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Elevated Endomyocardial Biopsy Macrophage-Related Markers in Intractable Myocardial Diseases

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Abstract—Tissue macrophages can be activated by endogenous danger signals released from cells that are stressed or injured, leading to infiltration of inflammatory macrophages and neutrophils. We postulated that macrophage-related markers might be closely associated with the existence of endogenous danger signals, reflecting ongoing tissue injury in the absence of foreign substances. This study was designed to assess the ability of macrophage-related markers in endomyocardial biopsies to predict ongoing cardiac injury in non-inflammatory myocardial diseases. We examined levels of macrophage-related markers (CD68, CD163, CD45) in endomyocardial biopsies from patients ($n=86$) with various myocardial diseases by quantitative reverse transcription-polymerase chain reaction ($n=78$) and immunohistochemistry ($n=56$). Thirty-three patients without inflammatory cardiac disease such as myocarditis and sarcoidosis were classified as "improved" or "non-improved" defined as a 10 % increase in left ventricular ejection fraction by echocardiograph and a value greater than 30 % at the time of follow-up. All macrophage-related (MacR) markers levels were not higher in non-improved dilated cardiomyopathy (DCM) patients than improved patients. However, patients with cardiac amyloidosis, cardiac Fabry disease, mitochondrial cardiomyopathy, and biventricular arrhythmogenic right ventricular cardiomyopathy (ARVC), which were categorized as "non-improvement diseases," had elevated macrophage-related markers compared to improved patients. Macrophage-related markers levels were increased in endomyocardial biopsy samples of patients with intractable myocardial diseases such as amyloidosis, mitochondrial disease, Fabry disease, and biventricular ARVC.

KEY WORDS: cardiomyopathy; endomyocardial biopsy; gene expression; inflammation; pathology.

INTRODUCTION

Recent studies in organisms have shown that even when foreign substances do not exist within a tissue, macrophages and dendritic cells, etc. can be activated by endogenous danger signals released from cells that are stressed or injured [1–4]. Endogenous danger signals are designated as damage-associated molecular patterns (DAMPS) in contrast to pathogen-associated molecular patterns (PAMPS). DAMPS can stimulate tissue resident macrophage and dendritic cells via pattern recognition receptors such as Toll-like receptors (TLR), as is also the case with PAMPS. When stimulated by DAMPS, cells produce proinflammatory cytokines and chemokines and then attract neutrophils and monocytes/macrophages to the lesion site [1, 2]. In such cases, it appears that these elevated cell-related markers indicate the existence of ongoing tissue damage and injury due solely to DAMPS in the absence of PAMPS.

In normal myocardium, similar to other organs, tissue resident macrophages and dendritic cells apparently exist [5, 6] and are reported to constitute 10–15 % of cells isolated from normal rodent hearts [7, 8], a fact not well appreciated by most cardiologists. When endomyocardial biopsy is performed for diagnosis of myocardial disease, the finding of numerous inflammatory cells generally

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indicates the presence of inflammatory myocardial disease such as myocarditis and cardiac sarcoidosis. In most cases, the existence of only a few inflammatory cells is thought to represent a relatively trivial change of little relevance in the diagnosis of myocardial disease. However, since DAMPS can stimulate tissue resident macrophages and dendritic cells and attract inflammatory cells as described above, finding a few inflammatory cells may be pathologically meaningful and indicate currently ongoing myocardial damage and injury. If so, inflammatory cell-related markers may be useful for evaluating myocardial disease progression. Almost all of various DAMPS are difficult to directly detect in endomyocardial biopsy samples because many nuclear and cytoplasmic molecules in cardiomyocytes are thought to be able to become DAMPS [2, 8]. Moreover, because DAMPS originally exist in normal cells and DAMPS released from injured cells initiate and extend sterile inflammation, it is difficult to detect only extracellular DAMPS in endomyocardial biopsy samples. Therefore, we focused not on DAMPS but on inflammatory cell-related markers.

It is known that myocardial diseases caused by various factors have different clinical courses, outcomes, and drug responsiveness. Some patients are improved by medication for heart failure such as β blocker, others are not improved, and others are worsened. Patients who experience little improvement may require intervention using devices such as implantable cardioverter-defibrillators and cardiac resynchronization therapy. Accordingly, prediction of response to drug therapy and progression of disease is important for managing patients with myocardial diseases [9]. Analysis of endomyocardial biopsy samples is a promising prognostic tool [10], but its utility for this purpose has not yet been fully established.

For the abovementioned reasons, in this study, we tested the hypotheses that analysis of macrophages in endomyocardial biopsy samples might distinguish progressive from non-progressive myocardial disease. We examined MacR markers such as CD68, CD163, and CD45 in myocardium samples by quantitative reverse transcription-polymerase chain reaction (qPCR) and immunohistochemistry (IH) and analyzed the association between MacR markers levels and the clinical courses of different types of myocardial disease.

MATERIALS AND METHODS

Study Subjects

The study subjects were 86 patients with myocardial disease admitted to Niigata University Medical and Dental Hospital ($n=85$) and Niigata Prefectural Shibata Hospital ($n=1$) between 1996 and 2014. All subjects underwent endomyocardial biopsy of the right ventricle ($n=84$) or left ventricle ($n=2$), and one to three biopsy samples were obtained. Gene expression analysis ($n=78$) using qPCR and IH analysis ($n=56$) was performed (Table 1), with both methods being employed in 48 patients. Sequential echocardiographic left ventricular ejection fraction (LVEF) was carried out in 33 patients at the time of biopsy (LVEF <55 %) and three or more months later (Table 2). None of these patients were treated with immunosuppressant drugs, coronary intervention, or cardiac resynchronization therapy. Biopsy samples of all 33 patients were analyzed by qPCR and in 21 patients (63.6 %) by IH. As shown in Table 2, 26 of the 33 patients (78.8 %) were diagnosed as having DCM including dilated phase of hypertrophic cardiomyopathy. We classified patients that showed both a

Table 1. Characterization of Patients Undergoing Endomyocardial Biopsy

	Total	qPCR	IH
	n= 86	*n= 78*	*n= 56*
Age (years)	52.2±14.0	52.7±13.1	52.1±14.3
Sex			
Male/female	64/22	56/22	41/15
Basic cardiac disease			
Acute myocarditis	8 (9.3 %)	6 (7.7 %)	6 (10.7 %)
Myocarditis associated with collagen disease	2 (2.3 %)	2 (2.6 %)	0 (0 %)
Post-myocarditis cardiomyopathy	1 (1.2 %)	1 (1.3 %)	1 (1.8 %)
Cardiac sarcoidosis	8 (9.3 %)	8 (10.3 %)	5 (8.9 %)
Cardiac amyloidosis	2 (2.3 %)	2 (2.6 %)	2 (3.6 %)
Mitochondrial cardiomyopathy	2 (2.3 %)	2 (2.6 %)	2 (3.6 %)
Cardiac FD	1 (1.2 %)	1 (1.3 %)	1 (1.8 %)
Biventricular ARVC	1 (1.2 %)	1 (1.3 %)	1 (1.8 %)
DCM	47 (54.7 %)	42 (53.8 %)	28 (50.0 %)
Hypertrophic cardiomyopathy	7 (8.1 %)	6 (7.7 %)	6 (10.7 %)
Restrictive cardiomyopathy	1 (1.2 %)	1 (1.3 %)	1 (1.8 %)
Tachycardia-induced cardiomyopathy	3 (3.5 %)	3 (3.8 %)	1 (1.8 %)
Peripartum cardiomyopathy	1 (1.2 %)	1 (1.3 %)	1 (1.8 %)
Takotsubo cardiomyopathy	1 (1.2 %)	1 (1.3 %)	1 (1.8 %)
Alcoholic cardiomyopathy	1 (1.2 %)	1 (1.3 %)	0 (0 %)

Values are mean±SD or *n* (%). *Italicized data* indicate number of patients analyzed by qPCR and IH

Table 2. Characterization of Improved and Non-Improved Patients without Inflammatory Cardiac Disease such as Myocarditis and Sarcoidosis

	Improvement		Non-improvement	
	n = 22 (66.7 %)		*n* = 11 (33.3 %)	
	qPCR	IH	qPCR	IH
	n = 22 (66.7 %)	*n = 11 (52.4 %)*	*n = 11 (33.3 %)*	*n = 10 (47.6 %)*
Age years	57.3 ± 9.8	58.2 ± 8.2	54.8 ± 12.6	53.5 ± 12.4
Sex				
Male/female	17/5	8/3	9/2	8/2
Observation period	14.6 ± 15.2	14.1 ± 13.2	13.8 ± 13.9	13.8 ± 14.7
LVEF at time of biopsy (%)	32.3 ± 10.9	30.8 ± 11.9	29.8 ± 11.2	29.6 ± 11.7
LVEF at time of follow-up evaluation (%)	57.6 ± 12.0	59.4 ± 11.2	25.0 ± 13.1***; 25.0 ± 13.1***; 25.0 ± 13.1***	24.2 ± 13.6***; 24.2 ± 13.6***; 24.2 ± 13.6***
Change of LVEF	25.3 ± 9.4	28.5 ± 9.8	−4.8 ± 15.7***; −4.8 ± 15.7***; −4.8 ± 15.7***	−5.4 ± 16.4***; −5.4 ± 16.4***; −5.4 ± 16.4***
BNP at time of biopsy (pg/ml)	292 ± 277	272 ± 290	627 ± 468*; 627 ± 468*; \627 ± 468*	581 ± 468
Drug				
β blocker	22 (100 %)	11 (100 %)	10 (90.9 %)	9 (90 %)
Bisoprolol	4 (18.2 %)	3 (27.3 %)	3 (27.2 %)	3 (30 %)
Bisoprolol (mg/day/person)	2.81 ± 0.6	2.9 ± 0.7	6.5 ± 2.1	6.5 ± 2.1
Carvedilol	17 (77.3 %)	7 (63.6 %)	8 (72.7 %)	7 (70 %)
Carvedilol (mg/day/person)	14.7 ± 6.4	13.9 ± 6.7	18.4 ± 10.6	18.2 ± 11.4
Metoprolol	1 (4.5 %)	1 (9.1 %)	0	0
Metoprolol (mg/day/person)	40	40	–	–
ACEI	11 (50.0 %)	7 (63.6 %)	7 (63.6 %)	7 (70 %)
ARB	9 (40.9 %)	5 (45.5 %)	3 (27.3 %)	2 (20 %)
Diuretic	18 (81.8 %)	10 (90.9 %)	11 (100 %)	10 (100 %)
Digitalis	1 (4.5 %)	1 (9.1 %)	1 (9.1 %)	0 (0 %)
Basic cardiac disease				
Cardiac amyloidosis			1	1
Mitochondrial cardiomyopathy			2	2
Cardiac FD			1	1
Biventricular ARVC			1	1
DCM	20	11	6	5
Tachycardia-induced Cardiomyopathy	2			

Values are mean ± SD or n (%). *Italicized data* indicate number and percentage of patients analyzed by qPCR and IH
ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker
Improved vs. non-improved patients analyzed by the same methods *$p < 0.05$, ***$p < 0.001$

greater than 10 % increase in LVEF and a value of LVEF exceeding 30 % at the time of follow-up as an "improved" and patients without them as "non-improved". Myocarditis and cardiac sarcoidosis were diagnosed by clinical and pathological findings. Mitochondrial cardiomyopathy and cardiac Fabry disease (FD) were diagnosed by gene analysis. Cardiac amyloidosis was diagnosed by pathological findings. Biventricular arrhythmogenic right ventricular cardiomyopathy (ARVC) was diagnosed by pathological findings, existence of ventricular arrhythmia of right ventricular origin, and imaging studies such as echocardiograph and nuclear magnetic resonance. Other myocardial diseases were diagnosed by clinical, pathological, and imaging findings.

Ethics and Informed Consent for Patient Studies

The ethics committee of Niigata University Medical and Dental Hospital approved the study, and all patients signed an informed consent form in relation to diagnosis by biopsy samples.

Gene Expression Analysis by qPCR

One or two biopsy samples were divided into two parts, one for gene expression analysis by qPCR and the other for histological examination. Total RNA was isolated from the materials described above using Trizol (Invitrogen, Tokyo, Japan). cDNA was synthesized from 0.5 to 5 μg of total RNA with random primers and murine

Moloney leukemia virus reverse transcriptase. To create the plasmids used for the standard, human CD45, CD68, CD163, and porphobilinogen deaminase (PBGD) mRNA were amplified from a heart-derived cDNA library using the primer pairs. Sequences of the primers were as follows: CD45, 5′-aagagagtgagcatgattcagatg-3′ (forward) and 5′-ttcaggtcaacttcaatatctcca-3′ (reverse); CD68, 5′-cagaaggttgtctacctgagctac-3′ (forward) and 5′-atgatgcagaaagcaataagcac-3′ (reverse); CD163, 5′-agaagtgagattccaaggagaatg-3′ (forward) and 5′-agtctctgaatctccacctcaact-3′ (reverse); and PBGD, 5′-ccatgtctggtaacggcaat-3′ (forward) and 5′-cttcaaggagtgaacaaccagg-3′ (reverse). PCR amplified cDNA inserts were directly inserted into the pGEM-T easy vector, and recombinant plasmids were isolated following transformation into Escherichia coli JM109-competent cells using a MagExtractor plasmid kit (Toyobo, Osaka, Japan). Absolute mRNA copy numbers were also measured by qPCR using a LightCycler instrument (Roche Diagnostics, Tokyo, Japan) together with the same primers and SYBR Premix Ex Taq (Takara, Otsu, Japan). After an initial denaturation step of 10 min at 95 °C, a three-step cycling procedure (denaturation at 95 °C for 10 s, annealing at 62 °C for 10 s, and extension at 72 °C for 13 s) was used for 45 cycles. The absolute copy numbers of particular transcripts were calculated by LightCycler software using a standard curve approach. Then the copy number ratio of each MacR marker mRNA to PBGD mRNA was calculated. PBGD mRNA in biopsy samples of 9 patients could not be detected, and we analyzed them in samples of remaining 78 patients.

IH Analysis

Biopsy samples for histological examination were fixed at room temperature in 10 % formalin. Samples were sequentially dehydrated through an alcohol series and embedded in paraffin. Sections that are 4-μm thick were cut, deparaffinized in xylene, and dehydrated in descending dilutions of ethanol. Specimens for CD45 and CD68 staining were treated by incubating them in trypsin solution at 37 °C for 15 min, and specimens for CD163 staining by incubating them in citrate buffer (pH 6.0) at 121 °C for 15 min in an autoclave. After washing in 0.01 mol/L phosphate-buffered saline (PBS), endogenous peroxidase activity was blocked by treatment for 20 min with 0.3 % hydrogen peroxidase in absolute methanol. Specimens were incubated with 10 % normal goat serum for 10 min at room temperature then reacted with mouse anti-human CD45 (clones, 2B11+PD7/26), mouse anti-human CD68 (clone, KP1) (DakoCytomation, Glostrup, Denmark), and mouse anti-human CD163 (clone, 10D6) (Leica Biosystems, Newcastle, UK) overnight at 4 °C. The samples were then incubated for 2 h at room temperature with appropriate secondary antibody (Nichirei, Tokyo, Japan). The specimens were carefully washed three times with PBS between each step of the procedure. Finally, they were visualized with 0.1 mg/ml 3,3′-diaminobenzidine (DAB) tetrahydrochloride (Dojin Chemical, Kumamoto, Japan) and counterstained with Mayer's hematoxylin. In cases with myocarditis, each MacR marker-positive cell was counted in 0.232 mm^2 biopsy sections in four random fields at ×600 magnification and in other myocardial diseases in all areas of biopsy sections at ×600 magnification. Then the numbers per unit area were calculated.

Statistical Analysis

Statistical assessment was performed using an unpaired Student's *t* test. The differences were considered significant at $p<0.05$. The data were expressed as mean ±standard error of the mean (SEM) or mean±standard deviation (SD). Numbers and ratios were transformed to a logarithmic scale for analysis. Correlations among log-transformed numbers of each of the MacR marker-positive cells per unit area and log-transformed ratios of copy number of each MacR marker mRNA to PBGD mRNA were evaluated by linear regression analysis. Data were analyzed using Pearson's correlation coefficient and Fisher's Z-transformation test.

RESULTS

Baseline Characteristics of Patients Undergoing Endomyocardial Biopsy

Patients undergoing endomyocardial biopsy were 74.4 % male, and ages ranged from 12 to 76 with a mean age of 52.2. Myocardial diseases were diagnosed as shown in Table 1, and 54.7 % were considered to have DCM, including dilated phase of hypertrophic cardiomyopathy, as characterized by diminished cardiac motion in the absence of critical coronary disease or of specific findings on clinical history, genetic, and histological examination.

Characterization of Improved and Non-Improved Patients Without Inflammatory Cardiac Disease

Of the 33 patients (LVEF <55 % at the time of biopsy) without inflammatory cardiac disease such as myocarditis

and sarcoidosis, 22 patients were classified as improved and 11 as non-improved (Table 2). Observation periods and LVEF at the time of biopsy did not differ significantly between the two groups. As shown in Table 2, plasma brain natriuretic peptide (BNP) levels at the time of biopsy were significantly higher in non-improved than in improved patients analyzed by qPCR ($p=0.0145$). During the follow-up period, patients were treated with conventional heart failure medications such as β blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, diuretics, and digitalis (Tables 2) but not with immunosuppressant drugs, coronary intervention, or cardiac resynchronization therapy. All patients, except for one individual with cardiac amyloidosis, were treated with β blockers. Cardiac amyloidosis, mitochondrial cardiomyopathy, cardiac FD, and biventricular ARVC were categorized as "non-improvement diseases", whereas tachycardia-induced cardiomyopathy were deemed capable of showing some resolution and categorized as "improvement diseases". In patients diagnosed as having DCM, 20 patients were improved and 6 patients were not improved (Tables 2).

Correlations Among MacR Markers Detected by qPCR and IH

To confirm the accuracy of our methods, we examined correlations between each of the MacR marker levels detected by qPCR and those detected by IH. Log-transformed numbers of each of the MacR marker-positive cells per unit area were significantly correlated with log-transformed ratios of copy numbers of each MacR marker mRNA to PBGD mRNA (Fig. 1). We also examined correlations among different MacR markers to elucidate the interrelationships of the markers in endomyocardial biopsy samples. Log-transformed ratios of copy number of MacR marker mRNA to PBGD mRNA were significantly correlated with those of other MacR marker mRNA to PBGD mRNA (Fig. 2, upper panels). Similarly, there were significant correlations between log-transformed numbers of MacR marker-positive cells per unit area and those of other MacR marker-positive cells (Fig. 2, lower panels).

MacR Marker Levels in Different Groups

We compared MacR marker levels among various myocardial diseases (Fig. 3). All MacR markers levels by both qPCR and IH were increased as anticipated in acute myocarditis (Fig. 3 and 4A). Endomyocardial biopsy samples of cardiac sarcoidosis patients diagnosed by ECG, cardiac imaging, and histological finding of sarcoidosis in other organs did not show granulomas or remarkable macrophages (Fig. 3 and 4B), and MacR markers were low (Fig. 3). However, MacR markers levels were slightly higher in some non-inflammatory myocardial diseases which were not inflammatory diseases such as myocarditis and sarcoidosis (Fig. 3).

To assess whether MacR markers can predict clinical courses in patients without inflammatory cardiac disease as listed in Table 2, we compared MacR marker levels between improved and non-improved patients (Fig. 5A). In gene expression analysis (Fig. 5A–i), ratios of MacR markers mRNA to PBGD mRNA were slightly higher but not significantly higher in non-improved than in improved patients. In IH analysis (Fig. 5A–ii), only CD45-positive cells per unit area were significantly higher in non-improved patients than those in improved patients ($p=0.0077$). CD68- and CD163-positive cells per unit area were slightly higher but not significantly so in non-improved vs. improved patients. Because all MacR markers levels tended to be higher in non-improved patients than in improved patients, we compared MacR markers levels in non-improved patients among diagnosed diseases (Fig. 5B). All MacR markers levels were not higher in non-improved DCM patients than improved

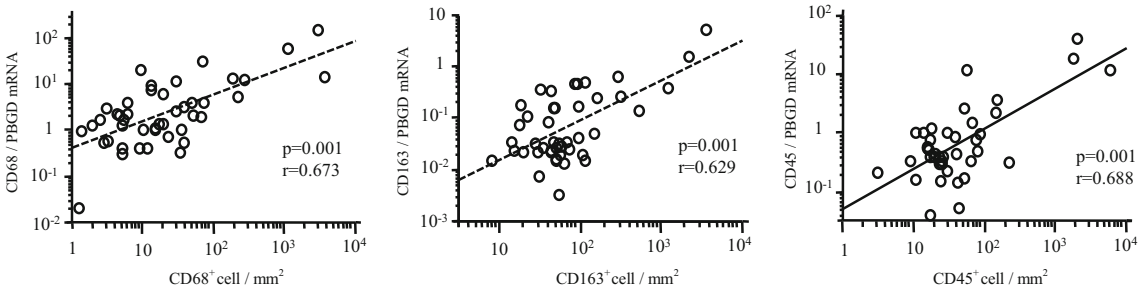

Fig. 1. Correlations between numbers of each MacR marker-positive cell per unit area and ratios of copy number of each MacR marker mRNA to PBGD mRNA. Numbers and ratios were transformed to a logarithmic scale for analysis. Data were analyzed using Pearson's correlation coefficient and Fisher's Z-transformation test.

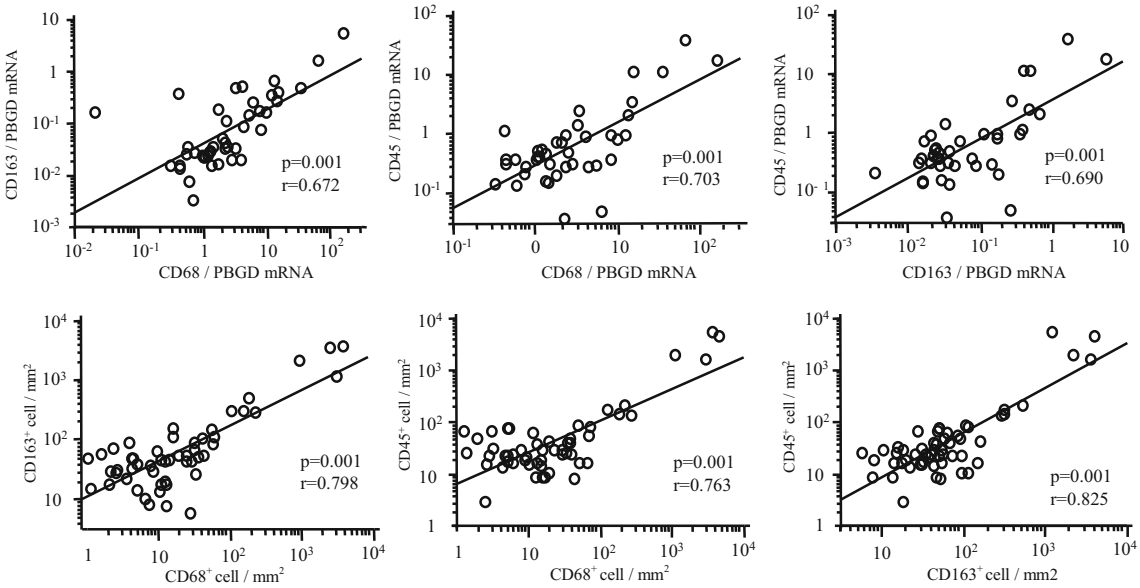

Fig. 2. Correlations between MacR markers. *Upper panels* show correlation between ratios of copy number of each MacR marker mRNA to PBGD mRNA and ratios of copy number of other MacR marker mRNA to PBGD mRNA by qPCR. *Lower panels* show correlation between numbers of MacR marker-positive cells per unit area and numbers of other MacR marker-positive cells per unit area by IH. Numbers and ratios were transformed to a logarithmic scale for analysis. Data were analyzed using Pearson's correlation coefficient and Fisher's Z-transformation test.

patients. On the other hand, all MacR marker levels measured by qPCR (Fig. 5B–i) and IH (Fig. 5B–ii) tended to be high in non-improved patients except for DCM, namely cardiac amyloidosis, mitochondrial cardiomyopathy, cardiac FD, and biventricular ARVC (Table 2).

Ratio of CD68 to CD163 Between Improved and Non-Improved Patients

Because CD163 has, in contrast to CD68, been reported to be a useful marker for anti-inflammatory or alternatively activated macrophages [11], we compared the ratio of CD68 to CD163 between improved and non-improved patients (Fig. 6). However, there was no significant difference between improved and non-improved patients (Fig. 6A). When we compared the ratio in non-improved patients among diagnosed diseases (Fig. 6B), myocardial diseases with high ratio of CD68 to CD163 by both qPCR and IH cannot be identified.

DISCUSSION

Elevated Macrophage Markers in Endomyocardial Biopsy of Intractable Myocardial Diseases

In this study, MacR markers were higher in non-improved patients except for DCM than in improved patients, but elevated MacR markers were not observed in non-improved DCM patients. These results imply that endogenous danger signals in a subset of non-improved patients except for DCM may have led to an increase in MacR markers, portending lack of responsiveness to treatment. Non-improvement diseases except for DCM in this study were intractable myocardial diseases such as cardiac amyloidosis, mitochondrial cardiomyopathy, cardiac FD, and biventricular ARVC. In this study, we showed that MacR markers were high in patients with these diseases not only by IH but also by qPCR. Analysis by qPCR of inflammatory markers for these diseases has not been reported yet. In all of these diseases, cardiac injury may gradually progress if the causes are not removed by proper therapy such as enzyme replacement for FD, etc [12–15]. Amyloidosis is a disorder of protein conformation and metabolism that results in the deposition of insoluble amyloid fibrils in tissues [16]. Amyloid fibrils formed by lysozymes have been reported to be a generic danger signal and to induce the secretion of proinflammatory cytokines through activation of the TLR-2 [17]. Moreover, mitochondrial DAMPS, which are recognized by specific pattern recognition receptors of the innate immune system, seem to be potent immunological activators [18]. Therefore, mitochondrial disease may frequently be associated with inflammation [19]. FD, which is a

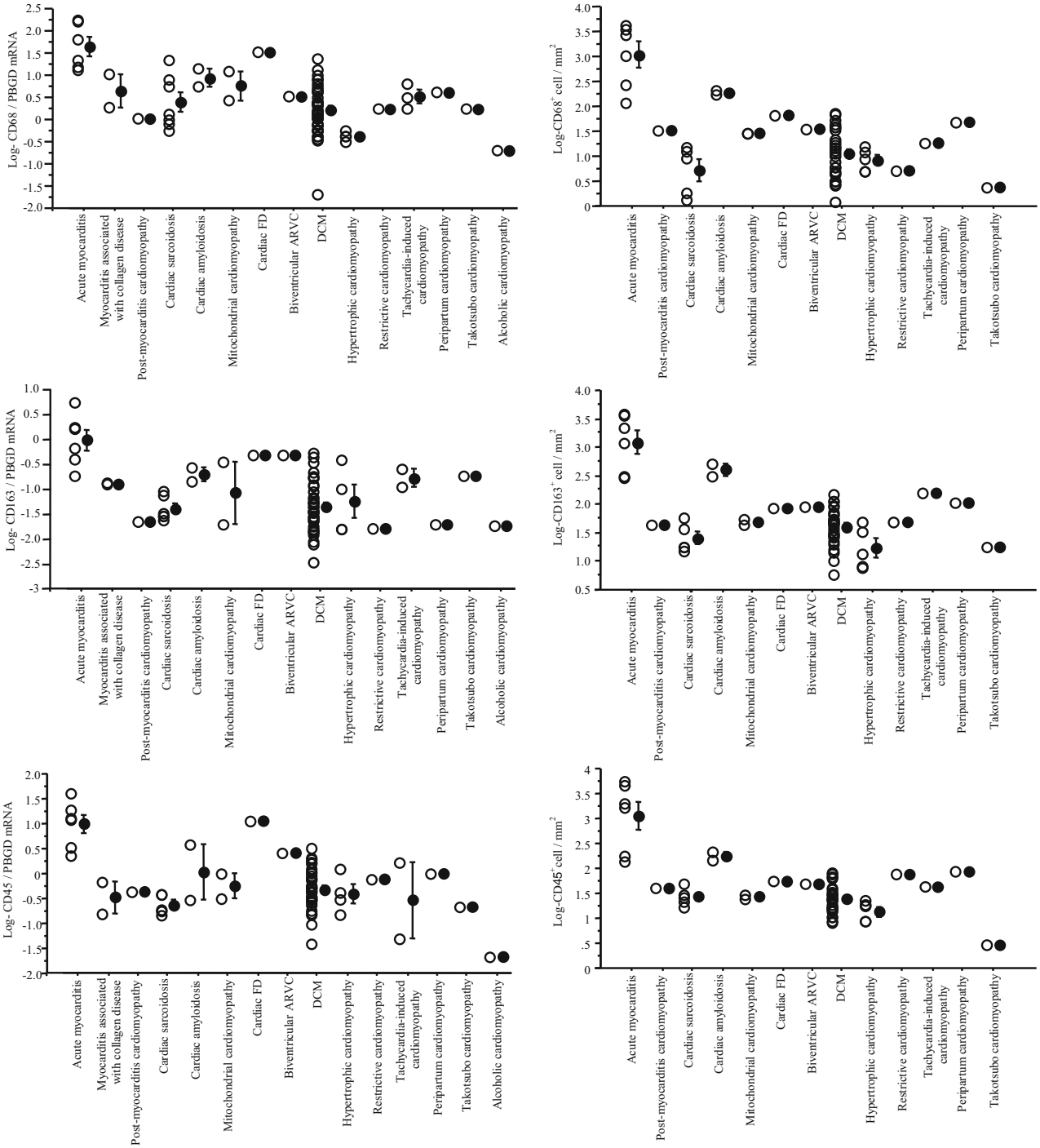

Fig. 3. MacR markers among various myocardial diseases. *Left panels* show ratios of copy number of each MacR marker mRNA to PBGD mRNA by qPCR. *Right panels* show numbers of MacR marker-positive cells per unit area by IH. Numbers and ratios were transformed to a logarithmic scale for analysis. *Closed circles* indicate the mean value, and *bars* indicate standard error of the mean (SEM) in each disease.

lysosomal storage disorder caused by a deficiency of the enzyme α-galactosidase A, results in accumulation of globotriaosylceramide, and its accumulation seems to have a proinflammatory role which is likely mediated

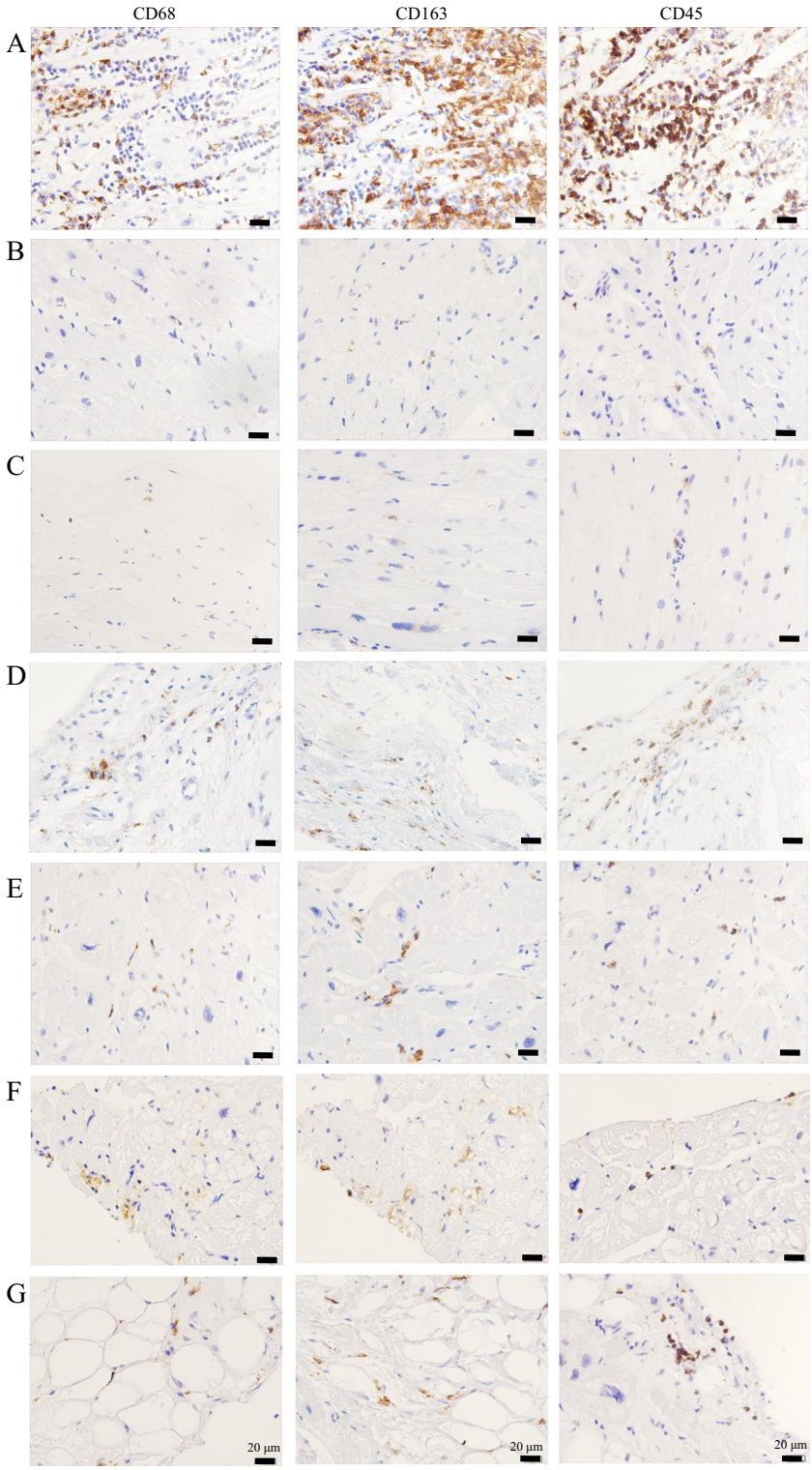

Fig. 4. Microscopic findings. Tissue sections were stained with each anti-MacR marker. **A** Acute myocarditis. **B** Cardiac sarcoidosis. **C** DCM. **D** Cardiac amyloidosis. **E** Mitochondrial cardiomyopathy. **F** Cardiac FD. **G** Biventricular ARVC. *Left panels*: sections stained with anti-CD68; *Middle panels*: sections stained with anti-CD163; *Right panels*: sections stained with anti-CD45.

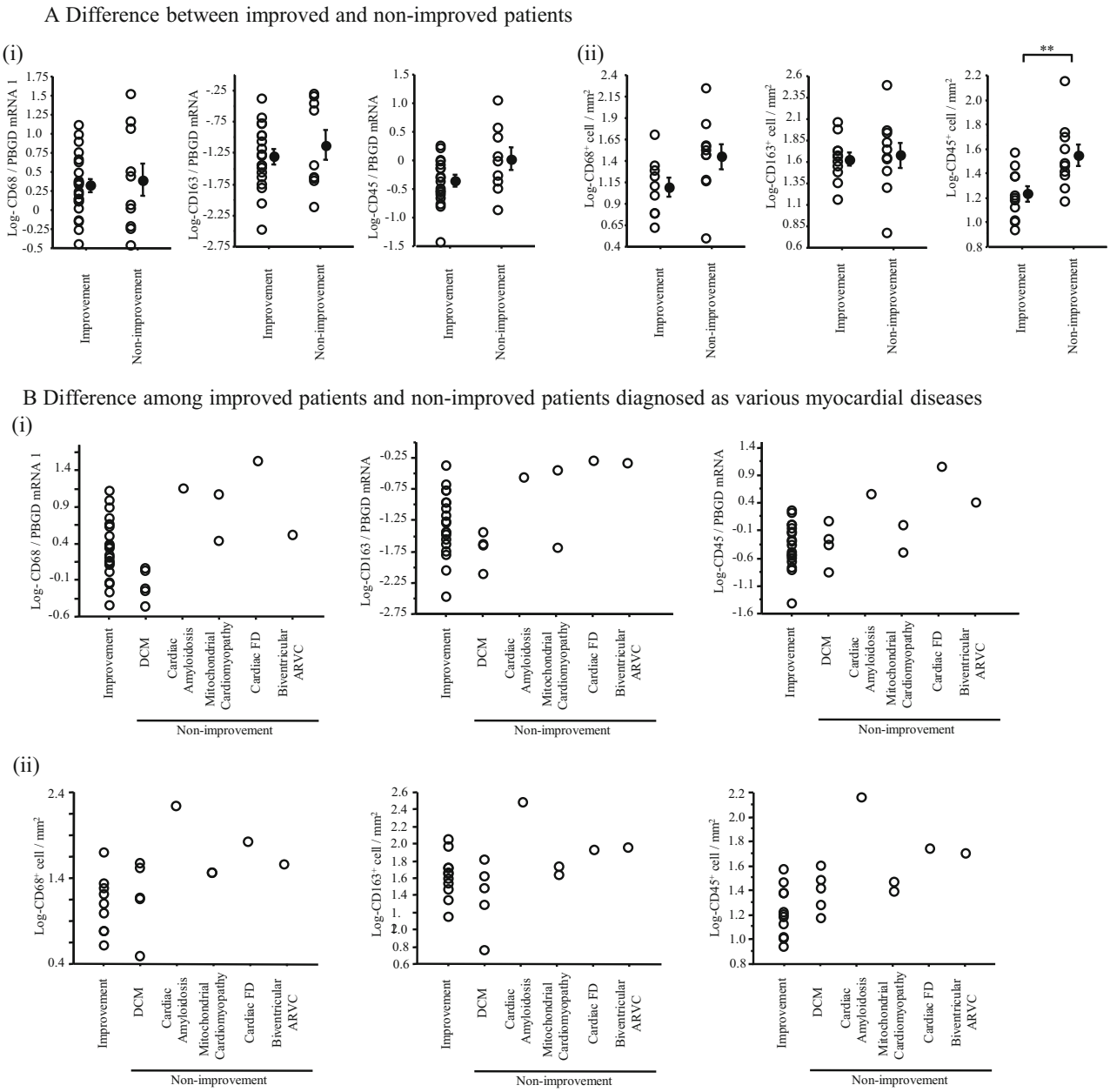

Fig. 5. MacR markers between improved and non-improved patients. Patients have myocardial diseases without inflammation such as myocarditis and cardiac sarcoidosis as shown in Table 2. **A** Levels of each MacR marker between all improved patients and all non-improved patients. Black-closed circles indicate the mean value, and bars indicate standard error of the mean (SEM) in each group. **B** Levels of each MacR marker between all improved patients and non-improved patients diagnosed as various myocardial diseases. **i** Ratios of copy number of each MacR marker mRNA to PBGD mRNA by qPCR. **ii** Numbers of MacR marker-positive cells per unit area by IH. Numbers and ratios were transformed to a logarithmic scale for analysis. Statistical analysis was performed by an unpaired Student's *t* test. $^{**}p < 0.01$.

by TLR-4 [20, 21]. A hallmark feature of ARVC is fibro-fatty replacement of right ventricular myocardium; however, inflammatory infiltrates in this chamber are also consistently reported [22, 23]. Similarly, the histology of non-alcoholic fatty liver disease, which shows fatty hepatic accumulation, also exhibits inflammatory infiltrates. The immune response in non-alcoholic fatty liver disease is thought to be initiated

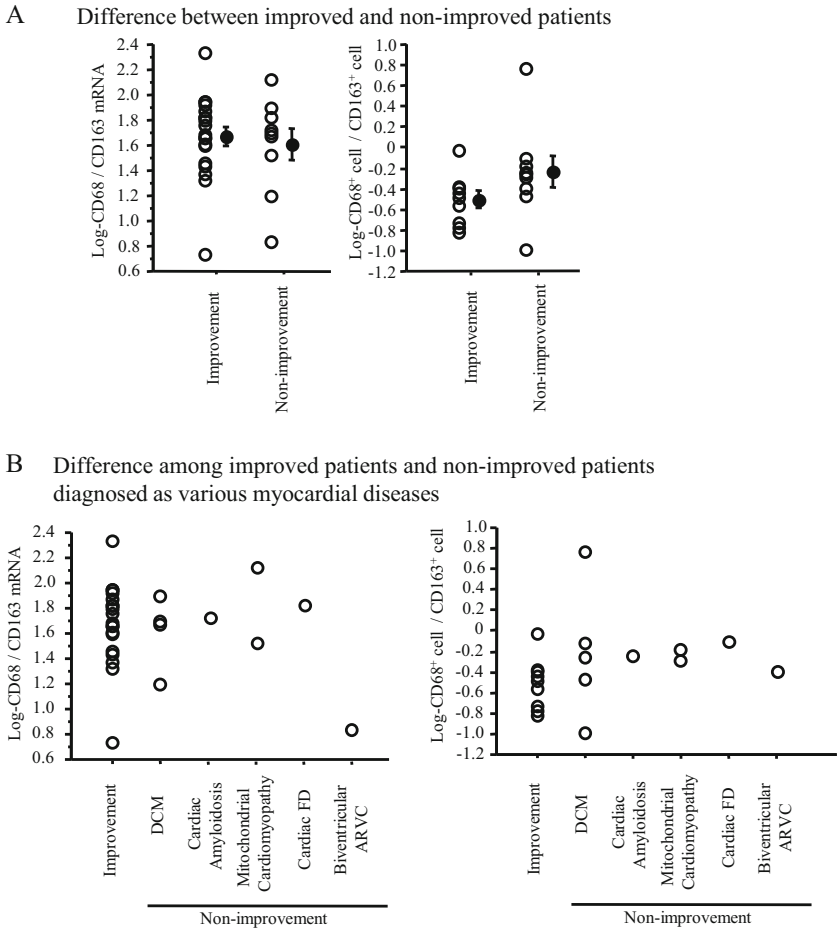

Fig. 6. Ratio of CD68 to CD163 between improved and non-improved patients. Patients have myocardial diseases without inflammation such as myocarditis and cardiac sarcoidosis as shown in Table 2. **A** Levels of ratio of CD68 to CD163 between all improved patients and all non-improved patients. *Black-closed circles* indicate the mean value, and *bars* indicate standard error of the mean (SEM) in each group. **B** Levels of ratio of CD68 to CD163 between all improved patients and non-improved patients diagnosed as various myocardial diseases. *Left panels* show ratio of CD68 to CD163 mRNA by qPCR. *Right panels* show ratio of CD68- to CD163-positive cells per unit area by IH. Ratios were transformed to a logarithmic scale for analysis. Statistical analysis was performed by an unpaired Student's *t* test.

not only by PAMPS but also by DAMPS such as free fatty acid, etc [24]. As mentioned above, intractable myocardial diseases such as cardiac amyloidosis, mitochondrial cardiomyopathy, cardiac FD, and biventricular ARVC are reported to cause inflammation via DAMPS. In this study, increased MacR markers levels are consistent with these reports. Our data may indicate that resident macrophages and inflammatory macrophages act as key players which are stimulated by DAMPS scattered from injured myocardium in these intractable myocardial diseases. Defining the role of these cells in intractable myocardial diseases may lead to the development of new diagnostic methods for determining prognosis and therapeutic outcomes.

MacR Marker in Endomyocardial Biopsy of DCM

MacR markers from non-improved DCM patients were not significantly higher than those from improved patients. We speculate about the reasons as follows: (1) There is the possibility that in this study most non-improved patients might not have had progressive myocardial diseases with continuous cardiac injury. It is known that in DCM caused by gene mutations such as lamin A/C, some sarcomeres may sustain continuous cardiac injury [25–27]. Inflammatory cellular infiltration is often observed in the patients with lamin A/C mutations [28]. However, the current study may not have included DCM patients caused by gene mutations, with the consequence that MacR markers in non-improved DCM patients were

not elevated. (2) Non-improvement of cardiac motion abnormalities may not be a good indicator of progressive DCM. A better comparison might have been the comparison between worsening and non-worsening patients. Non-improved patients may include worsening patients, but the two conditions are not synonymous. It was difficult to define worsening DCM in this study, particularly since only 6 DCM patients were in the non-improved group. Accordingly, the numbers were insufficient to analyze the difference in MacR markers between worsening and non-worsening DCM patients. (3) DCM patients in the recovery phase of myocarditis might exist in the improvement group. In this study, MacR markers in a few improved DCM patients were slightly elevated, and cardiac motion in myocarditis patients is known to improve spontaneously. Such patients in the improvement group may have been misdiagnosed and perhaps should have been properly classified as having myocarditis. To exclude DCM patients in the recovery phase of myocarditis, endomyocardial biopsy or imaging studies in lieu of biopsy could be performed at the time of recovery of cardiac motion, with remeasurement of levels of MacR markers to determine if they were decreased. It appears that several types of myocardial diseases may have been included in the DCM group. Cautious interpretation of MacR markers in DCM patients seems warranted, has pending categorization by gene analyses, has detailed clinical course, and has repeated examination.

Detailed Analysis of Immunological Molecules in Endomyocardial Biopsy Samples

Several studies reported that macrophages may be classified as M1 or M2 macrophages, namely the "M1-M2 paradigm" [29] with M2 macrophages also playing a role in immunoregulation [30]. However this concept has often been overinterpreted in a rigid functional classification of macrophages, and by adhering to this model tissue, resident macrophages may sometimes be classified as "M2-like" [31]. In the current study, ratios of CD68 as a pan-macrophage marker to CD163 as an M2-macrophage marker [32, 33] were compared between improved and non-improved patients. The ratio of CD68- to CD163-positive cells per unit area tended to be high in non-improved patients except for DCM, but the ratio of CD68 to CD163 mRNA was not high in these patients. Since the measurements were made only at one point in time, the results must be considered as inconclusive. Further studies are needed to evaluate the role played by different types of macrophages in biopsy samples of myocardial disease.

In this study, analysis by qPCR in endomyocardial biopsy samples provided measurements of mRNA levels of MacR markers. The reasons that levels of MacR markers were examined by two methods using different samples were not only to check reliability of the data by qPCR but also to reduce misunderstanding due to sampling errors. Because these data by qPCR were consistent with those obtained by IH, we considered the data to be reliable, although levels of MacR marker mRNA measured by qPCR are thought to be somewhat less elevated. The technique may permit measurement of various other immunological molecule mRNA levels in endomyocardial biopsy samples. It is hoped that these analyses can predict the clinical outcome of myocardial diseases in the future.

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Competing Interests. The authors declare that they have no competing interests.

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