0.33 $\pm$ 0.16	.011	.154
NM_002755	MAP2K1	-0.15 $\pm$ 0.19	-0.03 $\pm$ 0.15	-0.02 $\pm$ 0.28	0.17 $\pm$ 0.41	.012	.154
NM_019846	CCL28	-0.04 $\pm$ 0.11	0.01 $\pm$ 0.08	0.07 $\pm$ 0.16	0 $\pm$ 0.06	.012	.154
NM_006538	BCL2L11	-0.07 $\pm$ 0.06	-0.1 $\pm$ 0.1	0.01 $\pm$ 0.08	-0.02 $\pm$ 0.08	.013	.154
NM_017442	TLR9	0.02 $\pm$ 0.14	0.06 $\pm$ 0.08	0.14 $\pm$ 0.09	0.16 $\pm$ 0.12	.013	.154
NM_001242	TNFRSF7	-0.13 $\pm$ 0.06	-0.06 $\pm$ 0.06	-0.07 $\pm$ 0.09	-0.13 $\pm$ 0.09	.013	.154
NM_000043	TNFRSF6	-0.04 $\pm$ 0.05	-0.01 $\pm$ 0.08	-0.06 $\pm$ 0.1	-0.18 $\pm$ 0.23	.014	.154
NM_000655.2	SELL	-0.15 $\pm$ 0.13	0 $\pm$ 0.18	-0.18 $\pm$ 0.1	-0.15 $\pm$ 0.16	.014	.154
NM_000543	SMPD1	0.13 $\pm$ 0.18	0.02 $\pm$ 0.2	0.26 $\pm$ 0.15	0.2 $\pm$ 0.21	.017	.155
NM_002720	PPP4C	-0.11 $\pm$ 0.15	0 $\pm$ 0.09	-0.06 $\pm$ 0.11	0.04 $\pm$ 0.04	.017	.155
NM_002188	IL13	0.23 $\pm$ 0.24	0.11 $\pm$ 0.17	0.37 $\pm$ 0.19	0.3 $\pm$ 0.14	.018	.155
NM_021975	RELA	-0.14 $\pm$ 0.14	-0.03 $\pm$ 0.07	-0.11 $\pm$ 0.11	-0.02 $\pm$ 0.09	.018	.155
NM_004834	MAP4K4	0.34 $\pm$ 0.29	0.22 $\pm$ 0.25	0.52 $\pm$ 0.19	0.53 $\pm$ 0.31	.018	.155
NM_001350	DAXX	-0.01 $\pm$ 0.09	-0.04 $\pm$ 0.12	0.08 $\pm$ 0.09	0.05 $\pm$ 0.14	.018	.155
NM_022740	HIPK2	-0.11 $\pm$ 0.08	-0.03 $\pm$ 0.09	-0.16 $\pm$ 0.1	-0.09 $\pm$ 0.13	.019	.155
NM_004315	ASAHI	-0.09 $\pm$ 0.1	-0.06 $\pm$ 0.13	0.03 $\pm$ 0.1	0.02 $\pm$ 0.16	.019	.156
NM_003376	VEGF	0.61 $\pm$ 0.42	0.43 $\pm$ 0.29	0.83 $\pm$ 0.31	0.85 $\pm$ 0.33	.020	.161
NM_004131	GZMB	-0.23 $\pm$ 0.11	-0.14 $\pm$ 0.06	-0.22 $\pm$ 0.1	-0.13 $\pm$ 0.1	.022	.162
NM_000256.2	MYBPC3	1.02 $\pm$ 0.43	0.92 $\pm$ 0.3	1.22 $\pm$ 0.21	1.35 $\pm$ 0.17	.022	.162
NM_001225	CASP4	-0.14 $\pm$ 0.11	-0.06 $\pm$ 0.06	-0.03 $\pm$ 0.08	-0.12 $\pm$ 0.12	.023	.162
NM_001824.2	CKM	0.79 $\pm$ 0.4	0.78 $\pm$ 0.44	1.1 $\pm$ 0.28	1.2 $\pm$ 0.39	.023	.162
NM_002704	PPBP	0.02 $\pm$ 0.15	0.17 $\pm$ 0.45	0.06 $\pm$ 0.16	-0.35 $\pm$ 0.69	.025	.170
NM_032964	CCL15.2	-0.03 $\pm$ 0.1	-0.05 $\pm$ 0.09	0.03 $\pm$ 0.07	0.05 $\pm$ 0.06	.025	.170
NM_000611	CD59	0.09 $\pm$ 0.1	0.04 $\pm$ 0.12	0.17 $\pm$ 0.12	0.17 $\pm$ 0.08	.026	.170

SD, Standard deviation; DCM, dilated cardiomyopathy; DCMi, inflammatory dilated cardiomyopathy.