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BASIC SCIENCE

Improved diagnosis of idiopathic giant cell myocarditis and cardiac sarcoidosis by myocardial gene expression profiling

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Aims Improvement of clinical diagnostics of idiopathic giant cell myocarditis (IGCM) and cardiac sarcoidosis (CS), two frequently fatal human myocardial diseases. Currently, IGCM and CS are diagnosed based on differential patterns of inflammatory cell infiltration and non-caseating granulomas in histological sections of endomyocardial biopsies (EMBs), after heart explantation or postmortem. We report on a method for improved differential diagnosis by myocardial gene expression profiling in EMBs.

Methods and results We examined gene expression profiles in EMBs from 10 patients with histopathologically proven IGCM, 10 with CS, 18 with active myocarditis (MCA), and 80 inflammation-free control subjects by quantitative RT–QPCR. We identified distinct differential profiles that allowed a clear discrimination of tissues harbouring giant cells (IGCS, CS) from those with MCA or inflammation-free controls. The expression levels of genes coding for cytokines or chemokines (CCL20, IFNB1, IL6, IL17D; $P < 0.05$), cellular receptors (ADIPOR2, CCR5, CCR6, TLR4, TLR8; $P < 0.05$), and proteins involved in the mitochondrial energy metabolism (CPT1, CYB, DHODH; $P < 0.05$) were deregulated in 2- to 300-fold, respectively. Bioinformatic analyses and correlation of the gene expression data with immunohistochemical findings provided novel information regarding the differential cellular and molecular pathomechanisms in IGCM, CS, and MCA.

Conclusion Myocardial gene expression profiling is a reliable method to predict the presence of multinuclear giant cells in the myocardium, even without a direct histological proof, in single small EMB sections, and thus to reduce the risk of sampling errors. This profiling also facilitates the discrimination between IGCM and CS, as two different clinical entities that require immediate and tailored differential therapy.

Keywords Idiopathic giant cell myocarditis • Cardiac sarcoidosis • Dendritic cells • Gene profiling • CCL20 • CCR6

Translational perspective:

Idiopathic giant cell myocarditis (IGCM) and cardiac sarcoidosis (CS), two frequently fatal human myocardial diseases, often present with similar clinical features. Current diagnostics is based on histological examination of endomyocardial biopsies (EMBs). Due to its focal appearance, a direct proof of multinuclear giant cells or sarcoid granulomas in EMB specimens is often unsuccessful in routine histology, but early and exact diagnosis is a prerequisite for an immediate and tailored therapy. The presented myocardial expression profiling facilitates the primary diagnosis and discrimination of IGCM and CS, even without a direct histological confirmation of focal multinucleated cells, and is suitable for monitoring of the treatment response.

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Introduction

Cardiac inflammatory processes involving giant cells comprise a diverse group of disorders.[1,2] The distinction between idiopathic giant cell myocarditis (IGCM), a life-threatening and rapidly progressing disease with fatal prognosis,[3] and cardiac sarcoidosis (CS) remains particularly challenging and is frequently delayed until autopsy.[2] Idiopathic giant cell myocarditis is regarded as a distinct clinical and pathological entity with exclusively cardiac manifestation and poor survival, whereas CS as a predominantly systemic disorder presents with less rapid progression of heart failure but nevertheless fatal outcome in patients with untreated heart involvement. Cardiac sarcoidosis is essentially a non-necrotizing process with gradual progression, whereas IGCM is associated with acute myocyte necrosis and extensive early scar formation.[2]

Idiopathic giant cell myocarditis and CS with cardiac involvement present as acute myocarditis or progressing idiopathic heart failure, respectively and, because the pathogenetic causes take place at the cellular and subcellular levels, it is impossible to discriminate between both diseases or active lymphocytic myocarditis, respectively, by non-invasive clinical investigations including imaging methods.[4] Endomyocardial biopsy (EMB) is the gold standard indicated in suspected myocarditis but has low sensitivity to exclude IGCM or necrotizing eosinophilic myocarditis, due to focal involvement of the myocardium.[2,5–8] A definite diagnosis of CS with exclusion of all other causes of non-caseating granulomas and of IGCM can only be made by EMB but 70–80% of biopsies are non-diagnostic.

Since both multinuclear giant cells and granulomas are easily missed by conventional histological evaluation, an improved method to reliably identify IGCM despite of above described difficulties and independent from histology is desirable. During the last decade, gene expression profiling has been successfully introduced in clinical diagnoses. To date, there exists only one published approach to identify IGCM-specific genes but clinical information is limited due to the low sample numbers (2 controls vs. 2 IGCM) and the fact that biopsies have been taken from patients under immunosuppressive therapy.[3] Immunosuppression is a condition which causes rapid reduction of myocardial inflammation.[2,3]

Patients and methods

Patients

This retrospective study evaluated EMB specimens of 4738 consecutive patients with clinically suspected myocarditis or idiopathic dilated cardiomyopathy. The tissue samples were obtained between 2003 and 2011 to evaluate a possible origin of symptoms of moderate-to-severe heart failure and non-ischaemic wall motion abnormalities of unknown cause. According to histomorphological and immunohistochemical analyses, we identified in total 20 patients with multinucleated giant cells (0.42%), and all were included in this study. Eighteen patients with completely available clinical information on history, clinical parameters and with adequate tissue for gene analyses were characterized as pronounced active myocarditis (MCA) with high numbers of infiltrating inflammatory cells and active myocyte necroses. Patients with MCA lacking clinical information on relevant comorbidities or sufficient material for genetic analyses, patients with borderline myocarditis or documented cardiotropic viruses such as enterovirus or adenovirus were not included in the study. Patients with giant cells in the myocardium were equally distributed within the 9-year interval of baseline analysis. For this study, patients without viral genomes detected in the routine molecular biological analysis and undetectable inflammation of heart muscle tissue[9] were defined as inflammation-free control group ($n = 80$). Control patients were selected from the same time period accordingly.

Clinically, IGCM and MCA patients presented with symptoms mimicking acute myocardial infarction including angina, ST-segment elevation, elevated creatine kinase or Troponin T/I and pro-BNP, and sudden onset of symptomatic heart failure including fatigue, reduced physical capacity, dyspnoea at rest or on exertion and new occurrence of global wall motion abnormalities. Fatigue, weakness, chest pain at rest or on exertion, dyspnoea on exertion, palpitations, reduced physical capacity, and progressive impairment of global systolic left ventricular function were the dominant persisting complaints of CS patients and controls. Control patients complained for persisting symptoms of heart failure despite unremarkable EMB information.

Symptomatic heart failure medication including ACE inhibitors/ARB, beta blockers, diuretics, and aldosterone antagonists was not different between the four cohorts of patients (*Table 1*). The same holds true for clinical and demographic parameters such as LV-EF, LV-EDD, NYHA classification, gender, and age. The demographic data of all patients are depicted in *Table 1*. The age distribution of all 20 patients at the onset of histopathological confirmation of giant cells is shown in Supplementary material online, *Figure S1*.

Analysis of myocardial inflammation

The EMB diagnosis of myocarditis was based on histomorphologic criteria according to the Dallas Classification[10–12] supplemented by quantitative immunohistochemical analyses.[7,8,13] Histological evaluations were performed on paraffin sections of two to three EMBs using standard procedures, i.e. formaldehyde or RNA-later fixation, paraffin embedding, staining with haematoxylin & eosin, periodic acid Schiff stain, Elastica van Gieson stain, and azan stain. Immunohistochemical analyses were carried out on frozen sections (two EMBs) in order to allow the detection of additional inflammatory cell subsets with non-paraffin staining antibodies, e.g. CD11a (LFA-1), CD11b (MAC-1), CD45R0 (memory or activated lymphocytes), Perforin-positive cytotoxic lymphocytes, CD54 (ICAM-1), CD106 (VCAM-1) and HLA-1. Inflammatory cells and cell adhesion molecules were analysed using quantitative digital imaging analysis as reported earlier.[13]

Nucleic acid isolation and reverse transcription to cDNA

Genomic DNA from EMBs ($n = 2–3$) was extracted by Puregene Mousetail Kit (Gentra, MN, USA). The detection of viral DNA was performed with primers specific for corresponding virus by nested PCR. Specificity of positive PCR products of viral genomes was confirmed by DNA sequencing.[7,9] Total RNAs were isolated from EMBs ($n = 2–3$) using Trizol reagent (Invitrogen, Karlsruhe, Germany), treated with DNAse (PeqLab, Erlangen, Germany) to remove any traces of genomic DNA and reverse transcribed to cDNA with the High Capacity Kit (Life Technologies, Darmstadt, Germany).[14]

Preamplification and gene expression analysis

The cDNA of all patients was immediately transcribed at the time of the baseline biopsy and stored at $-80^{\circ}C$ until use for gene expression analyses. The gene expression data of control patients were compared with those of patients presenting different forms of myocarditis. The demographic data are depicted in *Table 1*.

Differential gene expression was determined by QPCR of generated cDNAs. Due to limited amounts of myocardial cDNA, a preamplification

Table 1 Baseline characteristics of patients with and without confirmed myocarditis, without and with detectable giant cells

Clinically diagnosis	MCA	CS	IGCM	Controls	*P*-Value
n	18	10	10	80	
Male, *n* (%)	14 (77%)	6 (60%)	4(40%)	54 (68%)	0.5781
Age (years ± SD)	51.3 (± 3.1)	46.6 (± 6.6)	52.1 (± 6.7)	48.4 (± 1.1)	0.8273
NYHA I/II/III/IV (%)	10/30/60/0	0/50/50/0	0/50/0/50	18.8/53.4/20.0/7.8	0.110
LV-EF (%)	30	31	48	48.5	0.0594
LV-EDD (mm)	61	61.5	53	55	0.3908
LV-ESD (mm)	56	46	43	40	0.1361
Diabetes (%)	7.7	25	0	11.6	0.7392
Digitalis (%)	27.3	0	0	10.5	0.3824
Diuretics (%)	63.6	50.0	50.0	49.36	0.8498
ACE inhibitors (%)	54.5	100	50.0	76.6	0.2685
Betablocker (%)	54.6	50.0	100	71.25	0.3940
Aldosteron antagonist (%)	45.5	100	50.0	40.2	0.2999
Antiarrhytmics (%)	8.3	0	50.0	9.3	0.4680
Marcumar (%)	33.3	0	0	18.42	0.3818

technique was applied which is a reliable method for the detection of altered gene expression from small samples.[14,15] The gene expression data were normalized to housekeeping gene HPRT as described previously.[15] All predesigned gene expression, QPCR test systems were purchased from Life Technologies (Darmstadt, Germany) and listed in *Table 2*. Due to storage condition, gene expression profiles were not altered significantly over time (Supplementary material online, *Figure S2*).

Statistics

Results for quantitative features are given as means ± SD or median (10–90% percentile) as indicated. All expression values of the controls were set as 100%, and the others were calculated in relation to it. Student's *t*-test, one-way analysis of variance, χ^2, and Fisher's exact tests were used when appropriate. All *P*-values were two-tailed. *P*-values of <0.05 were considered to indicate statistical significance. The statistical analyses were performed using the JMP software version 7.0 (SAS Institute Inc., Cary, NC, USA).[14]

Results

Histological and immunohistological classification of patients

Histological features

Examples of the histological and immunohistological findings are given in *Figure 1*. In the virus and inflammation-free control group, regular histological structures and only few lymphocytes in the surrounding of capillaries were identified (*Figure 1A*). In MCA, necrotic myocytes adjacent to infiltrating lymphocytes and histiocytes were identified by cytoplasmic dissolution of fibrillar contractile structures (*Figure 1B–D*) which resembled myocytolysis bordering cellular infiltrates in IGCM (*Figure 1B*). Idiopathic giant cell myocarditis displayed a rather diffuse infiltration of cardiac tissue with a mixture of inflammatory cells, e.g. lymphocytes, macrophages, multinuclear giant cells, some eosinophiles, and disseminated necrosis of myocytes, whereas CS was characterized by a circumscribed process with non-caseating granulomas in which comparable cellular infiltrates, including multinuclear giant cells, were present while myocyte necrosises were exclusively localized at the periphery of the inflamed areas. Fibrosis of granulomas could be observed in older foci of CS patients.

The most important feature to discriminate IGCM from CS resulted from the different histological patterns. In IGCM, this constituted a diffuse infiltration by T-lymphocytes, macrophages, or histiocytes and the presence of multinuclear giant cells with necrosis or apoptosis of neighbouring myocytes. Fibroblasts might be present, however, without a tendency to build granular structures. In CS, the inflammation was more focally distributed with collagen fibres that built up granular structures. Necrotic or apoptotic myocytes were predominantly present at the periphery of the inflammatory cell foci (*Figure 1E* and *F*). Examples of CD3- or CD11b-positive cells in an active stage of CS are depicted in *Figure 1G* and *H*. Patients were considered to have MCA, IGCM, or CS if one of the submitted samples met the criteria in histological or immunohistochemical analyses in EMBs without any proof in other organ tissues.

Distinctive gene expression profiles in myocarditis presenting giant cells

Aim of this EMB-based study was to identify a characteristic gene expression pattern in myocardial tissue that differentiates giant cells from giant cell negative and, of course, from inflammation-free tissue.

Differentially expressed genes encoded for cellular receptors including Toll-like receptors (TLRs), regulators of the immune response, and genes of the mitochondrial energy metabolism (*Figure 2*).[16,17] Genes coding for distinct receptors (ADIPOR2, CCR5, CCR6, TLRs) expressed on cardiomyocytes, T-cells, and dendritic cells (DCs) were significantly dysregulated in comparison to the control cohort. Whereas the ADIPOR2 gene was up-regulated in CS ($P < 0.05$), genes coding for CCR5 and CCR6 on T-cells, and

Table 2 List of used gene expression test systems (Life Technologies)

Nr.	Gene symbol	Gene name	Possible functions	Assay nr. Life Technologies
1	A4GALT	4-α-Galactosyltransferase	P-globoside, viral receptor	Hs00213726_m1
2	ADIPOR2	Adiponectin receptor 2	Adiponectin pathway	Hs00226105_m1
3	CCL20	Chemokine (C−C motif) ligand 20/MIP3 alpha	DCs, chemokine	Hs01011368_m1
4	CCR5	Chemokine (C−C motif) receptor 5	T-cell receptor	Hs00152917_m1
5	CCR6	Chemokine (C−C motif) receptor 6	T-cell receptor	Hs00171121_m1
6	CPT1	Carnitine palmitoyltransferase 1B (muscle)	Fatty acid oxidation	Hs00993896_g1
7	DHODH	Dihydro-orotat-dehydrogenase	Mitochondrial respiratory chain	Hs00361406_m1
8	FOXP3	Forkhead box P3	Regulatory T-cells	Hs00203958_m1
9	HPRT1	Hypoxanthine phosphoribosyltransferase 1	Housekeeping gene	Hs99999909_m1
10	IL10	IL10	Cytokine	Hs99999035_m1
11	IL17D	IL17D	Th17 immunotype	Hs00370528_m1
12	IL1B	IL1, β	Cytokine	Hs00174097_m1
13	IL23R	IL23 receptor	Th17 immunotype	Hs00332759_m1
14	IL6	IL6	Cytokine	Hs00174131_m1
15	IL6R	IL6 receptor	Cytokine receptor	Hs01075667_m1
16	INFB1	Interferon β	Cytokine	Hs00277188_s1
17	ATP6	Mitochondrially encoded ATP synthase 6	Mitochondrial respiratory chain	Hs02596862_g1
18	CYB	Mitochondrially encoded cytochrome *b*	Mitochondrial respiratory chain	Hs02596867_s1
19	ND1	Mitochondrially encoded NADH dehydrogenase 1	Mitochondrial respiratory chain	Hs02596873_s1
20	ND4	Mitochondrially encoded NADH dehydrogenase 4	Mitochondrial respiratory chain	Hs02596876_g1
21	TGFB1	Transforming growth factor, β1	Cytokine	Hs00998133_m1
22	TLR3	TLR3	TLR	Hs01551078_m1
23	TLR4	TLR4	TLR	Hs01060206_m1
24	TLR7	TLR7	TLR	Hs00152971_m1
25	TLR8	TLR8	TLR	Hs00607866_mH
26	TLR9	TLR9	TLR	Hs00928321_m1
27	TNF	Tumour necrosis factor (TNF superfamily, member 2)	Cytokine	Hs00174128_m1
28	UQCR	Ubiquinol-cytochrome *c* reductase, complex III subunit XI	Mitochondrial respiratory chain	Hs00199138_m1

activated DCs were up-regulated in IGCM (*Figure 2A*). Toll-like receptor 8, an intracellular receptor on plasmacytoid DCs and monocytes, that responds to single-stranded RNA of viral origin and has been reported to become activated in autoimmune settings[18–20] was strongly overexpressed in IGCM ($P < 0.001$) suggesting activation of TLR8-dependent innate immune signalling (*Figure 2A*). In contrast to TLR8, the expression levels of TLR3, TLR4, and TLR9 were significantly suppressed in all inflammatory cardiomyopathies (*Figure 2B*).

The extensive infiltration of myocardial tissue by inflammatory cells may be responsible for the strong up-regulation of several key genes involved in the adaptive immune response (CCL20, FOXP3, IFNB1, IL6, IL10) (*Figure 2C*) in IGCM and was not detected in the other inflammatory disorders. This massive induction of CCL20 in conjunction with that of CCR5 and CCR6 (*Figure 2A*) is consistent with a particularly strong activation of DCs in IGCM.[20] A similarly pronounced CCL20 up-regulation necessary to attract CCR6+ T lymphocytes to inflamed tissues has been reported in response to a microbial infection via synergistic effects of the pro-inflammatory cytokines IL1B and TNF.[21,22]

In giant cell arteritis (GCA), DCs mediate T helper 1 (Th1) and Th17 cell activation.[22] Even though IL17D was significantly reduced in IGCM, expression of pro-inflammatory cytokines secreted by macrophages (IL1B, IL10, IL6, TNF) upon IL17 stimulation was elevated as shown for GCA (*Figure 2C*).[22] It has been reported that TLR4 induces GCA via enhanced production of CCL20 followed by attraction of CCR6 positive, media-invasive T cells in an arteritis mouse model.[23] In contrast, TLR4 was significantly suppressed in our human EMBs (*Figure 2B*).[20,22]

Cardiac sarcoidosis but not IGCM displayed a significant deregulation of CPT1 ($P < 0.01$), DHODH ($P < 0.05$), and CYB ($P < 0.05$). These genes coding for proteins of the mitochondrial respiratory complexes were not differentially expressed in MCA and controls (*Figure 2D*).

Due to their distinct differential expression, the obtained expression profiles suggest the presence of giant cells in small myocardial tissue samples even in the absence of an unequivocal histopathologic evidence of these cells. Furthermore, genes (CPT1, IFNB1, IL17D, CYB, DHODH) that are differentially regulated between CS and IGCM facilitate their delimitation.

Correlation of immunohistochemical markers with deregulated genes

The immunohistochemical analysis of inflammatory cell populations and the expression of adhesion molecules showed significant

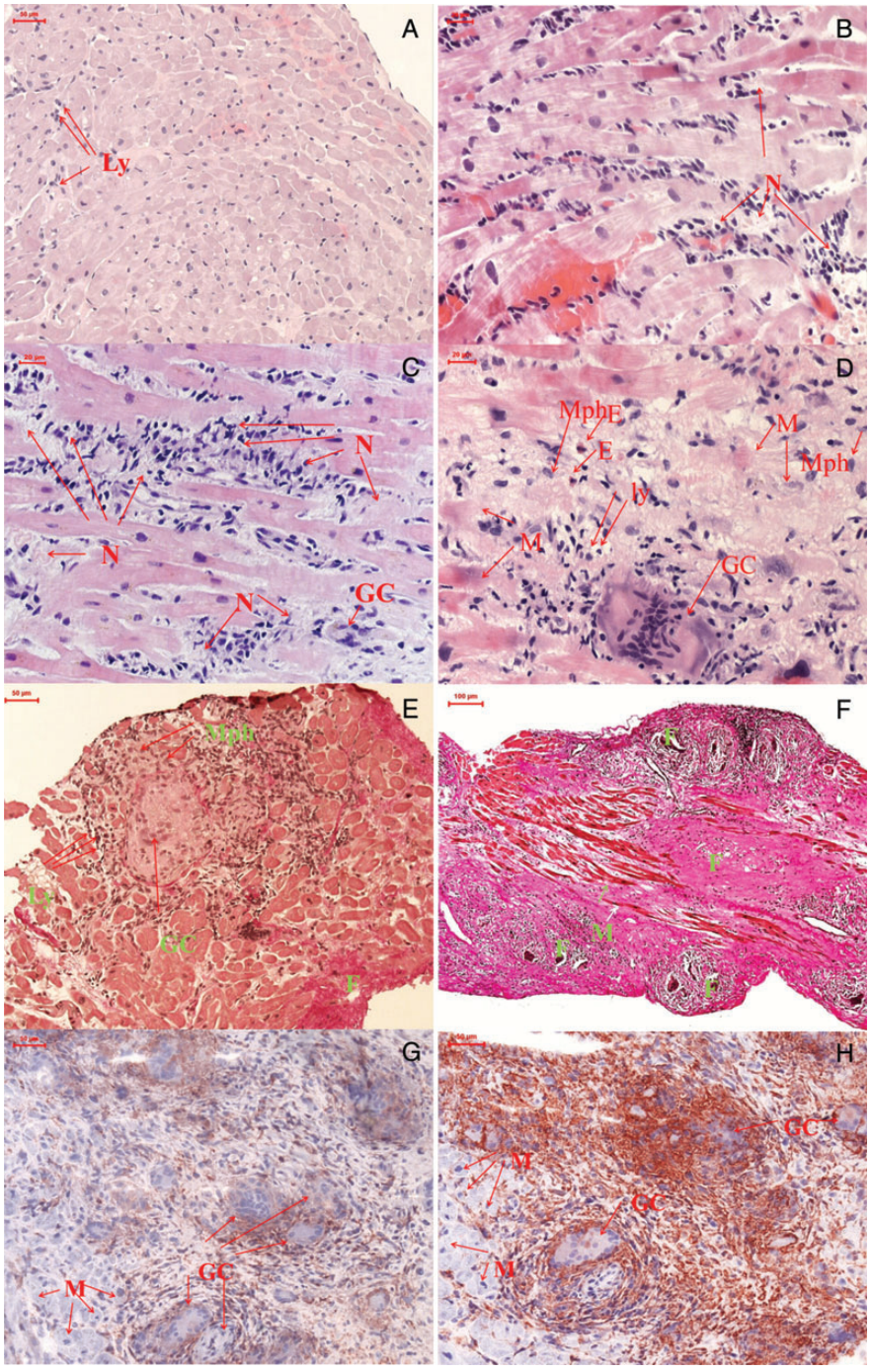

Figure 1 Histological findings in endomyocardial biopsies. A: Virus-and inflammation-free control. Few lymphocytes (Ly) near capillaries. H&E stain. B: MCA with widespread infiltration of lymphocytes, macrophages and necrosis or apoptosis of myocytes (N). H&E stain. C: IGCM: Lytic necrosis (N) or apoptosis of myocytes in the central area with dense cellular infiltration of lymphocytes and some macrophages or histiocytes, some eosinophiles and a multinuclear giant cell (GC) H&E stain. D: IGCM: In the center lytic necrosis or apoptosis of myocytes (M), in the surroundings infiltrates of lymphocytes (Ly) and some macrophages (Mph) or histiocytes, some eosinophiles (E) and a multinuclear giant cell (GC) H&E stain. E: Granuloma of CS with circumscribed infiltrate of lymphocytes (Ly), macrophages (Mph), multinuclear giant cell (GC) and fibroblasts producing collagen fibers stained red. Scar at the periphery of the focus (F). EvG stain. F: Multiple granulomas in CS with increasing sclerosis and fibrosis of the foci (F). Multinuclear giant cells, lymphocytes, macrophages and fibroblasts near myocytes (M). EvG stain. G: Granuloma of CS with multinuclear giant cells (GC), macrophages and lymphocytes. Immunohistological stain for CD3 positive cells (T-lymphocytes). In the periphery of the granuloma myocytes (M) partially on the way to necrosis or apoptosis. Positive lymphocytes with brown reaction product. H: Granuloma of CS immunohistology stain for CD 11b (MAC-1). Brown reaction product in the cytoplasm of activated macrophages. Multinuclear giant cells partially negative. Myocytes (M), multinuclear giant cells (GC).

differences between MCA, IGCM, and CS. The highest numbers of inflammatory infiltrates (CD3, LFA1, CD11b, CD45R0) and cell adhesion molecules (HLA-1, CD54/ICAM-1, CD106/VCAM1) were measured in IGCM (*Table 3*). These findings suggest that the different clinical features in the three inflammatory processes may not primarily be based on massive infiltration by inflammatory cells *per se*.[10,13] Correlation analysis of myocardial gene expression with the immunohistochemical markers revealed a strong association of regulated genes with T-lymphocytes (CD3), activated T-cells (CD45R0), and Perforin-positive cytotoxic lymphocytes in CS. In contrast, gene expression in IGCM and MCA was mainly correlated with activated leukocytes and macrophages (LFA1, MAC-1) (*Table 3*). Such differences point to distinctions between CS, IGCM, and MCA with respect to the types and composition of the cellular infiltrates as well as the interactions of these cells with the myocardium.

In a second approach, the numbers of inflammatory cells detected by immunohistochemistry were correlated with the expression levels of deregulated genes (*Table 3*) in order to identify a possible correlation of individual cell types with specific genes. Of note, the correlation pattern of specific genes with corresponding inflammatory cells was different for IGCM, CS, MCA and controls (*Table 3*). These data again support the assumption that the origin as well as the cellular and molecular triggers of IGCM and CS are different and constitute distinct disease entities.

Idiopathic giant cell myocarditis and cardiac sarcoidosis show different pathway regulation

The classification of intracellular and systemic pathways that are possibly involved in the pathogenesis of IGCM, CS, and MCA was

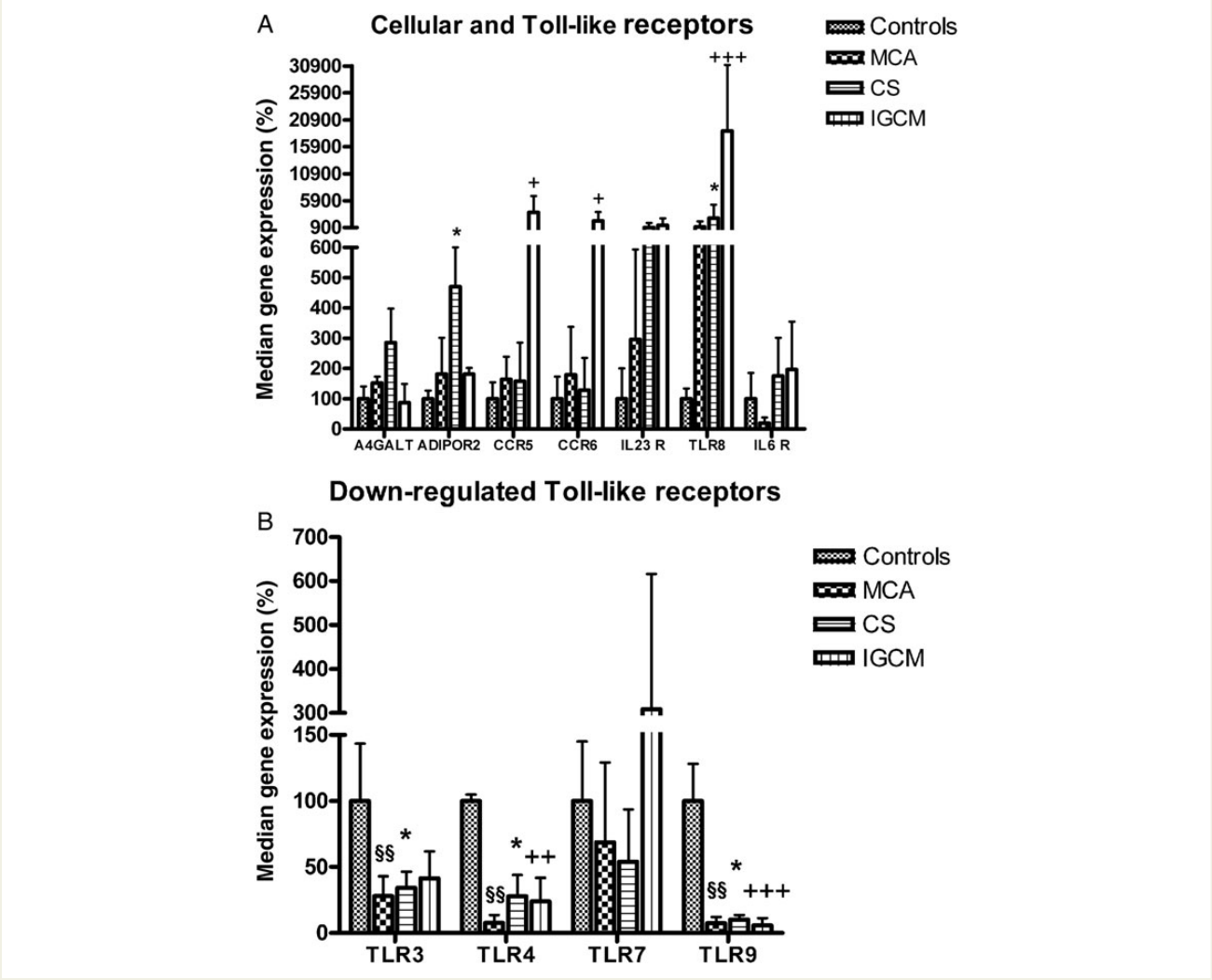

Figure 2 Cardiac gene expression shifts associated with presence of giant cells. Expression profiles of patients with active myocarditis (MCA), cardiac sarcoidosis (CS), and idiopathic giant cell myocarditis (IGCM) compared with controls for genes of cellular and Toll-like receptors (*A*), down-regulated Toll-like receptors (*B*), chemokines and cytokines (*C*), and energy metabolism (*D*). All expression values of controls are given as 100% and the others in relation to it. MCA vs. controls: $^{\S}P < 0.05$; $^{\S\S}P < 0.01$, CS vs. controls: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, IGCM vs. controls: $^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$, CS vs. IGCM: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

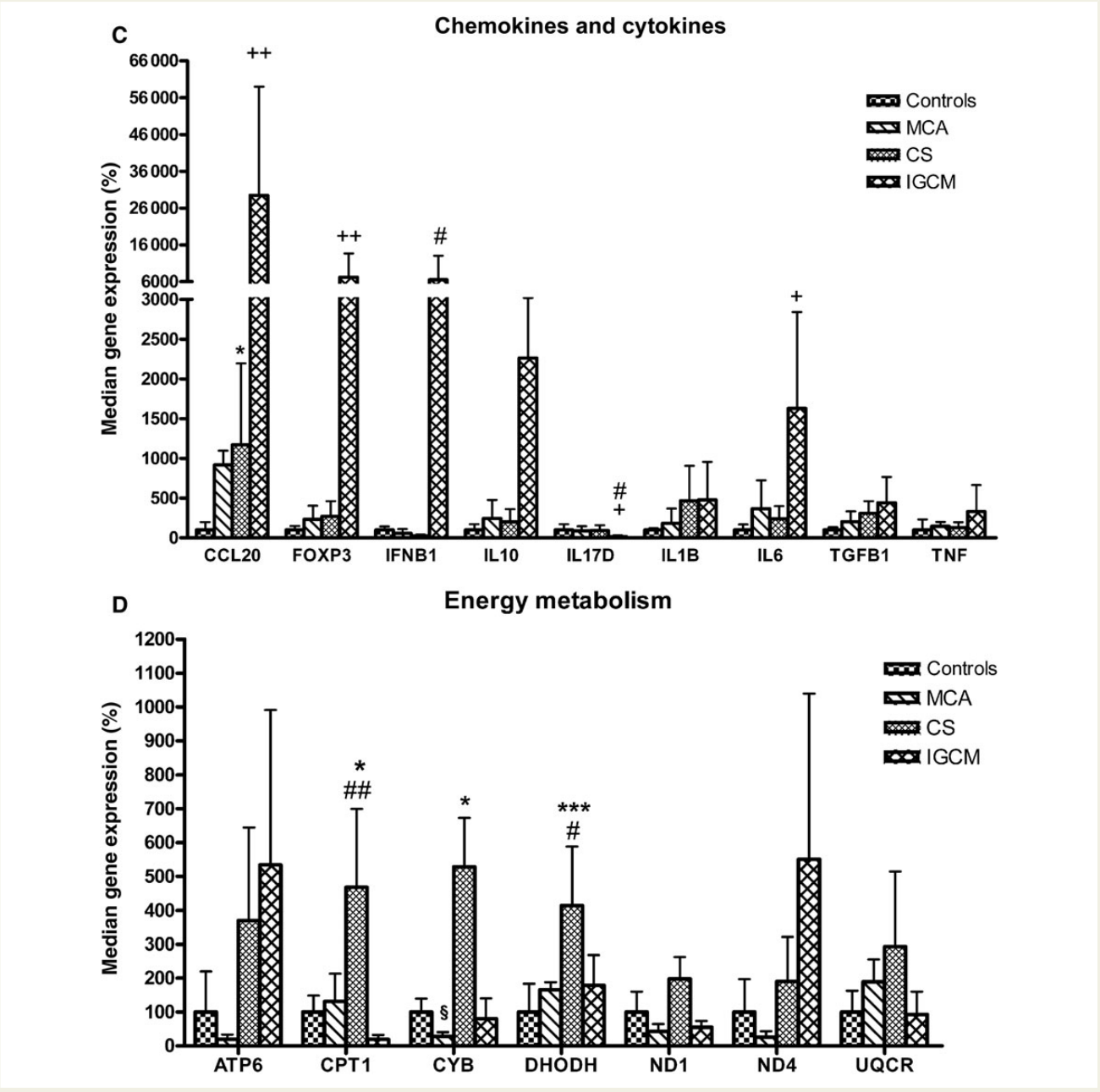

Figure 2 (*Continued*).

performed by a bioinformatic approach using determined gene profiles and ingenuity pathway analysis software (Ingenuity, Redwood City, CA, USA).[16] Based on the analysed gene set, 206 transcription factors, 1171 molecular functions, 7 top pathways, 393 associated genes, 51 canonical pathways, and 1317 disease-related functions were predicted (Supplementary material online, *Table S1A–D*).

Bioinformatic extraction of measured gene levels showed an activation of different transcription factors clustering MCA and CS in one group and IGCM in a second one (Supplementary material online, *Table S1A*). A comparable similarity was seen by predicted activated functions in myocardial inflammatory processes (Supplementary material online, *Table S1B*). The prediction of involved cellular networks combines both inflammatory disease harbouring giant cells in myocardium (CS, IGCM) and separate them from MCA (Supplementary material online, *Table S1C*). The complete separation of all three disease entities is made by extraction of disease-related functions (Supplementary material online, *Table S1D*).

The combined analysis of gene expression profile characteristics, immunohistochemical markers, and bioinformatic predictions of cellular pathways and associated functions, clearly demonstrates that MCA, IGCM, and CS have to be considered as distinct entities with few overlapping features.

Table 3 **Correlation of immunohistochemical markers with gene expression in endomyocardial biopsies**

Disease group	CD3/mm^{2a}	LFA1/mm^2	Perforin/mm^2	Mac1/mm^2	CD45R0/mm^2	HLA1 (%AF^b)	CD54 (%AF)	CD106 (%AF)
Significant differences of disease groups by immunohistochemical markers (*P*-value at least <0.05)								
Controls	5.7	14	1	28.4	17.4	7.5	2.1	0.04
MCA	26.2	57.5	2.9	49.79	51.9	9	2.9	0.11
CS	9.4	26.4	1.3	43.75	24.6	11.6	3.6	0.095
IGCM	141.5	308	2.5	178.3	214.5	14.5	12.4	0.5
P-values								
CS vs. controls	$P < 0.05$	$P < 0.001$		$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.001$	
CS vs. MCA				$P < 0.05$	$P < 0.05$		P < 0.05	
CS vs. IGCM	$P < 0.001$	$P < 0.01$					$P < 0.001$	$P < 0.01$
IGCM vs. Controls	$P < 0.001$	$P < 0.001$		$P < 0.001$	$P < 0.001$	$P < 0.01$	$P < 0.001$	$P < 0.001$
IGCM vs. MCA	$P < 0.01$	$P < 0.001$		$P < 0.01$	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.01$
MCA vs. controls	$P < 0.001$				$P < 0.01$			$P < 0.05$
Significant correlation of gene expression to immunohistochemical markers (*P*-value at least <0.05)								
Controls			A4GALT, ADIPOR2, ATP6, CCR6, CPT1, IL6R, IL17D, ND4, TGFB			CCR6		IL23R
MCA	CCL20, CCR5, TNF	ATP6, CCL20, CCR5, CYB, IL6R, ND4, TLR3, TNF	A4GALT	ATP6, CCL20, CCR5, CYB, ND4, TLR3	CCL20, TNF		DHODH	
CS	CCL20, CCR6, FOXP3, IFNB1, IL10, IL1B, IL6, IL6R, TLR4, TLR8, TLR9, TNF,		A4GALT, ADIPOR2, CCL20, CCR6, DHODH, FOXP3, IFNB1, IL10, IL1B, IL23R, IL6, IL6R,TGFB, TLR4, TLR8,TLR9,TNF, UQCR		CCL20, CCR5, CCR6, FOXP3, IL10, IL1B, IL6, TLR7, TLR8, TLR9, TNF			Il1B, IL10
IGCM	CCR6	DHODH, FOXP3, IFNB1, IL6	IL23R	DHODH, FOXP3, IFNB1, IL6			TGFB	
Overall	A4GALT, CCL20, CCR5,FOXP3, IFNB1, IL6, IL6R, IL10, IL23R, TGFB,TNF	CCL20, CCR5,FOXP3, IFNB1, IL6, IL10, IL23R, TGFB,TNF	CCL20, IL23R, TLR7	CCL20, CCR5,FOXP3, IFNB1, IL6, IL10, IL23R, TGFB,TNF	CCL20, FOXP3, IL1B, IL10, IL23R, TLR5, TLR7, TLR8,TNF	CCL20, CCR5, IL6, IL10, IL23R, TLR8, TNF	ATP6, CCL20, CCR5, IFNB1, IL6, IL10, TLR8, TLR9, TNF	FOXP3, IL1B, IL10,TNF

[a]Number of positively stained cells per mm^2 digitally analysed myocardial tissue.
[b]Area fraction (percentage in % of positively stained, digitally analysed myocardial tissue).

Discussion

Idiopathic giant cell myocarditis and cardiac involvement of sarcoidosis (CS) often escape diagnosis until autopsy or transplantation because of their unspecific clinical presentation and focal appearance which both contribute to the considerable sampling error in the histologic analysis. Both diseases cause severe heart failure with fatal outcome if untreated but development of cardiac impairment in IGCM occurs much faster than in CS. This demands a reliable diagnostic method that allows an unambiguous separation of IGCM from CS.

In this retrospective study, 20 consecutive patients (10 IGCM, 10 CS) with multinucleated giant cells in their myocardium were identified out of those patients that were submitted to our institution for routine EMB diagnostics. Mean age of patients with IGCM and CS was 52.1 ($\pm$6.7) and 46.6 ($\pm$6.6) years, respectively. The reason for the higher mean age of patients in our study, when compared with earlier publications, could be caused by the fact that we analysed EMB samples but no explanted hearts of autopsy material from patients with end-stage disease.[1,2] Idiopathic giant cell myocarditis is a condition with short median survival from onset of symptoms.[1–3] The younger IGCM patients are more likely to die before the clinical diagnostics including EMB is completed and therefore are often diagnosed postmortem.

Since IGCM is a rapidly progressing myocardial disease with a high early mortality of ~40% within the first 2–4 weeks it demands combined immunosuppression with anti-thymocyte globulin, ciclosporin, and prednisolon immediately after diagnostic confirmation in order to improve short- and long-term prognosis. Due to the often fatal outcome, the prognostic influence of immunosuppression, however, remains poorly known.[5]

Cardiac involvement occurs in 20–27% of sarcoidosis patients in the USA, ~11% in Finland and may be as high as 58% in Japan with a high mortality of nearly 50% in 5 years.[11,24,25] The diagnosis, although circumstantial, is strongly suspected if cardiac manifestation occurs in a patient with multi-systemic sarcoidosis. However, when cardiac dysfunction is the only manifestation of sarcoidosis, the diagnosis is frequently not entertained. Only 40–50% of patients with CS diagnosed at autopsy have the diagnosis made during their lifetime and in 37% of those patients no clinical signs or symptoms of the disease were present. This is in line with our observation that the first hints of a sarcoidosis were obtained from EMB in 70% of our positive patients. Systolic heart failure due to cardiac involvement in CS often responds to monotreatment with corticosteroids or other immunosuppressive agents.[26,27]

Despite the limitations of EMB with respect to sensitivity, the confirmation of IGCM and isolated CS confined to the heart solely depends on histology.[11,12] The sensitivity of EMB for non-caseating granulomas is low, usually <20%, and a consequence of patchy myocardial involvement which frequently involves the interventricular septum and the left ventricle. In the absence of detectable granulomas, it is difficult to distinguish CS from IGCM[1,2] since both diseases possess giant cells and are associated with ventricular tachycardia or heart block. Due to their focal appearance, a direct proof of giant cells demands multiple biopsies and examination of all sections of the multiple tissue samples, but even then focally distributed multinuclear giant cells are generally detected in not >2–6 sections of a biopsy. Of note, the giant cells were detected in only 1 of 5–10 histologically examined specimens in the reported IGCM patients. In this view, the probability that a second independent biopsy with giant cells has been extracted for gene expression profiling has to be considered very low. Although we cannot exclude this in single rare cases, we assume that the changed gene expression profiles represent alterations within the entire myocardium and also apart from areas with infiltrating giant cells as shown by the simultaneous analysis of three EMB specimens of a single patient (Supplementary material online, *Figure S3*).

In CS deregulated genes affect the cellular receptor ADIPOR2 and CCL20 as well as components of the mitochondrial respiratory chain such as CPT1, CYB, or DHODH. In contrast, IGCM gene expression is deregulated for genes of immune and TLRs CCR5, CCR6, TLR8 and to distinct acquired immune response genes such as CCL20, FOXP3, IFNB1, IL6, IL10, and IL17D.

In IGCM, the pronounced increase of CCR5 and CCR6 may be mediated by the massive T-cell infiltration[21,22] and the consecutive up-regulation of the CCR6 ligand CCL20 and pro-inflammatory cytokines IL6 and IL10 by macrophages, DCs, multinucleated giant cells, or epitheloid cells. Similarly, TLR8 ligands result in a synergistic release of pro-inflammatory mediators which also promote the activation of IL17-producing T cells.[28] Last but not least, TLR8 is an intracellular receptor that responds to single-stranded viral RNA[18–20] and the massive up-regulation of TLR8 in IGCM could be caused by a viral component associated with this disease entity.[2]

DHODH and CPT1, two genes of the mitochondrial respiratory chain, are good discriminators of the two forms of myocarditis presenting with multinuclear giant cells, IGCM and CS. DHODH is a key enzyme involved in pyrimidine biosynthesis and inhibition of DHODH leads to activation of p53 and induction of p53-dependent apoptosis.[29] Down-regulation of IL17D, as seen in IGCM, may be caused by an inhibition of this enzyme that also reduces the proliferation of lymphocytes and thereby blocks the production of IL-17 by lymphocytes in inflammatory diseases.[30,31] CPT1 is the rate-limiting enzyme for fatty acid β-oxidation in adipocytes. Cardiac sarcoidosis may display lower expression of immune response genes since the isoform CPT1A exerts anti-inflammatory effects by suppression of pro-inflammatory adipokines such as TNFα and IL6.[32]

Some patients displayed slightly variable patterns in their gene expression levels, but expression profiles allow a clear cut distinction of MCA, IGCM, and CS (*Figure 2A–D*). Although gene expression levels do not necessarily correlate with the protein levels, this is no obstacle for the use of the above described gene expression profiles as diagnostic tools. Reported differences in gene profiles allow for the first time a prediction of giant cells in myocardium even without their direct histological proof. Additionally, the identified gene profiles clearly discriminate between inflammation-free controls and MCA, IGCM, or CS. This provides a valuable additional diagnostic information, providing that no immunosuppressive treatment has been administered before.

Recently, published data have compared an only limited number of healthy controls with two IGCM patients under immunosuppressive treatment. Since other pronounced inflammatory conditions such as MCA or CS have not been taken into consideration and immunosuppression is known to rapidly alter gene expression profiles, the obtained gene expression data are of limited clinical relevance.[3] In untreated patients, deregulated gene levels are persisting and some gene expression differences are even increasing during follow-up

(data not shown). Consequently, diagnostic gene profiling has to be applied before initiation of any immunosuppression in order to get reliable clinical information.[3]

The aim of the presented study was the identification of specific diagnostic gene profiles in EMBs rather than to elucidate information on mechanisms of IGCM and CS that are correlated with biopsy independent parameters, e.g. cytokines in plasma fractions. This molecular approach provides additional information on the aetiology and pathogenesis of IGCM and CS in addition to histologic information facilitating therapy decision-making according to recent consensus statements for EMB diagnostics of both diseases.[11,12] The generation of valid gene expression data from single small biopsy specimen requires optimal specimen procurement, adequate sample storage, and preamplification techniques for specific gene sets[15] with reverse transcription of cellular RNA into cDNA immediately after biopsy processing. The RNA amount of one to two EMBs is sufficient to perform reliable and repeatable expression profiling studies including multiple genes,[14,15] also in archived samples.

Conclusion

Direct histological proof of multinuclear giant cells or sarcoid granulomas successes rarely in routine histology. Differential myocardial expression profiling using a set of 10–15 of 27 altered genes is suitable to differentiate giant cell myocarditis from active lymphocytic myocarditis (MCA) and facilitates the discrimination between IGCM and CS. Correlation and bioinformatic analysis demonstrates the pronounced differences between CS, IGCM, and MCA regarding the types and composition of the cellular infiltrates and their influence on myocardial gene expression.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: D.L., H.P.S., M.R., and U.K. have a pending patent (DE 102012101557.0) including presented gene profiles.

References

1. Cooper LT Jr, Berry GJ, Shabetai R. Idiopathic giant cell myocarditis – natural history and treatment. Multicenter Giant Cell Myocarditis Study Group Investigators. *N Engl J Med* 1997;**26**:1860–1866.
2. Okura Y, Dec GW, Hare JM, Kodama M, Berry GJ, Tazelaar HD, Bailey KR, Cooper LT. A clinical and histopathologic comparison of cardiac sarcoidosis and idiopathic giant cell myocarditis. *Am J Coll Cardiol* 2003;**15**:322–329.
3. Kittleson MM, Minhas KM, Irizarry RA, Ye SQ, Edness G, Breton E, Conte JV, Tomaselli G, Garcia JG, Hare JM. Gene expression in giant cell myocarditis: altered expression of immune response genes. *Int J Cardiol* 2005;**102**:333–340.
4. Pierre-Louis B, Prasad A, Frishman WH. Cardiac manifestations of sarcoidosis and therapeutic options. *Cardiol Rev J* 2009;**17**:153–158.
5. Cooper LT Jr, Hare JM, Tazelaar HD, Edwards WD, Starling RC, Deng MC, Menon S, Mullen GM, Jaski B, Bailey KR, Cunningham MW, Dec GW; Giant Cell Myocarditis Treatment Trial Investigators. Usefulness of immunosuppression for giant cell myocarditis. *Am J Cardiol* 2008;**102**:1535–1539.
6. Noutsias M, Pauschinger M, Gross U, Lassner D, Schultheiss HP, Kühl U. Giant cell myocarditis in a patient presenting with dilated cardiomyopathy and ventricular tachycardias treated by immunosuppression: a case report. *Int J Cardiol* 2008;**18**: 58–59.
7. Schultheiss HP, Kühl U, Cooper LT. The management of myocarditis. *Eur Heart J* 2011;**32**:2616–2625.
8. Caforio AL, Pankuweit S, Arbustini E, Basso C, Gimeno-Blanes J, Felix SB, Fu M, Heliö T, Heymans S, Jahns R, Klingel K, Linhart A, Maisch B, McKenna W, Mogensen J, Pinto YM, Ristic A, Schultheiss HP, Seggewiss H, Tavazzi L, Thiene G, Yilmaz A, Charron P, Elliott PM; European Society of Cardiology Working Group on Myocardial and Pericardial Diseases. Current state of knowledge on aetiology, diagnosis, management, and therapy of myocarditis: a position statement of the European Society of Cardiology – Working Group on Myocardial and Pericardial Diseases. *Eur Heart J* 2013;**34**:2636–2648.
9. Kühl U, Pauschinger M, Noutsias M, Seeberg B, Bock T, Lassner D, Poller W, Kandolf R, Schultheiss HP. High prevalence of viral genomes and multiple viral infections in the myocardium of adults with 'idiopathic' left ventricular dysfunction. *Circulation* 2005;**22**:887–893.
10. Aretz HT. Myocarditis: the Dallas criteria. *Hum Pathol* 1987;**18**:619–624.
11. Leone O, Veinot JP, Angelini A, Baandrup UT, Basso C, Berry G, Bruneval P, Burke M, Butany J, Calabrese F, d'Amati G, Edwards WD, Fallon JT, Fishbein MC, Gallagher PJ, Halushka MK, McManus B, Pucci A, Rodriguez ER, Saffitz JE, Sheppard MN, Steenbergen C, Stone JR, Tan C, Thiene G, van der Wal AC, Winters GL. 2011 consensus statement on endomyocardial biopsy from the Association for European Cardiovascular Pathology and the Society for Cardiovascular Pathology. *Cardiovasc Pathol.* 2012;**21**:245–274.
12. Thiene G, Bruneval P, Veinot J, Leone O. Diagnostic use of the endomyocardial biopsy: a consensus statement. *Virchows Arch.* 2013;**463**:1–5.
13. Noutsias M, Pauschinger M, Ostermann K, Escher F, Blohm JH, Schultheiss H, Kühl U. Digital image analysis system for the quantification of infiltrates and cell adhesion molecules in inflammatory cardiomyopathy. *Med Sci Monit* 2002;**8**:59–71.
14. Kuhl U, Lassner D, Dorner A, Rohde M, Escher F, Seeberg B, Hertel E, Tschope C, Skurk C, Gross UM, Schultheiss HP, Poller W. A distinct subgroup of cardiomyopathy patients characterized by transcriptionally active cardiotropic erythrovirus and altered cardiac gene expression. *Basic Res Cardiol* 2013;**108**:372.
15. Noutsias M, Rohde M, Block A, Klippert K, Lettau O, Blunert K, Hummel M, Kühl U, Lehmkuhl H, Hetzer R, Rauch U, Poller W, Pauschinger M, Schultheiss HP, Volk HD, Kotsch K. Preamplification techniques for real-time RT-PCR analyses of endomyocardial biopsies. *BMC Mol Biol* 2008;**9**:3.
16. Wittchen F, Suckau L, Witt H, Skurk C, Lassner D, Fechner H, Sipo I, Ungethüm U, Ruiz P, Pauschinger M, Tschope C, Rauch U, Kühl U, Schultheiss HP, Poller W. Genomic expression profiling of human inflammatory cardiomyopathy (DCMi) suggests novel therapeutic targets. *J Mol Med* 2007;**85**:257–271.
17. Bobbert P, Scheibenbogen C, Jenke A, Kania G, Wilk S, Krohn S, Stehr J, Kuehl U, Rauch U, Eriksson U, Schultheiss HP, Poller W, Skurk C. Adiponectin expression in patients with inflammatory cardiomyopathy indicates favourable outcome and inflammation control. *Eur Heart J* 2011;**32**:1134–1147.
18. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004;**303**: 1529–1531.
19. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 2004;**303**:1526–1529.
20. Prinz N, Clemens N, Strand D, Pütz I, Lorenz M, Daiber A, Stein P, Degreif A, Radsak M, Schild H, Bauer S, von Landenberg P, Lackner KJ. Antiphospholipid-antibodies induce translocation of TLR7 and TLR8 to the endosome in human monocytes and plasmacytoid dendritic cells. *Blood* 2011;**118**:2322–2332.
21. Wu YY, Tsai HF, Lin WC, Hsu PI, Shun CT, Wu MS, Hsu PN. Upregulation of CCL20 and recruitment of CCR6+ gastric infiltrating lymphocytes in Helicobacter pylori gastritis. *Infect Immun* 2007;**75**:4357–4363.
22. Facco M, Baesso I, Miorin M, Bortoli M, Cabrelle A, Boscaro E, Gurrieri C, Trentin L, Zambello R, Calabrese F, Cassatella MA, Semenzato G, Agostini C. Expression and role of CCR6/CCL20 chemokine axis in pulmonary sarcoidosis. *J Leukoc Biol* 2007; **82**:946–955.

23. Deng J, Ma-Krupa W, Gewirtz AT, Younge BR, Goronzy JJ, Weyand CM. Toll-like receptors 4 and 5 induce distinct types of vasculitis. *Circ Res* 2009;**104**:488–495.
24. Newman LS, Rose CS, Bresnitz EA. A case control etiologic study of sarcoidosis: environmental and occupational risk factors. *Am J Respir Crit Care Med* 2004;**170**: 1324–1330.
25. Matsui Y, Iwai K, Tachibana T. Clinicopathological study of fatal myocardial sarcoidosis. *Ann N Y Acad Sci* 1976;**278**:455–469.
26. Nunes H, Freynet O, Naggara N, Soussan M, Weinman P, Diebold B, Brillet PY, Valeyre D. Cardiac sarcoidosis. *Semin Respir Crit Care Med* 2010;**31**:428–441.
27. Ishimaru S, Tsujino I, Takei T, Tsukamoto E, Sakaue S, Kamigaki M, Ito N, Ohira H, Ikeda D, Tamaki N, Nishimura M. Focal uptake on 18F-fluoro-2-deoxyglucose positron emission Tomography images indicates cardiac involvement of sarcoidosis. *Eur Heart J* 2005;**26**:1538–1543.
28. Schwarz H, Posselt G, Wurm P, Ulbing M, Duschl A, Horejs-Hoeck J. TLR8 and NOD signaling synergistically induce the production of IL-1β and IL-23 in monocyte-derived DCs and enhance the expression of the feedback inhibitor SOCS2. *Immunobiology* 2013;**218**:533–542.
29. Khutornenko AA, Roudko VV, Chernyak BV, Vartapetian AB, Chumakov PM, Evstafieva AG. Pyrimidine biosynthesis links mitochondrial respiration to the p53 pathway. *Proc Natl Acad Sci USA* 2010;**20**:12828–12833.
30. Starnes T, Broxmeyer HE, Robertson MJ, Hromas R. Cutting edge: IL-17D, a novel member of the IL-17 family, stimulates cytokine production and inhibits hemopoiesis. *J Immunol* 2002;**15**:642–646.
31. Fitzpatrick LR, Deml L, Hofmann C, Small JS, Groeppel M, Hamm S, Lemstra S, Leban J, Ammendola A. 4SC-101, a novel immunosuppressive drug, inhibits IL-17 and attenuates colitis in two murine models of inflammatory bowel disease. *Inflamm Bowel Dis* 2010;**16**:1763–1777.
32. Gao X, Li K, Hui X, Kong X, Sweeney G, Wang Y, Xu A, Teng M, Liu P, Wu D. Carnitine palmitoyltransferase 1A prevents fatty acid-induced adipocyte dysfunction through suppression of c-Jun N-terminal kinase. *Biochem J* 2011;**1**: 723–732.