Activated Circulating Myeloid-Derived Suppressor Cells in Patients with Dilated Cardiomyopathy

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Key Words
Dilated Cardiomyopathy • Myeloid-derived Suppressor Cells • Inflammation • Autoimmune Abnormalities

Abstract
Background/Aims: Myeloid-derived suppressor cells (MDSCs) are increased in inflammatory and autoimmune disorders. This study aims to evaluate the significance of MDSCs in dilated cardiomyopathy (DCM) patients. Methods: In total, 42 newly hospitalized DCM patients and 39 healthy controls were enrolled in the study. The frequencies of circulating CD14+HLA-DR-/low MDSCs were determined by flow cytometry. Then, the functional properties of MDSCs in suppressing T cell proliferation and interferon-gamma (IFN-γ) production were measured in a co-culture model. Then, mRNA expression levels of various important molecules in peripheral blood mononuclear cells were measured by real time polymerase chain reaction. Furthermore, correlation analyses between MDSC frequencies and cardiac function parameters were also performed. Results: The frequencies of circulating CD14+HLA-DR-/low MDSCs were significantly elevated in DCM patients compared with healthy controls. It showed that MDSCs from DCM patients more effectively suppressed T cell proliferation and IFN-γ production compared with those from healthy controls, which was partially mediated by arginase-1 (Arg-1). In addition, the correlation analysis suggested that MDSC frequencies were negatively correlated with left ventricular ejection fraction (LVEF), while positively with N-terminal pro-brain natriuretic peptide (NT-proBNP) in patients with DCM. Conclusions: Circulating activated MDSCs might play significant immunomodulatory roles in the pathogenesis of DCM.
Introduction

Dilated cardiomyopathy (DCM) is a primary myocardial disorder characterized by ventricular chamber enlargement and systolic dysfunction of one or both ventricles for which appropriate treatment remains a major clinical challenge [1]. Inflammatory processes induced by viral infection have been discussed as one of the main mechanisms in the development and progression of DCM [2, 3]. Abnormal immune responses, even without irretrievable cell injury, can fatally disrupt cardiac function. T lymphocytes are critical mediators of disease pathogenesis and contribute to the progression of contractile dysfunction via mechanisms that include inducing direct cytotoxicity, enhancing the inflammatory functions of other cells, and helping B cells produce pathogenic antibodies [4]. Moreover, several antibodies against antigens within the myocardium, whether they are microbial, self, or alloantigens, impair cardiac function, cause permanent damage to the cardiac tissue, and often lead to ventricular dilation [5, 6]. Several trials have indicated that immunomodulation has the potential to offer a novel and future therapeutic strategy for the management of myocarditis and DCM patients [7-9].

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of immature myeloid cells that can be activated to suppress T cell functions through cell surface interactions and the release of soluble mediators [10-12]. Their phenotype differs vastly between humans and mice. However, in common, these cells contain precursors at earlier stages of differentiation, such as granulocytes, macrophages, dendritic cells, and myeloid cells. They also have defects in differentiating into mature myeloid cells [13]. In humans, MDSCs lack the markers of mature myeloid and lymphoid cells and MHC-class-II HLA-DR. These cells can also be divided into monocytic and granulocytic populations with potentially distinct functions according to the expression of monocytic marker CD14 or granulocytic marker CD15 [12, 14]. We and others have demonstrated that CD14⁺HLA-DR⁻/low cells share multiple characteristics with MDSCs [15] and expand under a variety of pathological conditions, such as cancer [16-19], inflammation [20, 21], and autoimmune diseases [22, 23]. Presently, the inhibitory properties of MDSCs are thought to be mediated by a number of different mechanisms, including arginase-1 (Arg-1), inducible nitric oxidase (iNOS), transforming growth factor-beta (TGF-β), and interleukin-10 (IL-10) as well as the induction of regulatory T cells [10, 23].

The accumulation of MDSCs in response to inflammation coupled with their well-characterized immunomodulatory activities under pathological conditions have prompted us to explore whether MDSCs participate in the immunomodulatory process to maintain immune homeostasis in the pathogenesis of DCM. Here, we analyzed the frequencies of MDSCs in the peripheral blood of DCM patients by flow cytometry and investigated the suppressive nature and the possible mechanisms of these MDSCs in a co-culture model.

Materials and Methods

Patients

In total, 42 DCM-diagnosed patients (31 men and 11 women, 49.7 ± 2.1 years old) without detectable etiology newly hospitalized at Union Hospital, Tongji Medical College, Huazhong University of Science and Technology from November 2012 to July 2014 were enrolled in this study. The diagnosis of DCM was based on the guidelines of the World Health Organization [24]. In this investigation, DCM was defined as systolic dysfunction [left ventricular ejection fraction (LVEF) < 45%] with ventricular dilation [left ventricular end-diastolic diameter (LVEDD) > 55 mm] in the absence of an apparent secondary cause of cardiomyopathy, such as coronary heart disease, hypertensive heart disease, or valvular heart disease. Eligible patients were excluded if they had received anti-inflammatory drug treatment, which was likely to impact their immune status, or if they had a medical history of autoimmune disease, serious infection, pregnancy, endocrine disease, collagen disease, or other inflammatory diseases. Moreover, 39 healthy volunteers (25 men and 14 women, 44.4 ± 1.8 years old) were enrolled as controls. This study was performed in strict accordance with
Sample Preparation and Peripheral Blood Mononuclear Cell (PBMC) Isolation

Blood samples from all subjects were obtained on the morning of the admission day in the recumbent position using a 21-gauge needle and collected in tubes containing 0.2 ml of sodium heparin. Blood samples were centrifuged at 300 ×g for 7 min, and then the plasma was frozen at −20°C for measurements. PBMCs from freshly obtained blood were isolated with Ficoll-Paque Plus and were subsequently washed twice using phosphate-buffered saline (PBS) before being used for flow cytometric analysis, magnetic cell sorting, real-time polymerase chain reaction (RT-PCR), or cell culture.

Flow Cytometry

PBMCs were labeled with the surface markers anti-human CD14-FITC (clone: 61D3, eBioscience), anti-human HLA-DR-PE (clone: L243, eBioscience), anti-human CD11B-PE (clone: M1/70, Biolegend), anti-human CD33-APC (clone: WM53, BD Biosciences), anti-human CD15-PE-Cy5 (clone: SSEA-1, Biolegend), anti-human CD4-FITC (clone: OKT4, Biolegend), anti-human CD25-PE (clone: BC96, Biolegend), and anti-human CD127-APC (clone: A019D5, Biolegend) antibodies, according to the manufacturer's protocol. In addition, isotype antibodies were used to ensure correct compensation and confirm the specificity of the staining. After washing with PBS, cells were fixed and detected using a FACS Calibur flow cytometer (LSR II, BD Biosciences, San Jose, CA, USA). The analyses of flow cytometry data were performed using FlowJo7.6.1 (Treestar Inc., USA). The estimated absolute numbers of MDSCs were calculated as previously described [20, 25].

Isolation of MDSCs and T Cells

As previously described [20], CD14+HLA-DR−/low MDSCs were isolated from PBMCs using CD14 and HLA-DR microbeads (Miltenyi Biotec, Germany). First, HLA-DR+ cells were depleted by negative selection using an LD column according to the manufacturers’ instructions. Then, HLA-DR- cells were further separated to obtain CD14+HLA-DR−/low cells using CD14 microbeads. The purity of the CD14+HLA-DR−/low cells was > 80% as assessed by fluorescence-activated cell sorting (FACS). These MDSCs were then used...
for function assays described below. In addition, autologous T cells were isolated using CD3 microbeads (Milenyi Biotec, Germany) according to the manufacturer’s instructions.

**T Cell Proliferation**

T-cell proliferation was evaluated using CFSE (5, 6 carboxyfluorescein diacetate succinimidyl ester; eBioscience, USA) dilution, and purified T cells were labeled with CFSE (2.5 μM). Quantitative functional analysis of the MDSCs was performed by co-culturing sorted CD14^+HLA-DR^−/low MDSCs with autologous CD3^+^ T cells stimulated with plate-bound anti-CD3 (10 μg/ml; eBioscience, USA) and soluble anti-CD28 (3 μg/ml; eBioscience, USA) at different suppressor-to-responder cell ratios (0, 1:8, 1:4, and 1:2). The cells were grown in complete RPMI-1640 medium at 37°C and 5% CO₂, in U-bottom 96-well plates for 96 h. Cells were stained for surface-marker expression with CD3-PE antibodies, and then T cell proliferation was analyzed on a flow cytometer (BD LSR II BD Biosciences, San Jose, CA, USA). Moreover, the concentration of interferon-gamma (IFN-γ) in the culture supernatant was detected using an enzyme-linked immunosorbent assay (ELISA) kit (Neobioscience, China) according to the manufacturer’s instructions. This kit exhibits a sensitivity of 8 pg/ml and each measurement was performed twice.

For mechanistic studies, Arg-1 inhibitor Nω-hydroxy-nor-L-arginine diacetate salt (nor-NOHA, Calbiochem, 200 μmol/l), iNOS inhibitor NG-methyl-L-arginine acetate salt (L-NMMA, Sigma-Aldrich, 200 μmol/l), neutralizing anti–TGF-β antibodies (R&D Biosystems, 10 μg/ml), or anti-IL-10 antibodies (R&D Biosystems, 10 μg/ml) were also added to various experiments.

**RT-PCR**

Total RNA was extracted from PBMCs by TRIzol lysis buffer (Takara, Japan), and the cDNA was synthesized by reverse transcription using a Reverse Transciptase kit (Takara, Japan) according to the manufacturer’s instructions. Quantitative RT-PCR was performed using a SYBR Green PCR Kit (Takara, Japan) on an ABI Prism 7900 sequence detection system (Applied Biosystems). The primers are listed in Table 2, and all samples were amplified in duplicate at 40 cycles. The gene expression levels were normalized to that of the housekeeping gene GAPDH using -2^ΔΔCT methods.

**Electrochemiluminescence Immunoassay of N-terminal Pro-B-type Natriuretic Peptide (NT-proBNP)**

The levels of NT-proBNP were detected with the Electrochemiluminescence Immunoassay NT-proBNP kit from Roche Diagnostics following the manufacturer’s protocols. This kit exhibits a sensitivity of 60 pg/ml with no cross-reactivity detected separately. Each sample was determined twice.

**Statistical Analysis**

The values are presented as the mean ± standard error of the mean (SEM) or as percentages in the text and figures. For the mean comparison, 2-tailed Unpaired Student’s t-tests were performed to detect significant differences between the two groups. For the ratio comparison, the Pearson chi-square test was performed. Spearman’s correlation analysis was used as a test of correlation between the variables. To exclude the influence of potential confounders to MDSCs frequencies, we adjusted for the Male/Female ratio, hypertension, diabetes, and drug treatments using a multiple linear regression model. For all tests, a probability value < 0.05 was considered statistically significant. For statistical analyses, SPSS 16.0 software was used.

**Table 2. The primer sequences used for real-time PCR**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Primer Sequences (5'-3')</th>
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<tr>
<td>Arg-1</td>
<td>Forward GGCTGGTCTGGTGGAGAACC</td>
</tr>
<tr>
<td>iNOS</td>
<td>Forward CTTTCGAGCAACATCTCACCA</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Forward TGAGGGCTTGGCTCGGGATG</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward GCCAGGGCCACCGATCT</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forward GAGAAGCTGGAGGGCATGCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward CCACATCGCTAGAGACACAT</td>
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Results

Clinical Baseline Characteristics

The clinical data of all participants are presented in Table 1. No significant differences in age, gender, hypertension, or diabetes mellitus status were noted. Compared with the controls, the DCM patients exhibited a significantly enlarged LVEDD, a reduced LVEF, an elevated NT-proBNP, and an increased utilization of angiotensin-converting enzyme inhibitor (ACEI) or angiotensin receptor blockers (ARBs), β–blockers, calcium blockers, digitalis, diuretics and nitrates.

Increased Levels of Circulating CD14+HLA-DR-/low in DCM Patients

To explore whether MDSCs participate in the pathogenesis of DCM, the frequencies of CD14+HLA-DR-/low MDSCs in the peripheral blood of DCM patients and controls were carefully analyzed. First, we observed that the percentages of CD14+ monocytes in the PBMC did not differ significantly between DCM patients and controls (P = 0.295, Fig. 1C). Then, the frequencies of CD14+HLA-DR-/low cells in CD14+ monocytes were significantly increased in DCM patients compared with controls (P = 0.002, Fig. 1D). In addition, comparative analyses of the estimated absolute numbers were performed based on the lymphocyte and monocyte count as previously described [20]. Importantly, the absolute number of CD14+HLA-DR-/low MDSCs was also significantly increased in the peripheral blood of DCM patients (P = 0.005, Fig. 1E) compared with controls. However, no significant differences in the percentages or absolute numbers of CD14+CD15+ and CD14+CD33+CD11B+ cells were noted between patients in the DCM and control groups (Fig. 2 and 3). To exclude the possibility that the difference in MDSC frequencies between the two groups was due to the Male/Female ratio, hypertension, diabetes, and drug treatments, we performed a multiple linear regression analysis. It showed that the Male/Female ratio, hypertension, diabetes, and the use of medications did not influence the frequencies of MDSCs in this study (Table 3).
Fig. 2. The frequencies and counts of circulating CD14⁺CD15⁻ cells in PBMCs of DCM patients and controls. (A) Representative FACS images of the frequency of CD14⁺CD15⁻ cells from a single patient in each group. The results from the statistical analysis of the frequencies (B) and numbers (C) of CD14⁺CD15⁻ cells in DCM patients and healthy controls. Values are means ± SEM.

Fig. 3. The frequencies and numbers of circulating CD14 CD33⁺CD11B⁺ cells in PBMCs of DCM patients and controls. (A) Representative FACS images of the frequency of CD14 CD33⁺CD11B⁺ cells from one patient in each group. The results from the statistical analysis of the frequencies (B) and counts (C) of CD14 CD33⁺CD11B⁺ cells in DCM patients and healthy controls. Values are means ± SEM.
The Suppressive Function of MDSCs from DCM Patients

To evaluate the suppressive function of MDSCs in DCM patients, T cell proliferation and INF-γ secretion assays were conducted in a co-culture model. In fact, CD14⁺HLA-DR⁻/low MDSCs were co-cultured with CFSE-labeled autologous CD3⁺ T cells for 96 h in different ratios (n = 4), and the CFSE dilution was assessed by flow cytometry as a measure for CD3⁺ T cell proliferation. Representative histograms of T cell proliferation from patients in each group. (B) CD14⁺HLA-DR⁻/low cells from DCM patients effectively suppress autologous CD3⁺ T cell proliferation (n = 4). (C) Increased suppressive function of the MDSCs from DCM patients was recommended by the IFN-γ production level (n = 4). NS-T cells, unstimulated T cells; S-T cells, stimulated T cells; Values are means ± SEM, *P < 0.05.

Table 3. Variables associated with MDSCs frequencies in all subjects (n = 81). Multiple adjustments were performed with linear regression models. β, standardized coefficients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>β</th>
<th>P</th>
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<tbody>
<tr>
<td>Sex (male/female)</td>
<td>-0.063</td>
<td>0.583</td>
</tr>
<tr>
<td>Hypertension</td>
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<td>0.877</td>
</tr>
<tr>
<td>Diabetes</td>
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<td>0.562</td>
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<tr>
<td>Medication</td>
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<tr>
<td>ACEI/ARBs</td>
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<td>0.217</td>
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<tr>
<td>β-blockers</td>
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<td>0.174</td>
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<tr>
<td>Calcium blockers</td>
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<td>0.390</td>
</tr>
<tr>
<td>Digitalis</td>
<td>0.083</td>
<td>0.612</td>
</tr>
<tr>
<td>Diuretics</td>
<td>-0.174</td>
<td>0.210</td>
</tr>
<tr>
<td>Nitrates</td>
<td>0.106</td>
<td>0.402</td>
</tr>
</tbody>
</table>

Fig. 4. The suppressive function of CD14⁺HLA-DR⁻/low MDSCs for autologous CD3⁺ T cell function. (A) Purified CD14⁺HLA-DR⁻/low MDSCs were co-cultured with CFSE-labeled autologous CD3⁺ T cells for 96 h in different ratios (n = 4), and the CFSE dilution was assessed by flow cytometry as a measure for CD3⁺ T cell proliferation. Representative histograms of T cell proliferation from patients in each group. (B) CD14⁺HLA-DR⁻/low cells from DCM patients effectively suppress autologous CD3⁺ T cell proliferation (n = 4). (C) Increased suppressive function of the MDSCs from DCM patients was recommended by the IFN-γ production level (n = 4). NS-T cells, unstimulated T cells; S-T cells, stimulated T cells; Values are means ± SEM, *P < 0.05.
Fig. 5. The mRNA expression levels of important molecules in PBMCs from DCM patients and controls. The results of statistical analysis for the mRNA expression levels of ARG-1 (A), iNOS (B), IL-10 (C), and TGF-β1 (D) in DCM patients (n=12) and healthy controls (n=10). Values are means ± SEM.

Fig. 6. The mechanism of MDSC-mediated T-cell suppression in DCM patients is primarily mediated by Arg-1. (A) Representative histograms of T cell proliferation in different conditions. (B) The merged histogram of T cell proliferation in different conditions. The effects of different chemical inhibitors and blocking antibodies on the CD14+HLA-DR− MDSCs suppression of autologous T-cell proliferation (C) and IFN-γ production (D). nor-NOHA, Nω-hydroxy-nor-L-arginine diacetate salt; L-NMMA, NG-methyl-L-arginine acetate salt; anti-TGF-β, neutralizing anti–TGF-β antibodies; anti-IL-10, neutralizing anti-IL-10 antibodies. Values are means ± SEM. *P < 0.05 compared with the non-treated groups.
The mRNA Expression Levels of Various Soluble Factors and Cytokines in PBMCs of DCM Patients

To investigate the main effector molecules in CD14+ HLA-DR−/low MDSCs from DCM patients, the mRNA expression levels of Arg-1, iNOS, TGF-β1, and IL-10 in the PBMCs were quantified by RT-PCR. The results indicate that Arg-1 mRNA expression was significantly enhanced in PBMCs from DCM patients compared with healthy controls, as normalized to the housekeeping gene GAPDH (Fig. 5A). However, no substantial differences in the mRNA expression levels of iNOS, TGF-β1, and IL-10 were observed between DCM patients and controls (Fig. 5B, C, and D).

The Mechanism of MDSCs-mediated T Cell Suppression in DCM Patients

To further delineate the possible immunomodulatory mechanisms of CD14+ HLA-DR−/low MDSCs in DCM patients, different chemical inhibitors or blocking antibodies were added in the co-culture model. Consistent with the mRNA results, the addition of the Arg-1 inhibitor...
nor-NOHA significantly restored T cell proliferation (Fig. 6C) and IFN-γ production by T cells (Fig. 6D). On the contrary, T cell suppression was still observed after pretreatment with the iNOS inhibitor L-NMMA, neutralizing anti-TGF-β antibodies, or anti-IL-10 antibodies (Fig. 6C and D). These results strongly imply that the suppressive effect of CD14⁺HLA-DR⁻/low MDSCs on T cells in DCM patients is primarily mediated by Arg-1.

The Correlation of CD14⁺HLA-DR⁻/low MDSCs Frequencies with Cardiac Function and NT-proBNP in DCM Patients

To explore the relationship between CD14⁺HLA-DR⁻/low MDSC frequencies and cardiac function, correlation analyses of CD14⁺HLA-DR⁻/low MDSCs and indices of cardiac function were performed in DCM patients. As shown in Fig. 7, the CD14⁺HLA-DR⁻/low MDSC frequencies were positively correlated with the NT-proBNP level (R = 0.448, P = 0.003, Fig. 7A) and negatively correlated with LVEF (R = -0.354, P = 0.023, Fig. 7B) in patients with DCM. No significant correlation with LVEDD was noted (R = 0.173, P = 0.273, Fig. 7C).

The Correlation between Treg Frequencies and MDSCs Frequencies

Recently, it was reported that MDSCs may contribute to Treg induction via various mechanisms. Consistent with previous published data, we found that the frequencies of circulating Tregs and the mRNA expression levels of Foxp3 in PBMCs from DCM patients were significantly reduced compared with healthy controls (Fig. 8C and D). In this study, correlation analyses between Tregs and MDSC frequencies were also performed. As shown in Fig. 8E, no significant association was noted between Treg and MDSC frequencies (R = 0.196, P = 0.213).

Discussion

DCM, which is associated with increased morbidity and mortality due to progressive heart failure and sudden cardiac death, remains an important clinical problem worldwide [26, 27]. DCM is a complex multi-step disorder in which several physiologic systems participate in its pathogenesis [28, 29]. Although immune activation and persistent inflammation are thought to contribute to the progression of DCM, the specific pathological mechanism and the exact cause of the immune activation remain to be elucidated. The active inflammatory status during the pathogenesis of DCM may cause increased levels of MDSCs that lead to maintaining immune homeostasis. This study aimed to evaluate the significance of MDSCs in DCM patients.

MDSCs, a heterogeneous population of cells, play a vital role in the subversion, inhibition, and downregulation of the immune response to cancer, autoimmune diseases, and inflammation-mediated diseases. However, little is known about the role of MDSCs in DCM. In this study, we observed a considerable increase in the frequencies and the suppressive function of circulating CD14⁺HLA-DR⁻/low MDSCs in DCM patients compared with healthy controls, indicating the participation of MDSCs in the immunomodulatory process of DCM. Increasing evidence demonstrates that chronic immune activation and systemic inflammation are critical factors in the development of DCM [4]. As a defensive response to the pathogens near or within cardiac myocytes, activated monocytes and lymphocytes migrate to the myocardium [30]. The presence of these cells induces the production of inflammatory mediators, which activate MDSCs, drive their accumulation, and strengthen their suppressive activity [20, 31]. In other words, the modified local microenvironment in the myocardium or systemic inflammation in DCM patients may motivate the expansion of MDSCs.

There is growing evidence to support a causative role of abnormal circulating T cell activation and chronic autoimmune T cell responses to cardiac self-antigens in the underlying pathophysiology of DCM [4, 32-34]. Cuervo et al. described that monocytic MDSCs expressing iNOS and Arg-1 are present in heart tissue in the acute phase of
Trypanosoma cruzi infections, which have the potential to suppress T lymphocytes present in the infiltrate [35]. In this study, we determined that the activated MDSCs from DCM patients inhibited T cell proliferation and IFN-γ production more efficiently than those from healthy controls, indicating that circulating MDSCs from DCM patients not only arise at a considerably enhanced frequency but also display an unregulated suppression on a per cell basis compared with those from healthy controls. This finding led us to hypothesize that MDSCs may participate in the immunomodulatory process through the suppression of uncontrolled T cell activation, which further leads to myocardial injury and aggravation of cardiac function, thereby preventing the development of a more severe and fatal immune response in DCM patients.

The inhibitory properties of immune-suppressive MDSCs are thought to be mediated by various mechanisms, including Arg-1, iNOS, IL-10, and TGF-β1 [31]. We explored whether CD14+HLA-DR−/low MDSCs suppressed T cell proliferation and IFN-γ secretion. As shown in Fig. 5, Arg-1 mRNA expression was significantly increased in PBMCs from DCM patients compared with those from healthy controls, providing evidence that Arg-1 may be indispensable for suppressive MDSCs. Furthermore, the suppression of T cell proliferation and IFN-γ production by MDSCs was partially recovered in the presence of the Arg-1 inhibitor nor-NOHA in the MDSC-T-cell co-culture system but not the iNOS inhibitor, L-NMMA, neutralizing anti–TGF-β antibodies, or anti-IL-10 antibodies. These findings are consistent with the RT-PCR results and further suggest that the suppression of T cell proliferation and IFN-γ production from MDSCs in DCM patients is partially mediated in an Arg-1-dependent manner. The Arg-1-dependent mechanism of MDSCs from DCM patients indicates that Arg-1 may be a promising target for DCM immunotherapy.

Since the initial characterization in the control of pathogenic T cell activation and systemic inflammation, MDSCs have been associated with a wide range of inflammation-associated pathological processes. To evaluate the possible effect of MDSCs in DCM patients, correlation analyses between MDSC frequencies and indices of cardiac function were performed in DCM patients. The correlation analyses revealed that the MDSC frequencies were positively correlated with NT-proBNP but negatively correlated with LVEF in patients with DCM. These data demonstrate that activated MDSCs are potentially correlated with the severity of heart failure in DCM patients, supporting the hypothesis that abnormal immune activation may stimulate bone marrow to release additional MDSCs into the blood and peripheral lymphoid tissues. Here, these cells function as immune suppressors by inhibiting other immune cells, preventing the development of severe information, and participating in the maintenance of immune homeostasis.

Previous reports have suggested that tumor-induced MDSCs may contribute to Treg induction via diverse mechanisms in several models [36-38]. However, Centuori et al reported that MDSCs were not only incapable of promoting iTreg differentiation but also hampered TGF-1-induced generation of these cells with a more pronounced effect noted with MDSCs isolated from tumor-bearing animals [39]. Therefore, the cross-talk between MDSCs and Tregs remains incompletely defined. In addition, accumulating evidence confirms the involvement of Tregs in the development and progression of DCM [39-41]. In this study, we found that the frequencies of CD4+CD25+CD127dim/− Tregs and the Foxp3 mRNA expression in PBMCs are statistically decreased in DCM patients compared with healthy controls. This finding is consistent with the previous studies [34, 40]. Then, we also explored the possible correlations between MDSC frequencies and Treg frequencies in DCM patients. Unfortunately, no significant association was noted. Given these findings, the interplay between these two immunosuppressive cell populations may be more complex than envisioned initially and additional details are also needed to understand the underlying molecular mechanisms.

The potential limitations in this study should be noted. Cardiac-specific autoantibodies secreted by B cells also contribute to progressive cardiac myocyte damage, and their pathogenic role has been suggested in in vitro and in vivo disease models as well as clinical studies [42, 43]. Unfortunately, we did not determine whether DCM-derived MDSCs could directly suppress B cell proliferation and IgG production because we don't have sufficient
numbers and time to carry out such assays. In addition, our study indicates the involvement
and the significance of MDSCs in DCM patients. However, viral infections and MDSCs
infiltration were not analyzed at myocardial tissue level on endomyocardial biopsy, our
hypothesis about the precise effects and mechanisms of MDSC expansion in DCM patients
may be incomprehensive and not all-inclusive and additional studies are required to enhance
the understanding of MDSCs' function in the pathogenesis of DCM using in vitro and in vivo
animal models as well as clinical trials, which might lead to novel therapies for DCM patients.

In summary, our study is the first to our knowledge to illustrate that the frequencies
of circulating CD14⁺HLA-DR⁻/low MDSCs are increased in DCM patients and that their
suppressive effects on T-cell responses are also upregulated partially via Arg-1, suggesting
the participation of MDSCs in the immunomodulatory process of DCM. Given these
findings, we hypothesize that the local contexts of inflammatory microenvironments
and chronic systemic inflammation may greatly affect the tissue recruitment, retention,
and immunomodulatory capabilities of MDSCs, which subsequently suppress abnormal immune
responses and prevent the development of a more severe and possibly fatal immune
response in DCM patients. Further studies are necessary to determine the role of MDSCs
in the pathogenesis of DCM and to evaluate whether these cells might serve as a therapeutic
option for DCM patients.

Acknowledgments
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in this study.

Disclosure Statement
The authors have no competing interests to declare.

References
2 Mobini R, Maschke H, Waagstein F: New insights into the pathogenesis of dilated cardiomyopathy: Possible
3 Pankuweit S, Portig I, Maisch B: Pathophysiology of cardiac inflammation: Molecular mechanisms. Herz
2002;27:669-676.
4 Barin JG, Cihakova D: Control of inflammatory heart disease by cd4⁺ t cells. Ann N Y Acad Sci
2013;1285:80-96.
5 Lichtman AH: The heart of the matter: Protection of the myocardium from t cells. J Autoimmun
2013;45:90-96.
6 Caforio AL, Mahon NJ, Tona F, McKenna WJ: Circulating cardiac autoantibodies in dilated cardiomyopathy
7 Caforio AL, Marcolongo R, Jahns R, Fu M, Felix SB, Illiceto S: Immune-mediated and autoimmune
8 Frustaci A, Russo MA, Chimenti C: Randomized study on the efficacy of immunosuppressive therapy in
patients with virus-negative inflammatory cardiomyopathy: The timic study. Eur Heart J 2009;30:1995-
2002.
R, Volker U, Felix SB: Myocardial gene expression profiles and cardiodepressant autoantibodies predict
response of patients with dilated cardiomyopathy to immunoadsorption therapy. Eur Heart J 2013;34:666-
675.
Zhang et al.: Activated MDSCs in DCM Patients