


Characterization of dendritic cells in human and experimental myocarditis

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Abstract

Aims Dendritic cells (DCs) are central mediators of adaptive immunity, and there is growing evidence of their role in myocardial inflammatory disease. We hypothesized that plasmacytoid and myeloid DCs are involved in the mechanisms of myocarditis and analysed these two main subtypes in human myocarditis subjects, as well as in a murine model of experimental autoimmune myocarditis (EAM).

Methods and results Circulating DCs were analysed by flow cytometry in patients with acute myocarditis, dilated cardiomyopathy, and controls. Myocardial biopsies were immunostained for the presence of DCs and compared with non-diseased controls. In a mouse model of acute myocarditis induced through synthetic cardiac myosine peptide injection, effects of immunomodulation including DC inhibition through MCS-18 versus placebo treatment were tested at the peak of inflammation (Day 21), as well as 1 week later (partial recovery). Circulatory pDCs and mDCs were significantly reduced in myocarditis patients compared with controls ($P < 0.01$ for both) and remained so even after 6 months of follow-up. Human myocarditis biopsies showed accumulation of pDCs (two-fold CD304+/three-fold CD123+, all $P < 0.05$) compared with controls. Myocardial pDCs and mDCs accumulated in EAM (P for both < 0.0001). MCS-18 treatment reduced pDC levels ($P = 0.009$), reduced myocardial inflammation (myocarditis score reduction from 2.6 to 1.8, $P = 0.026$), and improved ejection fraction ($P = 0.03$) in EAM at Day 21 (peak of inflammation). This effect was not observed during the partial recovery of inflammation on Day 28.

Conclusions Circulating DCs are reduced in human myocarditis and accumulate in the inflamed myocardium. MCS-18 treatment reduces DCs in EAM, leading to amelioration of inflammation and left ventricular remodelling during the acute phase of myocarditis. Our data further elucidate the role of DCs and their specific subsets in acute inflammatory cardiomyopathies.

Keywords Myocarditis; Dilated cardiomyopathy; Dendritic cell; Heart failure; Experimental autoimmune myocarditis

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Introduction

Myocarditis, a common condition generally attributed to cardiotropic viruses, is an inflammation of the myocardium and a major cause of non-ischaemic heart failure (HF), as it often results in dilated cardiomyopathy (DCM).^{1,2} Despite

progress in medical and device therapy, there is still a major unmet need for curative therapies in myocarditis and DCM due to incomplete understanding of underlying pathomechanisms in these conditions. Special attention has been directed at inflammation and autoimmunity, as inflammation in myocardial biopsies has been identified as

prognostic marker.^{3–6} Inflammatory mediators such as tumour necrosis factor and interleukin-6 have been shown to have a prognostic impact on the progression of HF.⁷ Identifying key inflammatory players in HF might elucidate the pathology of disease and potentially lead to novel therapeutic strategies.

Dendritic cells (DCs) are antigen presenting cells that play a central role in adaptive and innate immunity.⁸ These key inflammatory cells are subject to intensive research in different organ systems, as understanding of their function has led, among others, to novel cancer therapies.^{9,10} While their role in HF is not sufficiently understood, there is growing evidence of their role in myocarditis and DCM.^{11–13} For instance, it is possible to induce myocarditis and DCM in mice by activating autoantibodies through immunization with cardiac myosin unleashing a DC-dependent T-cell reaction.^{14–17} Also, mice can be made resistant to experimental autoimmune myocarditis (EAM), if manipulated DCs are unable to activate T lymphocytes.¹² Our study group also found that circulating DCs decrease in the context of an inflammatory response during an acute myocardial infarction, while they accumulate into the myocardia of patients with acute myocardial infarction.¹⁸

Despite the above evidence (mainly in animal models), the role of DCs in human myocarditis and cardiomyopathies needs further clarification and deeper understanding. In several experimental animal studies of ischaemic and non-ischaemic HF, DCs were either shown to enhance disease progression, or in the contrary, to mediate protection from disease.^{18–31} We previously showed DCs to be significantly reduced in the myocardium of patients with DCM.³² Our research on circulating blood DCs also found the myeloid subtype (mDCs) to be significantly reduced in patients with chronic HF due to DCM or ischaemic cardiomyopathy (ICM).³³ In both studies, the reduction of these cells corresponded to the severity of cardiomyopathies.

In the present study, we focused on the role of DCs in myocarditis. First, circulating DCs of both the myeloid and the plasmacytoid subtype (mDCs and pDCs) were measured in patients with acute myocarditis and compared with control subjects at the time of diagnosis, as well as after a follow-up period. The same analysis was also performed in patients with newly diagnosed DCM, in order to possibly reveal differences between DCM and myocarditis. We then analysed myocardial DCs in biopsies of patients with acute myocarditis and compared them with non-diseased myocardium. Furthermore, we assessed myocardial DCs in a murine model of EAM and analysed the effect of immune modulation through MCS-18. This substance, which is extracted from the plant *helleborus purpurascens*, has immunomodulatory properties and has been previously used for preventing experimental autoimmune encephalitis,³⁴ as well as autoimmune diabetes³⁵ in animal models. Although not a specific DC inhibitor, it has been shown that MCS-18 inhibits the production of functionally important DC surface proteins, as well as DC-mediated T-cell production.³⁶

Methods

Patient cohorts

This study was designed in a multi-step fashion using two different patient cohorts, as well as a murine model of EAM, thus analysing DCs in the peripheral blood of patients with myocarditis and DCM (*circulating DCs in human myocarditis*), in myocardial biopsies of patients with myocarditis (*myocardial DCs in human myocarditis*), as well as in the myocardium of EAM mice (*DCs in EAM and inhibition through MCS-18*).

Circulating dendritic cells in human myocarditis

Patients with acute myocarditis as well as newly diagnosed DCM were prospectively recruited between April 2010 and April 2013 shortly after admission at the University Hospital Jena, Germany. Myocarditis was clinically suspected and diagnosed according to usual clinical criteria such as typical anginal symptoms, elevated cardiac enzymes, unspecific ST segment elevation on electrocardiogram (EKG), then confirmed in cardiac magnetic resonance tomography via positive late gadolinium enhancement. DCM was first diagnosed clinically via echocardiography then confirmed on histopathological and immunohistochemical examination of endomyocardial biopsies, in accordance to the World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies.³⁷ All patients with DCM underwent coronary angiography in order to exclude coronary artery disease (CAD, defined as $\geq 50\%$ stenosis in at least one coronary artery). The control group of this cohort consisted of individuals with excluded structural heart disease who underwent a cardiac assessment including coronary angiography (CAD excluded for all these controls), mainly because of suspected angina pectoris and CAD. Excluded were all patients with systemic or malignant disease, active infection, immunosuppressive therapy, advanced kidney failure (glomerular filtration rate < 30 mL/min) or with other reasons for HF such as valvular, hypertensive, tachycardia related, or other forms of cardiomyopathy.

Fluorescence-activated cell sorting (FACS) analyses were undertaken at the time of recruitment (baseline), as well in a 6 month follow-up visit at our outpatient clinic. Patients with DCM were also analysed in a second 12-month follow-up. For each measurement, clinical assessment included an evaluation of symptoms and standard echocardiography. In each echocardiogram, left ventricular (LV) end-diastolic dimension were measured by M-mode in the parasternal long-axis view using the leading-edge method, whereas ejection fraction (EF) was measured using the planimetric (Simpson) method. All patients and controls gave written informed consent, and the study was approved by the local

Ethics Committee and performed in accordance with the Declaration of Helsinki.

Blood samples were collected in EDTA tubes and immediately cooled (4–10°C). Samples were analysed by flow cytometry for circulating DC precursors within 8 h after collection. The same blood samples were used for routine analyses, in particular leukocyte count. Routine blood analyses were performed by standardized techniques in the central laboratories of the University Hospital of Jena. Using the Blood Dendritic Cell Enumeration kit™ (BDCA kit; MiltenyiBiotec), circulating mDCs and pDCs were analysed by four-colour staining and FACS analyses in fresh blood samples collected in tubes containing EDTA. Circulating mDCs and pDCs were identified according to their expression of BDCA-1 and BDCA-2, respectively, and the absence of the expression of other peripheral blood mononuclear cell markers. Thus, the cells were classified according to CD303, CD1c, CD14, CD19, and CD141. For this purpose, 300 µL of blood were mixed with 20 µL of the control cocktail for isotype control and 300 µL of blood were mixed with 20 µL of the anti-BDCA cocktail for cell staining. In order to discriminate dead cells, 10 µL of a fluorescent cell-impermanent dye (which binds to nucleic acids of dead cells) were added, and samples were incubated for 10 min under 60 W light bulb. After cell staining, erythrocytes were lysed using the red blood cell lysis solution from the Blood Dendritic Cell Enumeration kit™ (BDCA kit; MiltenyiBiotec). Cells were washed and fixed using fix solution. Another solution was added to the samples for optimal dead cell discrimination even after prolonged storage (MiltenyiBiotec). Finally, cells were analysed using FACSCalibur flow cytometer with CellQuest software (Becton Dickinson). As circulating DCs comprise only 0.1–1% of white blood cells (WBC), a special gating strategy (Figure 1E) to exactly analyse the number of mDCs and pDCs was used. In Region R1 2*10⁵ WBC were registered defined by forward scatter and side scatter. In Region R2, granulocytes were excluded according to their high side scatter, and lymphocytes, monocytes, and dead cells were excluded according to their CD19, CD14, and propidium iodide staining, respectively. Circulating mDCs and pDCs were detected due to their specific staining for BDCA-1 and BDCA-2 in regions R3 and R4 as described previously.³⁸ The relative cell numbers of circulating DCs were thus determined as percentage of WBC.

Myocardial dendritic cells in human myocarditis

Left ventricular endomyocardial biopsies of 18 patients with acute myocarditis were previously obtained for diagnostic purposes and stored in TissueTek medium at –80°C at our tissue bank. Endomyocardial biopsies were performed when at least one of the specific clinical scenarios stated

in ESC guidelines were present.³⁹ Active myocarditis was diagnosed using histological criteria.⁴⁰ As controls, myocardial specimens from the left ventricle of 10 accident or suicide victims were embedded in TissueTek (Sakura, Finetek) and crash-frozen in liquid nitrogen during autopsy and subsequently preserved at –80°C. In all subjects, no past history of cardiac or systemic disease was known. Autopsy showed no macroscopical signs of cardiac structural abnormalities, ischaemia or coronary lesions.

Immunohistochemistry of dendritic cells

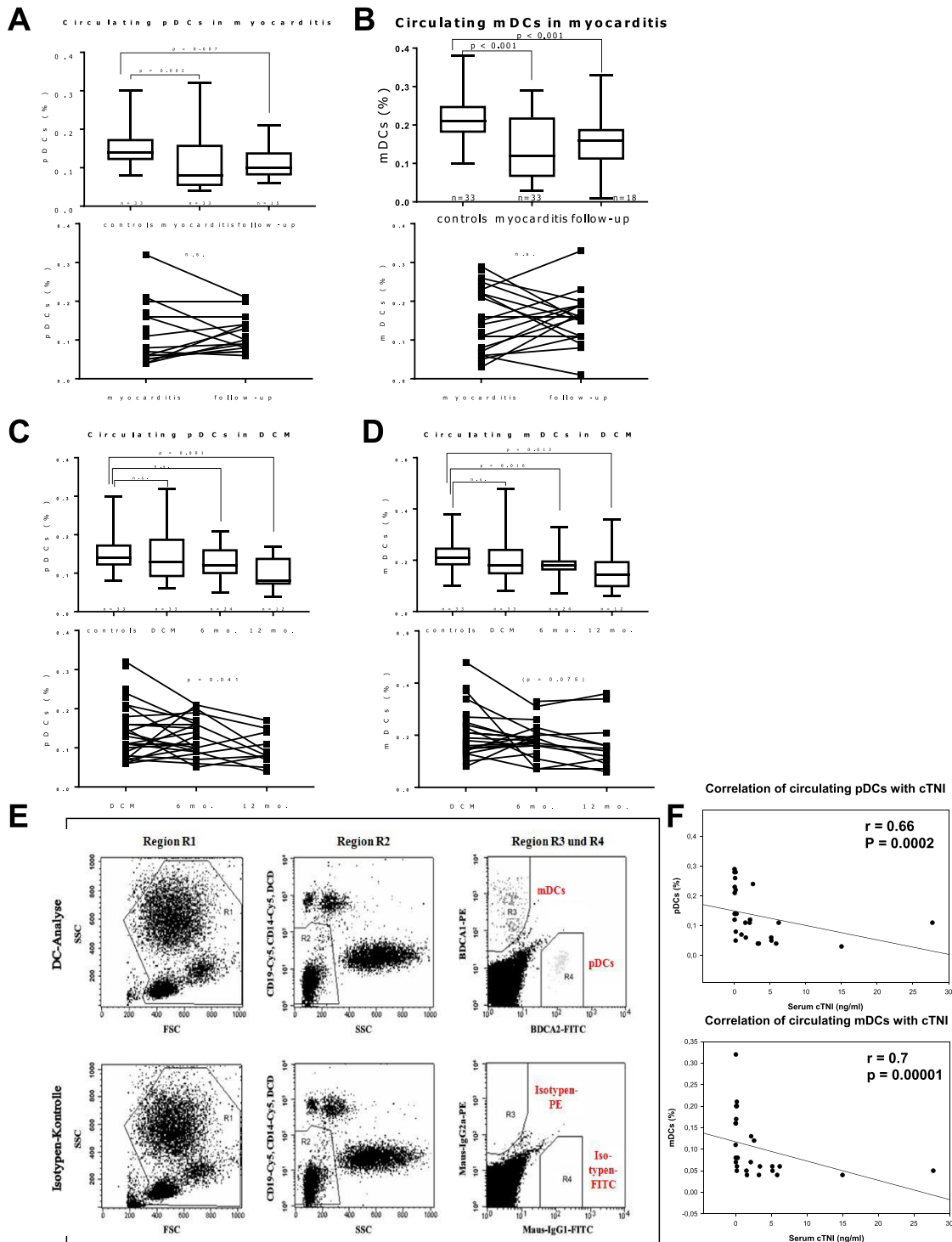
Acetone-fixed cryosections of biopsies and control specimens were stained with antibodies specific for pDCs (CD123 and CD304) and mDCs (fascin and CD209), as well as for T-cells and macrophages (see Table 2 for a detailed description of used antibodies) using the catalysed signal amplification technique (CSA System™, DAKO, Hamburg, Germany) as previously described.²³ The sources, applications, and specificities of the antibodies used for immunohistochemical analyses are listed in Table 2. Digital images of immunostained sections were taken with a CCD camera (Zeiss AxioCamHRc, Jena, Germany) at a magnification of 100×. For each analysis, the colour threshold for immunostained cells was manually adjusted in the images until the computerized detection matched the visual interpretation. Stained cells were counted digitally in a defined area of 0.2 mm², using a digital image processing software (ImageJ 1.43u, Wayne Rasband, NIH, USA). If biopsy sections were smaller than or shaped in a way that they could not fully cover the prespecified area, the cell count was proportionally corrected for tissue coverage.

Dendritic cells in experimental autoimmune myocarditis and immune modulation through MCS-18

Experimental autoimmune myocarditis was induced in 6- to 8-week-old male Balb/c mice (Janvier Labs, France) on Day 1 through subcutaneous injection of 150 µg synthetic myhc-α peptide (CASLO, Denmark). A second boost injection was followed on Day 7. For the inhibition of DCs, 100 µg of MCS-18 were injected intraperitoneally on Days 0, 2, and 4. Control mice were injected with PBS. MCS-18 was isolated as previously described at the University of Erlangen, Germany.³⁴

Echocardiography was performed on Day 21 (peak of inflammation) and Day 28 (partial recovery) on anaesthetized mice, which were consequently sacrificed. Animals were anesthetized with isoflurane (5% for induction, 0.5–2% for maintenance). Chests were shaved, and the mice were examined in supine position with a 30 MHz scanhead and a high-resolution micro-ultrasound imaging system especially

Figure 1 Flow cytometry analysis of circulating plasmacytoid (p) and myeloid (m) dendritic cells (DCs), as percentage of total white blood cells. (A–D) Boxplots showing relative circulating m and pDCs at baseline and follow-up compared with controls; *P*-values indicate significant difference to controls (Mann–Whitney *U* test); error bars indicate minimal and maximal values. Both DC subsets are reduced in myocarditis at baseline compared with controls (A and B). In patients with dilated cardiomyopathy (DCM), circulating DCs are reduced gradually during the course of the disease, reaching statistical significance after 6 or 12 months (C, D). Individual longitudinal (paired) patient data of myocarditis and DCM at baseline and follow-up are shown in the respective graphs below. *P* values in longitudinal DCM data indicate significance (or near significance) in non-parametric variance analysis (Friedman test). (E) Typical gating strategy for flow cytometry analysis of circulating DCs. (F) Circulating DCs correlate with serum cardiac Troponin I (cTNI) in myocarditis patients (Spearman rank order test).



designed for mice (Vevo 770, VisualSonics, Ontario, Canada). All measurements were averaged from three separate recordings. Two-dimensional short-axis views of the left ventricle at the papillary muscle level were obtained. Two-dimensional guided M-mode tracings were recorded with a sweep speed of 100 mm/s. Ejection fraction was determined according to the Teichholz method.

After sacrifice, hearts were sectioned and fixed with formaldehyde and embedded in paraffin for histological evaluation or embedded in TissueTek and snap frozen in liquid nitrogen for immunohistochemistry of DCs. Haematoxylin and eosin stained heart sections were scored according to a semi-quantitative scale (0, indicated no inflammatory infiltrates; 1, small foci of inflammatory cells between myocytes; 2, larger foci of >100 inflammatory cells; 3, <10% of a cross-section involved; 4, >30% of a cross-section involved), as previously described.^{41,42} Immunohistochemistry was performed using specific antibodies for pDCs and mDCs (CD304 and CD11c, respectively, see supplement section for information of sources, applications, and specificities) as described above. All animal experiments were approved by the local state authority for animal experimentation.

Statistical analysis

Data were tested for normal distribution. The results are presented either as mean values \pm standard error of the mean in the case of continuous data or as median values with their respective 25th and 75th percentiles in the case of non-continuous data. All comparisons between groups with unpaired data were performed using Student's *t*-test when normal distributed, otherwise with the Mann-Whitney *U* test. For the FACS analysis, besides comparison of controls with respective myocarditis and DCM data, a further analysis of longitudinal paired data within groups was performed using either the Friedman test (analysis of variance) when at least three data sets were available (i.e. for the three DCM measurements at baseline and follow-ups 1 and 2), or the Wilcoxon signed-rank test when less than three data sets were available (i.e. for the two myocarditis measurements at baseline and the single follow-up). Correlation analyses were performed using the Spearman rank order test. For all tests, a *P*-value <0.05 was considered statistically significant. Correlation analysis including correlation graphs were performed with Sigma Plot Software Version 13.0 (Systat Software Inc.) All other statistical analysis and graphs were performed using GraphPad Prism Version 7.03 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Circulating dendritic cells are reduced in human myocarditis with distinct kinetic patterns

The demographical and clinical characteristics of the 99 recruited eligible patients with either acute myocarditis ($n = 33$) or DCM ($n = 33$), as well as controls ($n = 33$) are shown in *Table 1*. Both myocarditis and DCM groups were significantly younger than controls, with patients with myocarditis being the youngest (mean age 38.5 ± 2.6 years) and mostly male (79%). Among the cardiovascular risk factors, hypertension was more predominant in the control group (78%, $P = 0.005$), while there were more smokers in the myocarditis and DCM groups ($P = 0.005$ and 0.27 , respectively). The main reason for hospital admission of patients with myocarditis was angina pectoris (73%), whereas patients with DCM presented mainly with HF symptoms including severe shortness of breath (77% NYHA III or IV) and peripheral oedema (30%). Accordingly, patients with DCM had severe LV impairment and dilation in echocardiography (EF $28.1 \pm 1.5\%$, LV end-diastolic diameter 62 ± 1.7 mm). LV dilation was not present in acute myocarditis, but this group showed a moderately reduced ejection fraction (mean EF $49.1 \pm 3.4\%$). Patients with DCM received significantly more HF medication than the other two groups (100% on betablockers and ACE inhibitors/ARB, 79% on diuretics and aldosterone antagonists).

Fluorescence-activated cell sorting analyses of patients with myocarditis showed significantly reduced relative numbers of both subsets of circulating DCs (% of WBC) compared with controls (for pDCs $P = 0.002$, mDCs $P < 0.001$), while there was no difference between WBC counts ($7.27 \times 10^9/L \pm 0.49 \times 10^9/L$ vs. $7.17 \times 10^9/L \pm 0.26 \times 10^9/L$, $P = 0.8$). After 6 months of follow-up period, circulating DCs remained significantly lower than controls ($P < 0.01$ for both DC subsets) (see *Figure 1*). Paired data analysis did not reveal a significant trend between the two measurements at baseline and at 6 months of follow-up in myocarditis.

We saw a different time kinetic pattern in DCM: while there was no significant difference with controls at baseline, both DC subsets deteriorated continuously during the 12 month follow-up period. Analysis of variance showed a significant reduction of pDCs from baseline to the 6 and 12 month follow-up measurements ($P = 0.041$). Such trend was statistically not significant for mDCs ($P = 0.075$), but of note, only 12 out of initially 33 patients of the DCM group were FACS-measured after 12 months. Compared with controls, both DC subsets were significantly lower at 12 months from baseline (both $P < 0.01$). Such result is consistent with our previous observation of mDCs being severely reduced in patients with chronic DCM and ICM.³³ In all groups, circulating mDCs varied between 0.03% and 0.45% of the WBC of peripheral blood, comparable with the results from previous publications.^{38,43}

Table 1 Clinical characteristics of myocarditis, DCM, and control subjects

	Myocarditis ¹	DCM ²	Controls ³	P-value	P-value	P-value
				1 vs. 2	2 vs. 3	1 vs. 3
<i>n</i>	33	33	33			
Age (years)	38.5 ± 2.6	46.5 ± 2.4	61.2 ± 1.9	0.024	<0.001	<0.001
Sex (male/female)	26/7	25/8	12/21	0.77	0.001	<0.001
BMI (kg/m ²)	29.2 ± 0.9	27.4 ± 0.7	26.6 ± 0.7	0.13	0.42	0.027
Cardiovascular risk factors (<i>n</i> (%))						
Hypertension	14 (42.4)	14 (42.4)	25 (75.8)	1	0.005	0.005
Diabetes	1 (3)	2 (6.1)	5 (15.2)	0.56	0.237	0.089
Smoking	22 (66.7)	21 (63.6)	12 (36.4)	0.36	0.027	0.005
Dyslipidaemia	9 (27.3)	9 (27.3)	12 (36.4)	1	0.436	0.436
Medication, <i>n</i> (%):						
Betablockers	13 (39.4)	33 (100)	19 (57.6)	<0.001	<0.001	0.144
ACE inhibitors/ARB	13 (39.4)	33 (100)	25 (75.8)	<0.001	0.002	0.003
Diuretics	11 (33.3)	26 (78.8)	17 (51.5)	<0.001	0.02	0.139
Aldosterone antagonists	9 (27.3)	26 (78.8)	0	<0.001	<0.001	0.001
Clinical presentation, <i>n</i> (%):						
Angina pectoris	24 (72.7)	8 (24.2)	18 (54.5)	<0.001	0.011	0.129
Dyspnoea on exertion, NYHA Class:						
I	8 (24.2)	3 (9.1)	26 (78.8)	0.102	<0.001	<0.001
II	12 (36.4)	8 (24.3)	5 (15.2)	0.29	0.36	0.05
III	9 (27.3)	14 (42.4)	2 (6.1)	0.202	<0.001	0.021
IV	4 (12.1)	8 (24.3)	0	0.207	0.002	0.04
Peripheral oedema	6 (18.2)	10 (30.3)	0	0.26	<0.001	0.01
Palpitations	10 (30.3)	9 (27.3)	5 (15.2)	0.78	0.14	0.146
Echocardiography:						
LVEF (%)	49.1 ± 3.4	28.1 ± 1.5	67.8 ± 1.7	<0.001	<0.001	<0.001
LVEDd (mm)	54.4 ± 1.6	62 ± 1.7	46.1 ± 1.5	0.002	<0.001	0.002

Data are presented as mean values ± standard error of the mean or number (%) of subjects. BMI, body-mass index; ACE, angiotensin-converting enzyme; ARB, angiotensin-receptor blocker; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter.

Correlation analyses revealed a strong correlation between the reduction of peripheral DCs of both subsets and serum cardiac troponin I ($r = 0.66$, $P = 0.0002$ for pDCs and $r = 0.7$, $P = 0.00001$ for mDCs) in patients with myocarditis (Figure 1). No other significant correlations were found, including the LV function parameters.

Increased expression of myocardial dendritic cells in human myocarditis

Biopsies from patients with myocarditis ($n = 18$) showed clear cellular infiltration of macrophages and T-cells (see immunostaining results at Table 2B). We saw a significant increase of pDCs with both used antibodies (>2-fold for CD304 and >3-fold for CD123, both $P < 0.05$, see Figure 2). Myeloid DCs on the other hand did not show any significant differences compared with controls. The marked increased expression of pDCs is in stark contrast with our previous observation on human DCM biopsies,³² as we found a significant decrease of both subunits.

Myocardial accumulation of dendritic cells in experimental autoimmune myocarditis and the impact of inhibition by MCS-18

Mice immunized with cardiac myosin peptide ($n = 10$) showed distinct histological and echocardiographical features of EAM (mean myocarditis score 2.6 ± 0.3 , mean EF reduced from 63.3% to 49.9%; both $P < 0.001$), with massive T-cell infiltration in HE histology and LV impairment on echocardiography (see Figure 3). Immunostaining with antibodies for pDCs and mDCs showed myocardial pDCs to be significantly increased compared with controls ($P < 0.0001$). MDCs were also significantly increased, but pDCs were much more abundant (mean 104 ± 10 vs. 10 ± 1.6 cells per immunostained area).

In animals injected with MCS-18, myocardial inflammatory infiltration measured by the myocarditis score, as well as LV function on echocardiography were both improved compared with untreated EAM mice at the peak of inflammation on Day 21 ($P = 0.026$ for myocarditis score, $P = 0.03$ for LVEF). Interestingly, the expression of pDCs was reduced (compared with uninhibited EAM, $P = 0.009$) but not of mDCs. Such could be explained by the smaller numbers of mDCs, but it is consistent with the aforementioned findings in human biopsies.

Table 2 (A) Antibodies used for immunohistochemical staining of human myocarditis and control biopsies and (B) their respective results in mean values \pm standard error of the mean

(A)							
Antibody	Clone	Against	Host	Ig-type	Dilution	Source	Specificity
CD 68	PG-M1	Human	Mouse	IgG3	1:500	DAKO	Macrophages
CD 69	FN50	Human	Mouse	IgG1	1:50	Acris	Activated T cells
CD 209 (DC-sign)	DCN46	Human	Mouse	IgG2b	1:50	BD Pharmingen	Immature mDCs
CD 304 (BDCA-4/neuropilin-1)	AD5-17F6	Human	Mouse	IgG1	1:100	Miltenyi Biotec	pDCs
CD 123	6H6	Human	Mouse	IgG1	1:100	Serotec	pDCs
Fascin	55 K-2	Human	Mouse	IgG1	1:100	DAKO	mDCs
(B)							
Epitome (target cells)			Myocarditis	Cells/0.2 mm ² area		P value	
				Controls			
Fascin (mDCs)			82 \pm 11.8	94.8 \pm 19			n.s.
CD 209 (mDCs)			30.4 \pm 2.8	33.7 \pm 4.6			n.s.
CD 304 (pDCs)			76.1 \pm 6.8	43.2 \pm 10.5			0.011
CD 123 (pDCs)			62.2 \pm 7.1	26.2 \pm 5			0.001
CD 69 (activated T cells)			10.1 \pm 2.7	3.1 \pm 0.6			0.025
CD 68 (macrophages)			13.5 \pm 2.6	6.2 \pm 1.9			0.036

P-values indicate significant differences (Student's *t*-test) between myocarditis and controls. MDCs, myeloid dendritic cells; pDCs, plasmacytoid dendritic cells.

At the partial recovery phase of EAM at Day 28, as both the myocarditis score and LVEF improved, MCS-18 treated mice showed paradoxically less improvement than untreated animals (see *Figure 3*, *P* not significant). The same was observed for pDC expression but again not for mDCs. Thus, DC inhibition leads to an amelioration of myocarditis but not to better recovery in mice.

Discussion

This study is a systematic investigation of circulating and cardiac DCs in myocarditis, in human subjects and in an animal model of EAM. We designed the study starting from the hypothesis that peripheral DCs would be reduced in patients with myocarditis, as they would infiltrate into the myocardium (*Figure 4*). We could show both major subtypes of circulating DCs to be decreased at the time of diagnosis of myocarditis with persistently reduced levels over an intermediate follow-up period. This differed from newly diagnosed DCM subjects in whom we found initially normal but progressively decreasing circulating DCs during the course of the disease. Along with this finding, we found DCs of the plasmacytoid type to be increased in the myocardium of patients with myocarditis. Both DC subtypes were overexpressed in the experimental mouse model, but pDCs were predominant. The inhibition of DCs led to an amelioration of myocarditis in terms of myocardial infiltration and LV ejection fraction at the acute phase but not during recovery. Such finding confirms the presumption that DCs play a major

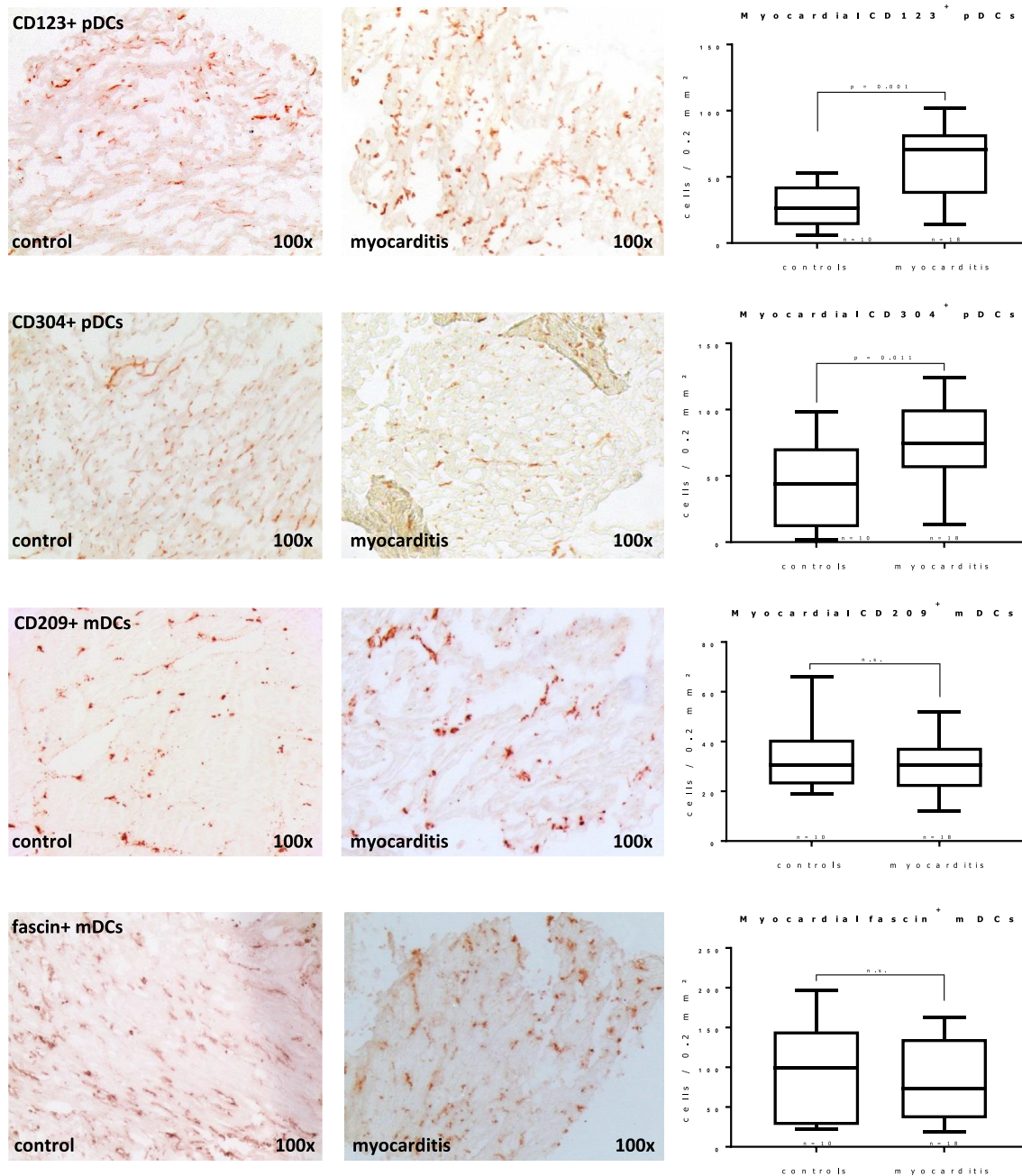
role in myocarditis during the acute inflammatory reaction and is in line with earlier histological findings of increased myocardial DCs in human myocarditis.^{44,45}

The role of DCs in human myocarditis is also in line with autoimmune processes in inflammatory heart disease.⁴⁶ DCs are known key mediators of autoimmunity. As DCs can initiate myocarditis via an autoimmune response in mice, such role in human hearts remains speculative but becomes more plausible with our present findings. DCs on the other hand are not solely linked to autoimmunity, as they are not only known to be activated through viruses but also modulate immune responses.^{24–31}

The notable fact that mDCs remained significantly reduced even after 6 months following the acute myocarditis episode should be further evaluated. Although such finding does not come as a surprise, as previous systematical studies recognized a viral persistence in human myocarditis following the resolution of disease.^{2,47} It is therefore possible that inflammatory processes persist long after clinical symptoms resolve in human myocarditis.

The involvement of DCs in myocarditis should not necessarily mean a pro-inflammatory role, but as it has been amply reported in animal experiments, DCs often modulate inflammation, with the net effect of amelioration of myocarditis.^{24–31} However, it will be a fruitful attempt to analyse in a larger study cohort whether patients who develop DCM after myocarditis have different changes of circulating DCs compared with patients with full recovery. Whether future techniques involving vaccination with modified DCs can alter the course of human disease remains to be seen.^{47,48}

Figure 2 Immunohistochemistry of endomyocardial biopsies shows increased plasmacytoid dendritic cells (pDCs) in human myocarditis, while myeloid DCs (mDCs) do not differ significantly from controls. Significant differences (Student's *t*-test) are indicated with the corresponding *P* values in the box plots; error bars indicate minimal and maximal values. See *Table 2* for a full description of used antibodies and staining results.



Another novel finding of this study was that in newly diagnosed patients with DCM circulating DCs were not different from controls. Their 'delayed' and gradual reduction did not resemble the time kinetic of patients with myocarditis. As circulating DCs in patients with DCM continued to decline in the short-term and medium-term follow-ups, they reached levels known from our previous study on patients with chronic HF

(DCM and ICM).³³ This difference to myocarditis is an interesting finding, because it is thought that myocarditis often precedes DCM and the two conditions often clinically overlap.^{1,2,49,50}

In our experimental mouse model of myocarditis, we could reduce the myocardial presence of DCs using MCS-18, a compound that has previously been used successfully to prevent

Figure 3 (A) Haematoxylin and eosin (H&E) and immunohistochemistry staining with antibodies for the two subtypes of dendritic cells (DCs) of myocarditis mouse heart sections showing massive T cell infiltration and accumulation of plasmacytoid (p) and myeloid (m) dendritic cells (DCs) in myocarditis compared to controls. Scatter plots on the right show myocarditis scores for H&E sections and numbers of stained DCs at peak of inflammation (Day 21) and at recovery phase (Day 28). T-cell inflammation and infiltration with pDC (but not mDC) at the acute phase is milder in animals treated with MCS-18. Scattered plots indicate individual values, as well as mean and standard deviations. *P* values indicate significant differences (Student's *t*-test) between MSC-18 treated and untreated groups. See Methods section for a full description of the myocarditis score. Used antibodies for pDCs: CD304, Neuropilin-1; for mDCs: CD11c, Integrin α -X. (B) Left: typical M-mode mouse echocardiography showing dilation and reduced ejection fraction of the left ventricle (LVEF) in experimental autoimmune myocarditis (EAM) compared with controls; right: graphic representation of LVEF reduction in EAM mice with and without MCS-18 treatment. Horizontal bar shows mean LVEF of control mice. MCS-18 treatment ameliorates EF reduction in peak myocarditis at Day 21 but not at recovery (Day 28). *P* value indicates significant difference between treated and untreated mice (Student's *t*-test).

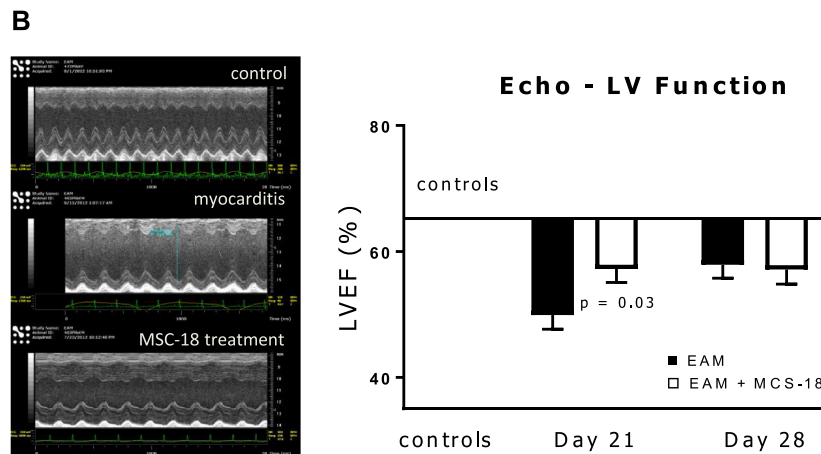
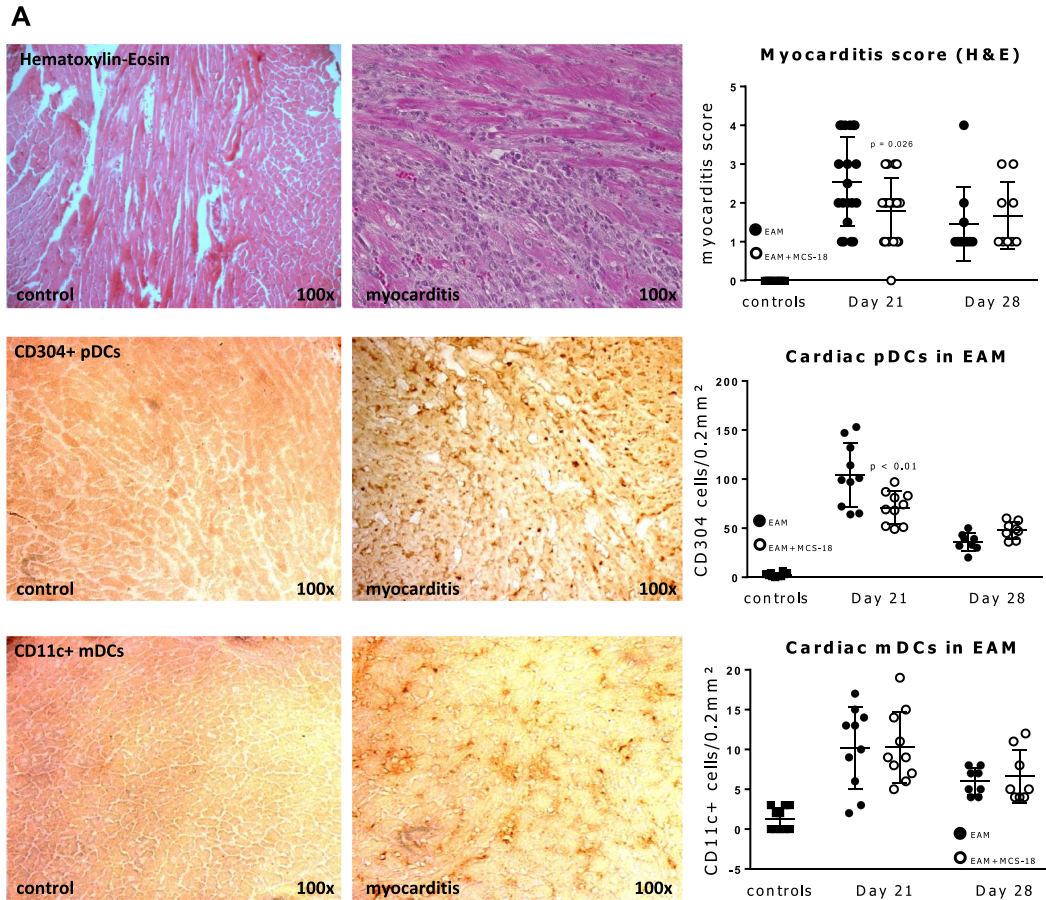
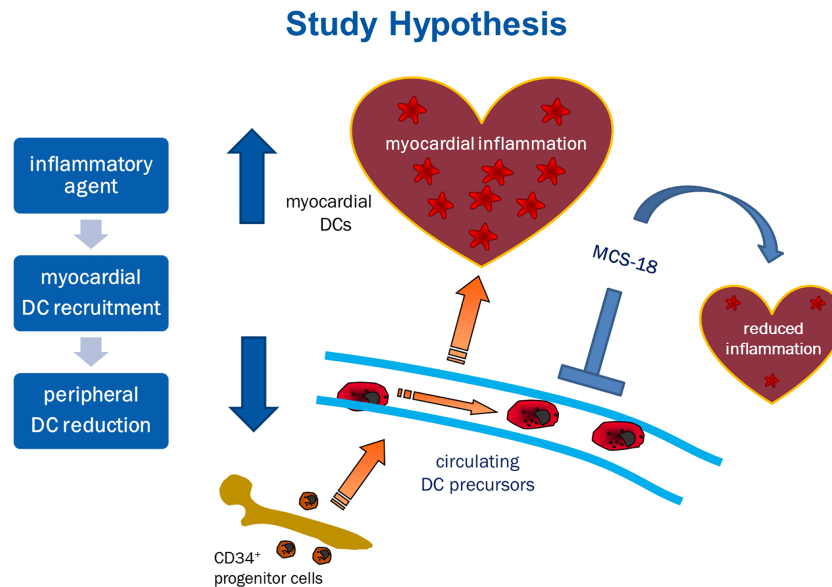


Figure 4 Study hypothesis. DC precursors originate from CD34⁺ progenitor cells in the bone marrow. Following antigen contact, circulating DC precursors mature and migrate to the site of inflammation, in this case the heart, unleashing myocardial inflammation through T-cell activation. MCS-18 treatment causes immune modulation and DC inhibition, resulting in milder inflammation.



autoimmune encephalitis and diabetes in mice via modulation of inflammation, including inhibition (though not specific) of DC maturation.^{34,36} To the best of our knowledge, the current study is the first application of this substance in a myocarditis model. Besides the significant reduction of myocardial DCs, we could show that MCS-18 treatment successfully reduces the acute inflammation in EAM. Furthermore, its effect on myocardial T-cell infiltration and LV function confirms the general assumption that EAM is a DC-dependent disease.^{11–17} It is tempting to speculate on possible effects of DC inhibition of substances such as MCS-18 beyond the animal EAM model. Yet the end-effect on myocardial inflammatory disease through DC inhibition or stimulation is far from obvious. We did not see a difference in myocardial DC expression or indeed inflammation and LV function at the partial recovery phase of the EAM. Although statistically not significant, LV impairment, inflammation, and DC expression tended to increase after the peak of myocarditis in MCS-treated mice. It is also unknown what effect DC stimulation has on the late phase of EAM. In a previous publication, Blyszczuk *et al.* successfully stimulated DCs with GM-CSF in EAM mice but could not change the course of myocarditis or tissue fibrosis.¹⁹

The discrepancy of the results concerning plasmacytoid and myeloid DCs in our study is not surprising. These subsets differ greatly in their immune responses, resulting from different Toll-like receptor expression profiles and antigen presenting properties.⁵¹ pDCs possibly play a greater role than mDCs in innate immunity as a response to viral infections and are capable

of activating T-cells through presentation of endogenous antigens, rather than extracellular substances like mDCs.^{52–54} Such key distinctions of pDCs might make them more capable to be involved in both antiviral and autoimmune responses in human, as well as autoimmune murine myocarditis.

Limitations

For the analysis of human peripheral and myocardial DCs, we used different patient collectives and, for obvious ethical reasons, different control groups (patients and post-mortem accident victims). The intermediate longitudinal character of the FACS analysis falls short of the impact of a long-term observational study with survival data. Although our patients were recruited as soon as their diagnosis was suspected, we cannot exclude a previous 'silent' disease. There were also age and gender differences between the groups, with patients with myocarditis representing the youngest collective. Presently, there is no consensus regarding peripheral DCs and aging (some authors observed similar numbers between young and old humans, others found a decline of either mDCs or pDCs with advancing age^{55–57}). Yet we are not aware of any reporting of increase of peripheral DCs with age; hence, our results (reduced peripheral DCs in myocarditis and DCM) are unlikely due to age difference between groups. We measured the relative, as opposed to absolute number of DCs. It is therefore possible that other leukocytes were increased, thus artificially reducing the relative DC count. Although we did not notice an increase of blood leukocytes in

our patients with myocarditis compared with controls, we are aware of the limitation and possible bias in our measurement method. We also lacked sufficient virus PCR data from our myocarditis biopsies, which could have possibly given our results an important prospective in relation to myocarditis aetiology. The protocols and specificity of the antibodies for DCs in human myocardial tissue were previously established and tested (including double staining) in one of our previous publications.³² Yet we can not exclude some unspecific staining of endothelial cells, and such must be regarded as a limitation. In this study, we used two different antibodies for each DC subset, yielding similar results.

In the myocarditis mouse model, LV function was only mildly reduced in myocarditis (EF from 63% to 50%). Such is in line with previous observations,⁴² while EF was even found to remain constant in another EAM study.³⁶ Although we found significant changes in EF in our model, other parameters such as fibrosis and haemodynamics should be considered in future studies.

Finally, although MCS-18 was shown to modulate the immune response in experimental animal models, it is by no means a specific DC inhibitor. We could show a reduction of myocardial DCs, as well as inflammation and myocardial damage in the acute phase of myocarditis. Yet MCS-18 is also known to inhibit B cell proliferation, as well as cytokine induced immunoglobulin production.⁵⁸ The reduction of inflammation and myocardial dysfunction in our model could have therefore resulted in part from other anti-inflammatory properties of MCS-18.

In conclusion, we report a reduction in circulating and increase in myocardial DCs in acute human myocarditis, suggesting an association between DC myocardial recruitment

and acute myocarditis in humans. The presence of DCs in myocarditis was confirmed in the animal experiment, and we further showed that immune modulation and inhibition of DCs led to myocardial DC reduction and amelioration of the acute phase of myocarditis in mice. We further report differences between myocarditis and DCM in circulating DCs during the course of disease. From the DC subsets, pDCs might play a specific role in myocarditis. Our study contributes to the growing evidence that DCs play a major role in myocarditis, in animal experiment, and in humans.

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Conflict of interest

None declared.

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