High Perforin-Positive Cardiac Cell Infiltration and Male Sex Predict Adverse Long-Term Mortality in Patients With Inflammatory Cardiomyopathy

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Background—The authors analyzed the effects of perforin-dependent infiltration on long-term mortality in patients with inflammatory cardiomyopathy (CMi). We previously demonstrated that left ventricular function deteriorates and progresses to substantial cardiac dysfunction in patients with perforin-positive cardiac cell infiltration.

Methods and Results—Between 2003 and 2013, 2389 consecutive patients with clinically suspected CMi who underwent endomyocardial biopsies were enrolled. Endomyocardial biopsies were performed at first admission after exclusion of ischemic or valvular heart disease, and CMi was confirmed in 1717 patients. Follow-up was up to 10.1 years (median 0.47 years; interquartile range, 0.03–2.56 years) and information on vital status was obtained from official resident data files. Multivariable statistical analysis was conducted for all patients with CMi regarding significant predictors of all-cause mortality or need for heart transplantation. Multiple Cox regression analysis revealed perforin above the calculated cutoff point of 2.9 cells/mm^2 as a strong predictor of impaired survival with a hazard ratio of 1.881 (95% confidence interval, 1.177–3.008; *P*=0.008), independent of left ventricular function and other myocardial inflammation markers (CD3, macrophage-1 antigen, leukocyte function–associated antigen-1, human leukocyte antigen-1, and intercellular cell adhesion molecule-1). Unexpectedly, male sex emerged as another strong adverse predictor of survival in CMi (hazard ratio, 1.863; confidence interval, 1.096–3.168 [*P*=0.022]). Whereas left ventricular ejection fraction course is adversely affected by myocardial perforin, multivariate analysis indicates that left ventricular ejection fraction explains only part of the observed overall mortality.

Conclusions—High perforin-positive cardiac cell infiltration and male sex are independent adverse predictors of long-term mortality in CMi. Furthermore, exact quantification of immunohistochemically detected infiltrates is necessary to assess the prognosis. (*J Am Heart Assoc*. 2017;6:e005352. DOI: 10.1161/JAHA.116.005352.)

Key Words: inflammatory cardiomyopathy • myocardial inflammation • perforin • survival

Inflammatory cardiomyopathy (CMi) represents a major cause of heart failure with potential for transition to the clinical picture of dilated cardiomyopathy.[1–3] The pathogenesis of CMi encompasses immune responses as well as autoimmune reactions involving autoantigen-specific T cells. Myocardial inflammation is reflected by infiltration of lymphocytes, macrophages, and cell adhesion molecules.[4,5] Several studies have shown that cytotoxic cells expressing cytotoxic effector molecules are also increased in this cardiac inflammatory process.[6–11] One key mediator of cytotoxicity is perforin, a molecule released by T cells and natural killer cells. Its expression on T-cell maturation is strongly regulated after

activation, and effector T cells have been shown to express the highest levels of intracellular perforin. Once conjugated to target cells, the cytotoxic secretory granules traffic to the immunological synapse and release the deadly protein perforin. We previously demonstrated in a cohort of 495 patients with endomyocardial biopsy (EMB)–proven myocardial inflammation that left ventricular function deteriorates in patients with detection of perforin-positive cardiac cell infiltration and progresses towards substantial cardiac dysfunction.[6] In the present study, in a series of 2389 patients with suspected CMi, we investigated the influence of high perforin-positive cardiac cell infiltration and other clinical and immunological parameters on long-term mortality or need for heart transplantation.

Patients and Methods

Patients

Between January 2003 and November 2013, we screened all patients admitted to our clinic for further evaluation of suspected CMi by the complete spectrum of clinical and EMB-based diagnostics. These patients complained about symptoms of heart failure with fatigue, reduced physical capacity or dyspnea on exertion, and cardiac dysfunction. Coronary artery disease and other possible causes of myocardial dysfunction (valvular heart disease) had been excluded by angiography and echocardiography before EMB in all patients.

Patients presenting with signs of acute myocarditis with recent onset of symptoms (eg, mimicking acute myocardial infarction with elevated serum markers of troponin T and creatine kinase/creatine kinase-MB) were excluded, as well as those with proof of intramyocardial genomes of enterovirus, adenovirus, human herpesvirus 6, Epstein-Barr virus, or erythrovirus (B19V) (primers used to test the presence of viral genome in Table S1). Other exclusion criteria were antiviral or immunosuppressive therapy in the past, clinical or biochemical evidence for concomitant chronic inflammatory disease (eg, rheumatological disorders), inability to understand the consent form, or participation or consent to participate in another study. EMBs from the right ventricular septum were obtained. Left ventricular ejection fraction (LVEF) was determined by echocardiography.

The follow-up period was up to 10.1 years (median 0.47 years; interquartile range, 0.03–2.56 years). Information on vital status was obtained from direct contact with the patient or official resident data files. The demographic and clinical characteristics of these 2389 patients (1655 men, 734 women) are summarized in Table 1.

Ethical Approval

The study was approved by the local ethics committees of the participating clinical centers and the committees of the respective federal states. Informed written consent was obtained from each study patient and the protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Myocardial Inflammation

Up to 8 EMBs were obtained from the right ventricular septum. Histology was developed by hematoxilin eosin staining in light microscopy. For immunohistological evaluation, specimens were embedded in Tissue Tec (SLEE Medical) and immediately snap-frozen in methylbutane, which had been cooled in liquid nitrogen, and then stored at $-80°C$ until processing. Embedded specimens were cut serially into cryosections of 5-mm thickness and placed on 10% poly-L-lysine-precoated slides. Immunohistochemistry was used for the characterization of inflammatory infiltrates. Myocardial inflammation was diagnosed by >14.0 lymphocytes/mm², including >7.0 $CD3^+$ lymphocytes/mm² according to the European Society of Cardiology guidelines.[1] Furthermore, we analyzed macrophages (threshold >35.0 $CD11b^+/Mac-1^+$ macrophages/mm²). Antibodies used included: $CD3^+$ lymphocytes (Dako; dilution 1:25), $CD11a^+/LFA-1^+$ lymphocytes (ImmunoTools; dilution 1:250), $CD11b^+/Mac-1^+$ macrophages (ImmunoTools; dilution 1:500), HLA-1 (Dako; dilution 1:2000), and ICAM-1 (ImmunoTools; dilution 1:800). Perforin-positive cardiac cell infiltration was defined by immunohistochemistry (clone $\delta G9$, BD Bioscience; dilution 1:150). As a secondary antibody we used enhancing EnVision peroxidase–conjugated anti-mouse antibody (DakoCytomation). Immunohistological staining was visualized using 3-amino-9-ethylcarbazole (Merck) as chromogenic substrate. Finally, slides were counterstained in hematoxylin and mounted with Kaiser's gelatinR

Table 1. Clinical and Hemodynamic Data of Study Groups

Patients	Group IA CMi (Perforin >2.9 cell/mm²)	Group IB CMi (Perforin ≤2.9 cell/mm²)	Group IC CMi (Without Perforin)	Group II Noninflammatory cardiomyopathy
No.	305	890	522	672
Age, y	51.7±15.0	50.2±14.6	48.8±14.7	50.2±14.5
Men/women	184/121	604/286	361/161	506/166
Preceding infection, No. (%)	113 (37.0)	360 (40.4)	181 (34.6)	206 (30.6)*
LVEF, %	52.0 (32.5–68.5)	46.0 (29.0–64.0)	48.0 (30.0–65.0)	46.0 (30.0–65.0)
LVEDD, mm	53.0 (47.0–61.0)	56.0 (49.0–63.0)	55.0 (49.0–63.0)	56.0 (49.0–65.0)
Dyspnea, No. (%)	249 (81.6)	719 (80.7)	380 (72.7)	385 (52.2)*
NYHA class I, No. (%)	…	…	…	…
NYHA class II, No. (%)	166 (54.4)	480 (53.9)	280 (53.6)	270 (40.1)
NYHA class III, No. (%)	83 (27.2)	239 (26.8)	100 (19.1)	115 (17.1)*
NYHA class IV, No. (%)	…	…	…	…
Fatigue/reduced capacity, No. (%)	217 (71.1)	705 (79.2)	412 (78.9)	490 (72.9)
ICD/pacemaker, No. (%)	36 (11.8)	98 (11.0)	71 (13.6)	89 (13.2)
Cardiovascular risk				
Smoking, No. (%)	110 (36.0)	297 (33.3)	180 (34.4)	252 (37.5)
Diabetes mellitus, No. (%)	31 (10.1)	98 (11.0)	50 (9.5)	60 (8.9)
Arterial hypertension, No. (%)	22 (7.2)	59 (6.6)	46 (8.8)	57 (8.4)

Data are presented as mean±SD, median and range (75–95%), or number (percentage) of patients. ICD indicates implantable cardioverter-defibrillator; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association.
*Significantly different inflammatory cardiomyopathy (CMi) (perforin >2.9 cell/mm²) vs non-CMi.

(Merck). The staining and peroxidase reactions in all samples were performed identically and in parallel for all samples. Specimens with perforin cellular infiltrates were classified as perforin positive.[6] Immunoreactivity was quantified by digital image analysis. The images for the quantification of infiltrates were grabbed at ×200 magnification. The calculated objects were related to the unit Heart Area (mm²).

We divided the overall cohort in group I with immunohistological evidence of myocardial inflammation and designated it "confirmed CMi". Group I with confirmed CMi was subdivided (group IA and group IB) at a cutoff value of 2.9 cells/mm² for perforin-positive cardiac cell infiltration, and group IC without perforin, according to our previous study.[6] There, we presented 95% confidence intervals (CIs) for sensitivity and specificity and calculated the optimal cutoff point (2.95 with 94.2% sensitivity and 80.4% specificity) according to the maximal Youden index with a high risk for LVEF deterioration. Group II included those without evidence of myocardial inflammation and were designated non-CMi (Table 2).

Statistical Analyses

Data are shown as means and SDs. The nonparametric Mann–Whitney *U* test was used for group comparisons. Variables

predicting survival were identified via Kaplan–Meier analysis and log-rank test in a first step. Adjustment of potential confounders such as baseline LVEF and parameters significantly differing in the univariate analyses was performed using multiple Cox regression analysis with backward and forward selection. Thus, with perforin as the independent variable, baseline LVEF, left ventricular end-diastolic diameter, CD3, CD45, sex, and age as potentially confounding covariates were included into the Cox regression model before backward and forward selection. In order to statistically confirm an observed mutually reinforcing effect of (male) sex and (high) perforin in the CMi group, a cox regression analysis with a perforin-sex interaction term, adjusted for the known additional predictors, age and LVEF, was performed. A probability value of <0.05 was considered statistically significant. No Bonferroni correction was been performed. All statistical analyses were performed with SPSS (version 22.0; IBM Corp), STATA.13 (Stata Corporation), and Prism7 (PRISM).

Results

Clinical and immunological data of the study patients (n=2389, mean age 50.5±14.7 years) are summarized in Table 1. Table 1 encompasses 2 patient groups. Patients in

Table 2. Histological and Immunohistological Data of Study Groups

Patient	Group IA CMi (Perforin >2.9 cell/mm²)	Group IB CMi (Perforin ≤2.9 cell/mm²)	Group IC CMi (Without Perforin)	Group II Noninflammatory cardiomyopathy
Cardiomyocyte diameter, mm	20.0 (18.0–22.0)	19.0 (17.0–22.0)	20.0 (16.0–22.0)	20.0 (17.0–23.0)
CD3⁺, cells/mm²	8.7 (5.3–13.4)	7.5 (3.9–11.0)	5.4 (2.5–9.4)	2.1 (1.0–3.6)*
Mac-1⁺, cells/mm²	44.6 (30.7–61.8)	36.8 (27.2–48.0)	32.4 (21.8–44.2)	15.7 (10.6–21.0)*
LFA-1⁺, cells/mm²	21.5 (13.9–29.3)	15.8 (10.7–25.3)	14.8 (9.0–23.0)	5.8 (3.4–8.0)*
Perforin⁺, cells/mm²	4.5 (3.4–7.0)	1.1 (0.7–1.8)	0	0 *
HLA-1/AF, %	8.4 (6.5–10.2)	7.5 (5.9–9.2)	6.9 (5.4–8.8)	5.1 (4.0–6.6)*
ICAM-1/AF, %	2.9 (1.9–3.9)	2.3 (1.6–3.2)	2.0 (1.4–2.8)	1.1 (0.9–1.9)*
VCAM-1/AF, %	0.09 (0.04–0.14)	0.05 80.02–0.11)	0.04 (0.02–0.08)	0.03 (0.01–0.06)*

Data are presented as mean±SD, median and range (75–95%), or number (percentage) of patients. AF indicates area fraction; Perforin⁺, perforin above the optimal cutoff value (2.9 cells/mm²) as previously defined[6]; HLA-1, human leukocyte antigen-1; ICAM-1, intercellular cell adhesion molecule-1; LFA-1, lymphocyte function–associated antigen-1; Mac-1, macrophage-1 antigen; VCAM-1, vascular cell adhesion molecule-1.
*Significantly different inflammatory cardiomyopathy (CMi; perforin >2.9 cell/mm²) vs non-CMi.

group I had immunohistological evidence of myocardial inflammation and were designated "confirmed CMi" (n=1717). Patients in group II included those without evidence of myocardial inflammation and were designated "non-CMi" (n=672). There were no significant differences between these 2 groups with regard to baseline LVEF (47.4±21.0% versus 48.1±20.3%, P=0.5), age (50.1±14.7 years versus 50.2±14.5 years, P=0.6), or sex. Group I with confirmed CMi was subdivided (group IA and group IB) at a cutoff value of 2.9 cells/mm² for perforin-positive cardiac cell infiltration according to our previous study,[6] where this value was statistically calculated as the optimal cutoff point regarding prediction of a high risk for LVEF deterioration. A third group (group IC) had evidence of myocardial inflammation but no perforin (Table 2). There was no significant difference between the 3 groups regarding clinical or hemodynamic data.

Multivariate statistical analysis (Table 3) of all examined clinical and immunological parameters was conducted for the entire CMi cohort of 1717 patients to identify predictors of all-cause mortality or need for heart transplantation. Multiple Cox regression analysis revealed (besides LVEF and age) that

Table 3. Multivariable Analysis of Factors Influencing Survival Rate

Group	Parameter	Hazard Ratio	95% CI for Hazard Ratio	P Value
I	Perforin >2.9	1.881	1.177–3.008	0.008
	Sex	1.863	1.096–3.168	0.022
	Age	1.031	1.014–1.049	0.000
	LVEF	0.989	0.979–1.000	0.042
II	LVEF	0.977	0.956–0.999	0.043

CI indicates confidence interval; LVEF, left ventricular ejection fraction.

perforin was a strong predictor of impaired survival, with a hazard ratio of 1.881 (95% CI, 1.177–3.008; P=0.008) (Table 3). Figure 1A shows 4 survival curves: first patients with CMi with perforin above 2.9 cells/mm², second CMi patients with perforin below 2.9 cells/mm², third patients with CMi without perforin, and fourth patients with non-CMi.

To investigate whether either the existence of intramyocardial inflammation with high perforin or the severity of baseline cardiac dysfunction were predictive of survival in univariate analyses, Figure 1B shows that during the follow-up period, patients with CMi with increased perforin but low cardiac dysfunction (LVEF >35%) had a significantly increased risk of cardiovascular death in contrast to those without intramyocardial inflammation. Of note, patients with CMi with increased perforin and severe cardiac dysfunction had the worst prognosis (non-CMi, LVEF >35% versus CMi perforin positive, LVEF >35% [P=0.004]; non-CMI, LVEF ≤35% versus CMi perforin positive, LVEF ≤35% [P=0.02]).

In addition, perforin and LVEF are significant predictors in patients with CMi as indicated by multivariable analysis (Table 3).

Unexpectedly, male sex emerged as another strong predictor of adverse survival in group I patients with confirmed CMi, with a hazard ratio of 1.863 (CI, 1.096–3.168; P=0.022) (Table 3, Figure 2). Age- and LVEF-adjusted Cox regression analysis revealed a significant mutually reinforcing interaction between inflammation and sex. Whereas LVEF course was adversely affected by myocardial perforin,[6] multivariable analysis shows that LVEF explains only part of the observed overall mortality.

Immunohistochemical staining verifies that perforin-positive cells attacked their targets, gradually beginning colocalized cardiomyocyte destruction. Representative aspects of immunohistologically detected infiltrates are shown in Figure 3.
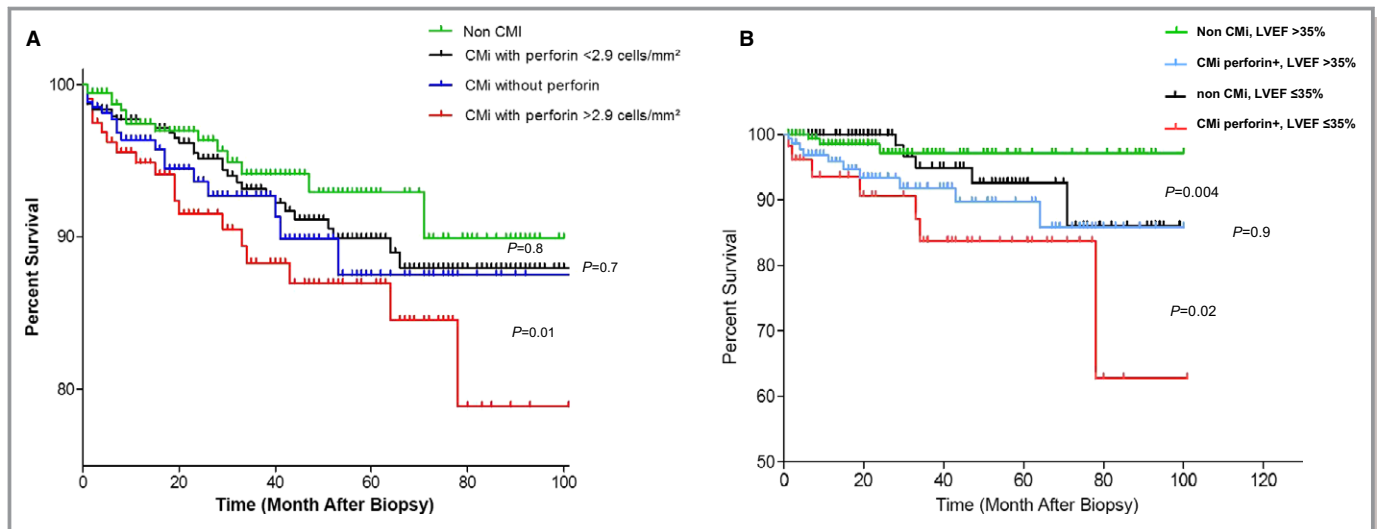
Figure 1. A, Kaplan–Meier analysis of patients with noninflammatory cardiomyopathy and inflammatory cardiomyopathy (CMi) for parameters with significant influence on survival by multivariable statistical analysis. Survival rate according to perforin analysis in endomyocardial biopsies of patients with CMi (group IA, IB, and IC) compared with patients with non-CMi (group II). Perforin at the optimal cutoff point of 2.9 cells/mm^2 was associated with increased mortality or need for heart transplantation. Non-CMi vs CMi without perforin, P=0.8 (not significant [ns]); CMi with perforin <2.9 cells/mm^2 vs CMi without perforin, P=0.7 (ns); CMi with perforin <2.9 cells/mm^2 vs CMi with perforin >2.0 cells/mm^2, P=0.01. B, Survival rate among patients with non-CMi and patients with CMi and high perforin (group IA) according to baseline left ventricular ejection fraction (LVEF) >35% vs LVEF ≤35%. Perforin and LVEF are significant predictors in this patient group as indicated by multivariable analysis (Table 3). Non-CMi, LVEF >35% vs CMi perforin$^+$, LVEF >35%, P=0.004; non-CMi, LVEF ≤35% vs CMI perforin$^+$, LVEF ≤35%, P=0.02.

Discussion

In a previous study,[6] we found in a large cohort of 495 patients with EMB-proven myocardial inflammation that left ventricular function rapidly deteriorates in patients with perforin-positive cardiac cell infiltration above a calculated critical cutoff value of 2.9 cells/mm^2 despite continued use

Figure 2. Survival rate among patients with inflammatory cardiomyopathy (CMi) according to sex. Male sex emerged as another strong predictor of adverse survival in patients in group I with confirmed CMi.

of heart failure medication. In contrast, perforin infiltration <2.9 cells/mm^2 or lack of perforin-positive cardiac infiltrates in first EMBs was associated with spontaneous LVEF improvement. However, the possible influence of perforin-positive cardiac cell infiltration on survival in patients with CMi has not yet been studied.

The current study shows that high perforin-positive cardiac cell infiltration (>2.9 cells/mm^2 perforin-positive status) is not only a risk factor for progression of cardiac dysfunction, but also a significant independent risk factor for mortality or need of heart transplantation. Whereas LVEF course is known to be adversely affected by myocardial perforin,[6] multivariable analysis in the current large CMi patient cohort revealed that LVEF explains only part of the observed overall mortality rate. The current study clearly demonstrates a significant influence of a peculiar cardiac inflammatory status (high perforin-positive cell infiltration) on survival in human myocardial disease. To our knowledge, this has been shown only in animal models of cardiomyopathies.[7] Unexpectedly, male sex emerged as another independent strong adverse predictor of survival in CMi (hazard ratio, 1.863; CI, 1.096–3.168 [P=0.022]).

Beyond this, the current data are consistent with the hypothesis that "local" cardiac perforin-positive status reflects a peculiar status of a patient's immune system in general. "General" perforin-positive status would then not only predispose the patient to accelerated perforin-positive cell migration into the heart, but also to a generally aggravated perforin-
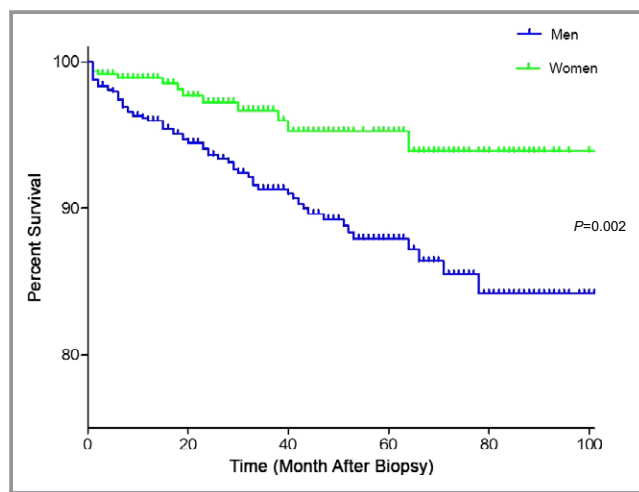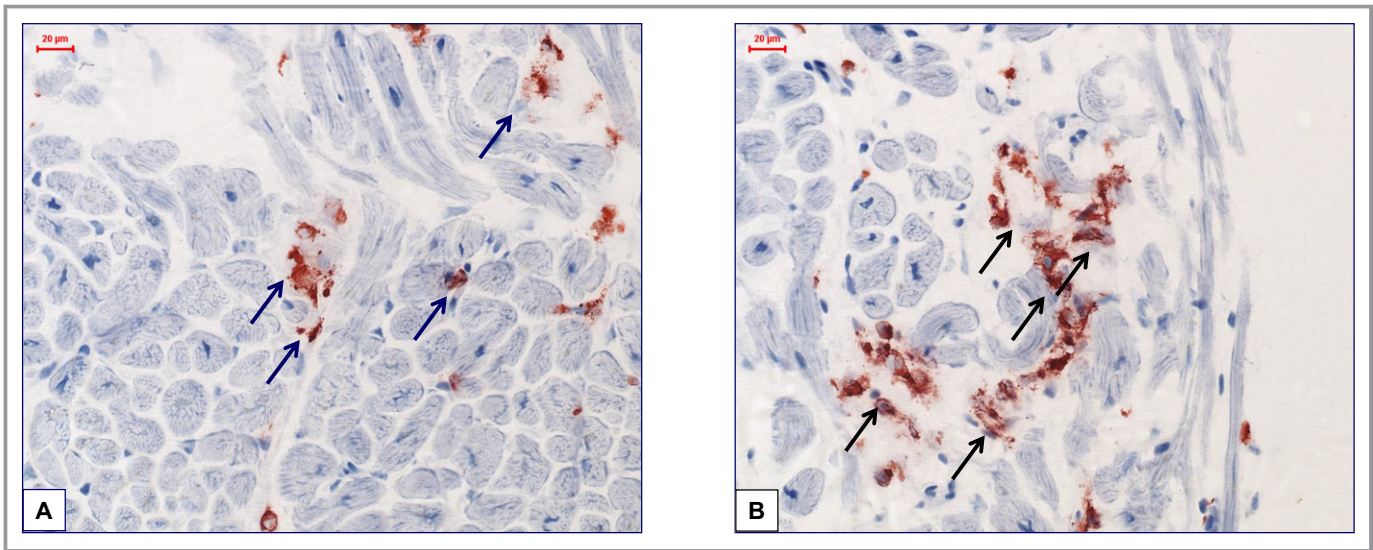
Figure 3. Representative images of immunohistological staining from frozen samples. A and B, Increased perforin-positive cardiac cell infiltration with focal infiltration pattern and partially beginning of cardiomyocyte destruction in a patient with perforin-positive inflammatory cardiomyopathy (×400).

associated response to diverse injuries. In fact, several important recent studies have shown aggravated perforin-associated immunological responses in genetically perforin-deficient or otherwise perforin-depleted animal models and in patient cohorts with myocardia,[6–13] vascular,[14–16] and other diseases.[17,18] Across several important cardiovascular pathologies, perforin-mediated mechanisms play important roles during pathogenesis. Thus, a perforin-mediated mechanism controls cardiac inflammation in Chagas disease,[12] perforin is an important marker of cardiac transplant rejection,[9,13] and CD4-positive natural killer T cells potently augment atherosclerosis by perforin- and granzyme B–dependent cytotoxicity.[14–16]

Additional knowledge regarding the immune cell populations that contribute to the complement of perforin-positive cells detected and quantitated in our patients' EMBs could provide novel insight into CMi etiology at the cellular level. In a post hoc subgroup analysis of 230 patients, there was no significant correlation between the numbers of CD8-positive cells and perforin-positive cells (P=0.3, r=0.07) (Figure S1), suggesting that natural killer cells are a key component of inflammatory activation in patients with CMi. The prognostic role of standard immunohistochemically markers (CD3-positive cells, macrophages, and human leukocyte antigen) was demonstrated in regard to cardiovascular death and need for heart transplantation, but perforin staining was not employed in these studies.[19,20] Regarding the possible origins for the observed spectrum of patients with CMi who had high to undetectable cardiac perforin, genetic polymorphisms may account for differences in perforin-associated immune processes.[21–23]

Thus, there are sex-associated differences of perforin polymorphisms in the susceptibility to multiple sclerosis,[22]

but there are no data thus far regarding a role in cardiovascular diseases. In addition to such genetically determined differences in an individual's immune constitution, there are well-known grave sex differences regarding inflammatory processes in general.[22,23,24,25,26] In mice, there are gross differences between male and female mice with identical genetic backgrounds regarding the extent of myocardial inflammation.[27] From these animal data, it might be deduced that the adverse survival effect of men with CMi (hazard ratio, 1.863; CI, 1.096–3.168 [P=0.022]) also reflects an unfavorable immune response in men. Statistical analysis did not, however, detect significant interaction between male sex and perforin-positivity in humans, but rather important sex differences in the general immunological responses leading to the observed outcome differences in patients with CMi.

Conclusions

Cardiac perforin-positive status is associated with progressive cardiac dysfunction and impaired survival in patients with CMi and should be routinely measured to improve prognosis assessment in these patients. Our study has high and immediate clinical impact. First, perforin-positive status should prompt the clinician to conduct clinical surveillance at narrow intervals. Beyond previous data regarding cardiac dysfunction progression, the new survival data reported here support inclusion of cardiac perforin-positive status into the routine evaluation of EMBs. Second, the indication for immunosuppressive therapy is thus far commonly based only on a standard set of myocardial inflammation markers.[28–32] Recent studies have demonstrated that immunosuppressive

treatment of patients with virus-negative CMi results in improved LVEF during long-term follow-up and significant reduction of cytotoxic cells.[33] Controlled prospective immunomodulating clinical trials are needed to clarify whether perforin is also a predictor of responsiveness to therapy, beyond being a marker of disease course and survival. From a scientific perspective, the origins of differential cardiac perforin infiltration need to be further investigated.

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Disclosures

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Supplemental Material

Table S1. Primer used to test the presence of viral genome.

name	virus	forward/reverse	sequenz 5´ - 3´
ADV1	ADV	forward	5´- ACT ACA AYA TTg gCT ACC Agg -3
ADV2	ADV	reverse	5´- CAA AAC ATA AAg AAg KgT ggg C -3
ADV3	ADV	forward	5´- AAC TTC CAg CCC ATg AgC Mg -3´
ADV4	ADV	reverse	5´- CTC AAA AgT CAT gTC BAg CgC -3´
COX1	Coxsackie	forward	5´-Cgg TAC CTT TgT gCg CCT gT -3´
COX2	Coxsackie	reverse	5´-CAg gCC gCC AAC gCA gCC-3´
COX3	Coxsackie	forward	5´-CCC Cgg ACT gAg TAT CAA TA-3´
COX4	Coxsackie	reverse	5´-ggC CgC CAA CgC AgC CAC Cg-3´
EBV1	EBV	forward	5'-CAA gCT TTT gAC CAA gCT ACC-3'
EBV2	EBV	reverse	5'-Cag Cag TTg CTT AAA CTT ggC-3'
EBV3	EBV	reverse	5'-Agg CCA gCT AAC TgC CTA TC-3'
EBV4	EBV	forward	5'-AAT gTC TgC TAg CTg TTg TCC-3'
U94A	HHV6	forward	5'-CATCgCATACgTCTCCCAg-3'
U94B	HHV6	reverse	5'-TCTCTAACgTgYCCgTgCC-3'
U94C	HHV6	forward	5'-CCCATTggAACTgTggTCT-3'
U94D	HHV6	reverse	5'-YAgRgATATgCACTCACCg-3'
PVB1	B19V	forward	5´- AgC ATg Tgg AgT gAg ggg gC -3´
PVB2	B19V	reverse	AAA gCA TCA ggR gCT ATA CTT CC
PVB3	B19V	forward	5'-gCC AAY TCT gTR ACY TgT AC-3'
PVB4	B19V	reverse	AAA TAT CTC CAT ggK gTT gAg

ADV – Adenovirus; COX – Coxsackievirus; EBV – Ebstein-Barr virus; HHV6 – Human Herpes virus 6; B19V – parvovirus B19.

Figure S1. Regression analysis of perforin-positive cells vs. CD8-positive T cells in a subgroup of 230 patients