BACKGROUND: Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that expand in cancer, inflammation, and infection and negatively regulate inflammation and the immune response. Heart failure (HF) is a complex clinical syndrome wherein inflammation induction and incomplete resolution can potentially contribute to HF development and progression. However, the role of MDSCs in HF remains unclear.

METHODS: The percentage of MDSCs in patients with HF and in mice with pressure overload–induced HF using isoproterenol infusion or transverse aortic constriction (TAC) was detected by flow cytometry. The effects of MDSCs on isoproterenol- or TAC-induced HF were observed on depleting MDSCs with 5-fluorouracil (50 mg/kg) or gemcitabine (120 mg/kg), transferring purified MDSCs, or enhancing endogenous MDSCs with rapamycin (2 mg·kg⁻¹·d⁻¹). Hypertrophic markers and inflammatory factors were detected by ELISA, real-time polymerase chain reaction, or Western blot. Cardiac functions were determined by echocardiography and hemodynamic analysis.

RESULTS: The percentage of human leukocyte antigen-D–related (HLA-DR)-CD33⁺CD11b⁺ MDSCs in the blood of patients with HF was significantly increased and positively correlated with disease severity and increased plasma levels of cytokines, including interleukin-6, interleukin-10, and transforming growth factor–β. Furthermore, MDSCs derived from patients with HF inhibited T-cell proliferation and interferon-γ secretion. Similar results were observed in TAC- and isoproterenol-induced HF in mice. Pharmaceutical depletion of MDSCs significantly exacerbated isoproterenol- and TAC-induced pathological cardiac remodeling and inflammation, whereas adoptive transfer of MDSCs prominently rescued isoproterenol- and TAC-induced HF. Consistently, administration of rapamycin significantly increased endogenous MDSCs by suppressing their differentiation and improved isoproterenol- and TAC-induced HF, but MDSC depletion mostly blocked beneficial rapamycin-mediated effects. Mechanistically, MDSC-secreted molecules suppressed isoproterenol-induced hypertrophy and proinflammatory gene expression in cardiomyocytes in a coculture system. Neutralization of interleukin-10 blunted both monocytic MDSC- and granulocytic MDSC–mediated anti-inflammatory and antihypertrophic effects, but treatment with a nitric oxide inhibitor only partially blocked the antihypertrophic effect of monocytic MDSCs.

CONCLUSIONS: Our findings revealed a cardioprotective role of MDSCs in HF by their antihypertrophic effects on cardiomyocytes and anti-inflammatory effects through interleukin-10 and nitric oxide. Pharmacological targeting of MDSCs by rapamycin constitutes a promising therapeutic strategy for HF.
Clinical Perspective

What Is New?

- The proportion of myeloid-derived suppressor cells (MDSCs) is linked to heart failure severity.
- Cardiac hypertrophy, dysfunction, and inflammation are exacerbated by MDSC depletion but alleviated by MDSC transfer.
- Monocytic MDSCs exert an antihypertrophic effect on cardiomyocytes through nitric oxide, but monocytic and granulocytic MDSCs display antihypertrophic and anti-inflammatory properties through interleukin-10.
- Rapamycin increases the accumulation of MDSCs by suppressing their differentiation, which in part mediates its cardioprotective mechanisms.

What Are the Clinical Implications?

- Increased frequency of MDSCs represents a compensatory mechanism in and might be a prognostic factor for heart failure.
- Pharmacological targeting of MDSCs (by increasing MDSC production, proliferation, and recruitment, as well as suppressing MDSC differentiation) represents a promising therapeutic strategy for heart failure.

Heart failure (HF) is a complex clinical syndrome and a progressive disorder characterized by high morbidity and mortality. The elevation of proinflammatory cells (monocytes/macrophages, neutrophils, and dendritic cells) and proinflammatory mediators (tumor necrosis factor–α, interleukin [IL]–1β, IL-6, interferon-γ, and C-reactive protein) is observed in either immune-based HF such as that associated with autoimmunity and infection (viral and bacterial) or non–immune-based HF such as that associated with ischemic injury, hypertension, and genetic cardiomyopathy. Resident and infiltrating immune cells activate inflammatory and reparative pathways that contribute to persistent heart damage and tissue remodeling. Chronic stress and tissue damage during HF development induce the release of danger-associated molecular patterns, which activate Toll-like receptors on cardiomyocytes and inflammatory cells. As a result, the ongoing inflammation and its incomplete resolution contribute to HF development and progression. The imbalance between proinflammatory and anti-inflammatory responses may play an important role in HF development because intramyocardial injection of bone marrow mononuclear cells after myocardial infarction improves cardiac function in an IL-10–dependent manner. In addition, immune-suppressive regulatory T (Treg) cells are significantly decreased in patients with HF, and adoptive transfer of Treg cells ameliorates cardiac damage and suppresses cardiac hypertrophy and fibrosis despite sustained angiotensin II–induced hypertension in mice.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature cells including precursors of macrophages, granulocytes, and dendritic cells that expand in cancer, inflammation, autoimmunity, and infection. In mice, MDSCs are characterized by coexpression of CD11b (a myeloid lineage marker) and GR1 (a granulocytic marker). In humans, MDSCs are most commonly identified by CD11b and CD33, with low levels of the major histocompatibility complex (MHC) class II molecule HLA-DR (human leukocyte antigen–D-related). MDSCs suppress immune responses by a number of mechanisms that include the following: expressing high level of arginase 1 and producing nitric oxide (NO) and reactive oxygen species to inhibit T-cell proliferation and to promote T-cell apoptosis and facilitating Treg cell induction by release of IL-10 and transforming growth factor–β, which downregulates cell-mediated immunity.

In the steady state, immature myeloid cells do not exhibit suppressive activity and are present in the bone marrow but absent in the lymph nodes. Accumulation of MDSCs was discovered in cancer and chronic inflammatory and autoimmune diseases such as diabetes mellitus, inflammatory bowel disease, pulmonary hypertension, and chronic hepatitis B. Nevertheless, little is known about the role of MDSCs in HF. Because MDSC accumulation and activation can be induced by the inflammation-associated factors vascular endothelial growth factor and prostaglandin E2 and proinflammatory cytokines, we hypothesized that MDSCs function as immune- and inflammation-suppressing cells in HF. Here, we show that MDSCs were significantly increased in both patients with HF and HF mouse models. Depletion of MDSCs exacerbated pressure overload–induced cardiac remodeling and inflammation. Conversely, adoptive transfer of MDSCs significantly reversed these pathological phenomena, and rapamycin treatment greatly improved HF by increasing MDSCs via suppression of their differentiation into mature myeloid cells. Our results indicate that MDSCs represent a promising target for HF treatment by enhancing inflammation resolution.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Patients

Fifty-five patients with dilated cardiomyopathy and HF from Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, and 11 healthy volunteers were
enrolled in this study. Diagnosis of chronic HF was based on the 2005 American College of Cardiology/American Heart Association guidelines. In accordance with the Declaration of Helsinki, this study was approved by the Ethics Committee of Tongji Hospital, and informed consent was obtained from all subjects before study initiation.

Animal Models
Male BALB/c mice and C57BL/6 mice (both 8 weeks old) weighing 25±1 g were purchased from Beijing HFK Bioscience Company (Beijing, China). C57BL/6 green fluorescent protein–positive transgenic mice were kindly provided by Professor Y.P. Hu (Department of Cell Biology, Second Military Medical University, Shanghai, China). Mice were bred in a specific pathogen-free barrier facility, and all animal experiments were approved by the Animal Care and Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology.

For HF disease models, isoproterenol (30 mg/kg; Sigma-Aldrich, St. Louis, MO) in saline containing 0.002% ascorbic acid was infused daily with Alzet osmotic minipumps (Alzet, Cupertino, CA) for 2 weeks.25 Transverse aortic constriction (TAC) surgery was performed as described previously.26 The detailed methodology is described in the online-only Data Supplement.

Flow Cytometry Analysis
Cells from fresh heparinized human blood were stained for 30 minutes at 4°C with the following antibodies: fluorescein isothiocyanate–labeled anti-human HLA-DR, phycoerythrin-labeled anti-human CD11b, allophycocyanin-labeled anti-human CD33, phycoerythrin-Cy7–labeled anti-human CD14, and phycoerythrin-Cy5.5–labeled anti-human CD15 (BD Biosciences, San Jose, CA). Red blood cells were then lysed with lysis buffer for 10 minutes, and the remaining cells were washed twice with PBS. For mice, single-cell suspensions from bone marrow, peripheral blood, spleen, and heart were prepared as described previously.27 Cells were then incubated for 1 hour at 4°C with the following monoclonal antibodies: phycoerythrin-Cy7–labeled anti–Gr1, allophycocyanin-labeled anti–CD11b, phycoerythrin-labeled anti–LY6C, fluorescein isothiocyanate–labeled anti–LY6G, phycoerythrin-labeled anti–CD68, fluorescein isothiocyanate–labeled anti–CD80 (eBiosciences, San Diego, CA), and fluorescein isothiocyanate–labeled anti–MHC-β (BD Biosciences). Stained cells were detected with an LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed with FlowJo software (FlowJo, Ashland, OR).

Echocardiography and Hemodynamic Measurements
Transtracheal echocardiography was measured 4 weeks after TAC or 2 weeks after isoproterenol infusion with a 30-MHz high-frequency scan head (VisualSonics Vevo770; VisualSonics, Toronto, Canada) as previously described.26 Hemodynamics analysis was evaluated using a pressure-volume catheter (Millar 1.4F, SPR 835; Millar Instruments, Houston, TX) as previously described.26

MDSC Isolation
For human samples, peripheral blood mononuclear cells were isolated with human lymphocyte separation medium (Tianging Haoyang, Biological Products Technology Co, Ltd, Tianjin, China) according to the manufacturer’s instructions. HLA-DR+ cells were removed from peripheral blood mononuclear cells by a negative selection using HLA-DR microbeads, followed by further isolation of CD14+HLA-DR−/low cells by positive selection with anti-CD14 microbeads. The purity of the CD14+HLA-DR−/low cell population was >80%, as detected by flow cytometry.

Murine MDSCs were separated from spleens 2 weeks after isoproterenol infusion as previously described.26 Briefly, mouse splenocytes were fractionated by Percoll (Amersham Biosciences, Little Chalfont, UK) density gradient centrifugation. Gr1+ MDSCs were isolated with Gr1 microbeads (eBiosciences) according to the manufacturer’s instructions. Monocytic MDSCs (M-MDSCs) or granulocytic MDSCs (G-MDSCs) were isolated with a Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The purity of MDSCs, M-MDSCs, and G-MDSCs was >90% according to flow cytometry results.

MDSC Depletion and Adoptive Transfer
For MDSC depletion, 5-fluorouracil (5FU; 50 mg/kg) or gemcitabine (120 mg/kg) (Sigma-Aldrich) was injected intraperitoneally every 5 days, which was initiated 3 days after TAC surgery or isoproterenol infusion and continued until the end of the experiments (ie, 2 weeks after isoproterenol infusion or 4 weeks after TAC surgery). The same volume of saline was injected as a control.

For adoptive transfer, MDSCs were isolated from spleens 2 weeks after isoproterenol infusion or 4 weeks after TAC surgery. MDSCs (1×10⁶) were injected through the tail vein of recipient mice 3 days after isoproterenol infusion or TAC surgery. At the end of the experiments, the recipient mice were euthanized.

The methods used for pharmaceutical elevation of endogenous MDSCs by rapamycin are described in the online-only Data Supplement.

RNA Extraction and Real-Time Polymerase Chain Reaction
Total RNA from heart left ventricle tissues and H9C2 cells was extracted with TRIzol (Invitrogen, Carlsbad, CA) and reverse-transcribed with MultiScribe Reverse Transcriptase (Thermo Fisher Scientific). Primers were synthesized by TsingKe (Wuhan, China), and their sequences are listed in Table I in the online-only Data Supplement. mRNA levels for ANP (atrial natriuretic peptide), α-MHC, β-MHC, IL-1β, IL-6, IL-10, inducible NO synthase (iNOS), arginase 1, and nicotinamide adenine dinucleotide phosphate-oxidase 2 (NOX2) were quantified by real-time polymerase chain reaction with the SYBR Select Master Mix (Life Technologies, Carlsbad, CA) on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). All reactions were performed in triplicate with 40 cycles of 15 seconds at 95°C, followed by 1 minute at 60°C. Gene relative expression level was calculated using...
the 2-ΔΔCT method and normalized to the corresponding level of GAPDH or α-MHC.

Western Blot Analysis
H9C2 cells were lysed in ice-cold lysis buffer and centrifuged at 12,000g at 4°C for 20 minutes. Total protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat milk for 3 hours, membranes were incubated overnight at 4°C with primary antibodies, including anti-IL-6, anti-IL-10, anti-BNP (B-type natriuretic peptide), anti-GAPDH (Abclone Biotechnology, Hurstbridge, Australia), and anti-ANP (Santa Cruz Biotechnology, Dallas, TX), followed by horseradish peroxidase–conjugated secondary antibodies. The bands were visualized with an enhanced chemiluminescence system (Thermo Fisher Scientific).

Isolation of Neonatal Rat Primary Cardiomyocytes
Hearts from neonatal rats (1 to 3 days old) were cut into small pieces in ice-cold Hanks' balanced salt solution and then digested with collagenase II and trypsin at 37°C. Cardiomyocytes were collected after centrifugation at 1000 rpm for 10 minutes, followed by removal of fibroblasts and endothelial cells through adherence to tissue culture dishes for 2 hours at 37°C.

Coculture of MDSCs and Cardiomyocytes
H9C2 cells were obtained from ATCC (Manassas, VA) and were routinely cultured in DMEM with 10% FBS at 37°C in a 5% CO2 atmosphere. MDSCs were cocultured with H9C2 cells or primary cardiomyocytes at a ratio of 5:1. For Transwell experiments, 2×10^5 H9C2 cells or primary cardiomyocytes were seeded in each upper chamber of a Transwell system (Corning, Lowell, MA) with a polycarbonate membrane (0.4-μm pore size), and 10^6 MDSCs were seeded in each lower chamber. Isoproterenol (10 μmol/L) was added to the upper chambers, followed by incubation for 24 hours.

Statistical Analysis
All results are expressed as mean±SEM. Data were analyzed with GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). Student t tests were used for comparisons between 2 groups. For multiple groups, 1-way ANOVA was used and followed by Tukey post-hoc tests. A value of P<0.05 was considered significant.

RESULTS

HLA-DR−CD33−CD11b+ MDSCs Are Increased in the Peripheral Blood of Patients With HF and Are Positively Correlated With Disease Severity
To examine whether MDSCs accumulate in HF, we detected the MDSC proportions in the peripheral blood of patients with dilated cardiomyopathy with HF (their basic clinical features are listed in Table II in the online-only Data Supplement). We found that the percentage of HLA-DR−CD33−CD11b+ MDSCs in peripheral blood was significantly elevated in patients with HF compared with healthy control subjects. In addition, the proportion of MDSCs was markedly higher in patients with New York Heart Association grades III and IV HF relative to patients with grades I and II HF (Figure 1A). HLA-DR−CD33−CD11b+ human MDSCs can be further divided into CD14+ M-MDSCs and CD15+ G-MDSCs. In patients with HF, M-MDSCs were significantly increased, whereas G-MDSCs remained unchanged, although there were more G-MDSCs than M-MDSCs (Figure 1B). The plasma levels of N-terminal pro-BNP (N-terminal pro-BNP), a marker of cardiac hypertrophy and failure, were elevated along with disease severity (Figure 1C) and positively correlated with the MDSC proportion (Figure IA in the online-only Data Supplement).

In addition, serum levels of inflammatory and anti-inflammatory cytokines, including IL-6, tumor necrosis factor–α, IL-10, and transforming growth factor–β (Figure 1D through 1G), were elevated with HF progression. Among them, increased levels of IL-6 and IL-10 were also positively correlated with MDSC proportion (Figure IB through IE in the online-only Data Supplement). To determine whether MDSCs from patients with HF exert a suppressive effect on the immune response, we cocultured MDSCs with carboxyfluorescein succinimidyl ester–labeled CD3+ T cells at a ratio of 2:1 in the presence of anti-CD3/anti-CD28 antibodies. As expected, these MDSCs strongly inhibited the proliferation of both CD4+ T cells and CD8+ T cells and the production of interferon-γ by CD3+ T lymphocytes (Figure 1H through 1K).

MDSCs Are Enhanced in TAC- or Isoproterenol-Induced HF Mouse Models
To further investigate whether the MDSC proportion is also elevated in mice experiencing HF, we established isoproterenol- and TAC-induced pressure-overloaded hypertrophy and HF models. As shown in Figure IIA through IIF in the online-only Data Supplement, isoproterenol infusion and TAC induced marked cardiac dysfunction and left ventricular dilation, which manifested as decreased ejection fraction and increased left ventricular internal diameter at end diastole, as detected by echocardiography. As expected, the percentages of CD11b+GR1+ murine MDSCs were significantly increased in the bone marrow, peripheral blood, spleen, and heart from both models (Figure 2A and 2B). The percentages of MDSCs peaked on day 6 in the bone marrow and blood and on day 9 in the spleen and heart after isoproterenol infusion and then remained constant over the experimental period (Figure IIG through IIJ in the online-only Data Supplement). The percentages of MDSCs peaked on day 14 in the bone marrow, day 3 in
the blood, day 21 in the spleen, and day 28 in the heart after TAC treatment and then remained constant in the bone marrow, spleen, and heart but declined slowly in the blood (Figure IIK through IIN in the online-only Data Supplement). Furthermore, both MDSC subsets in the 4 sites were enhanced in HF mice, although the ratio of G-MDSCs to M-MDSCs did not change significantly (Figure IIO through IIR in the online-only Data Supplement). In
addition, immunofluorescence staining showed that infiltration of CD11b+ (green) and GR1+ (red) coexpressing MDSCs (yellow) was observed in heart and spleen tissues from isoproterenol- or TAC-treated mice (Figure 2C and 2D). Purified MDSCs from spleens of isoproterenol-induced HF mice could suppress the proliferation of autologous CD3+ T cells and their production of interferon-γ (Figure 2E and 2F) rather than induce T-cell apoptosis.

Figure 2. Increase in GR1+CD11b+ myeloid-derived suppressor cells (MDSCs) in pressure overload–induced heart failure in mice. CD11b+GR1+ MDSCs in the bone marrow, peripheral blood, spleen, and heart from mice 2 weeks after isoproterenol (ISO) infusion or 4 weeks after transverse aortic constriction (TAC) surgery (n=5 each group) were detected by flow cytometry. A and B, Representative cytograms (top) and quantitative analysis of MDSCs (bottom). C and D, CD11b+GR1+ MDSCs (yellow) infiltrated in spleen and heart were detected by immunofluorescence (×400 magnification). E, Purified MDSCs were cocultured with carboxyfluorescein succinimidyl ester–labeled splenic cells from ISO-treated mice at a ratio of 2:1 in the presence of anti-CD3/anti-28 Dynabeads for 72 hours, and proliferation of CD3+ T cells was analyzed by flow cytometry. F, Interferon (IFN)–γ released in the supernatants of coculture was detected by ELISA. Data represent mean±SEM. Quantitative data of E and F are from 3 independent experiments. **P<0.01. ***P<0.001.
MDSC Depletion Aggravates Cardiac Hypertrophy and Inflammation In Mice With TAC- or Isoproterenol-Induced HF

In contrast to the decreased number of Treg cells in patients with HF,9 our results showed that MDSC numbers increased along with HF severity. Furthermore, MDSCs can differentiate into inflammatory cells such as macrophages, which could aggravate the disease in vivo, although MDSCs show a suppressive effect on lymphocyte proliferation in vitro.11 To determine the role of MDSCs in HF progression, we used 5FU and gemcitabine to deplete endogenous MDSCs without significant effects on T cells, natural killer cells, dendritic cells, and B cells.31–33 As shown in Figure IIIA through IIID in the online-only Data Supplement, treatment with 5FU or gemcitabine markedly decreased MDSCs in bone marrow, peripheral blood, and spleen on days 2 and 4 compared with MDSC levels after treatment with isoproterenol alone. On day 6 after the treatment, the MDSC proportion exhibited varying degrees of recovery. Therefore, we injected these 2 drugs every 5 days after isoproterenol infusion (Figure 3A) or TAC surgery (Figure IVA in the online-only Data Supplement) and found complete blockade of isoproterenol- and TAC-induced increases in MDSCs in the bone marrow, peripheral blood, and spleen (Figure IIIE through IIIJ in the online-only Data Supplement), as detected on day 14 after isoproterenol infusion and day 30 after TAC surgery.

Depletion of MDSCs by 5FU and gemcitabine exacerbated isoproterenol- and TAC-induced cardiac hypertrophy. Heart size, heart weight to body weight ratio, myocyte area, β-MHC/α-MHC transcript ratio, and ANP mRNA levels increased significantly compared with these measurements in the corresponding control groups (Figure 3B through 3E and Figure IVB through IVE in the online-only Data Supplement). In addition, MDSC depletion by 5FU and gemcitabine further aggravated isoproterenol- and TAC-induced cardiac dysfunction, as indicated by reduced ejection fraction (Figure 3F and Figure IVF in the online-only Data Supplement), fractional shortening, peak instantaneous rate of left ventricular pressure increase (dP/dtmax), peak instantaneous rate of left ventricular pressure increase decline (dP/dtmin), and reduced left ventricular internal diameter at end diastole (Tables VII and VIII in the online-only Data Supplement). Moreover, adoptive transfer of MDSCs lowered the levels of the proinflammatory cytokine IL-6 and further enhanced the amounts of the anti-inflammatory cytokine IL-10 in plasma and myocardial tissues of mice with isoproterenol- or TAC-induced HF (Figure 4F through 4H and Figure VF through VH in the online-only Data Supplement). In addition, isoproterenol- or TAC-induced accumulation of CD3+ T lymphocytes was significantly decreased in the heart tissue by MDSC transfer (Figure 4I and Figure V, part I, in the online-only Data Supplement). These data imply that MDSCs protect against pressure overload–induced cardiac hypertrophy, cardiac dysfunction, and inflammation.
beginning 3 days after isoproterenol infusion or TAC surgery and examined whether the drug modulates HF progression through MDSC accumulation. We found that rapamycin treatment resulted in marked increases in MDSCs, M-MDSCs, and G-MDSCs in the peripheral blood, spleen, and heart, but not in the bone marrow, in mice with isoproterenol- (Figure 5A through 5E) and TAC- (Figure VIA through VIE in the online-only Data Supplement) induced HF. Therefore, we hypothesized that rapamycin affects MDSC differentiation or prolif-
To test this hypothesis, purified MDSCs from the spleens of mice with isoproterenol-induced HF were pretreated with rapamycin (100 μmol/L) or vehicle for 30 minutes and then stimulated with lipopolysaccharide (1 μg/mL) to induce their differentiation. Compared with that of vehicle control, lipopolysaccharide alone significantly promoted the expression of MHC-II and the costimulatory molecules CD80 and CD86, indicating a mature phenotype (Figure 5F through 5H), whereas rapamycin markedly suppressed lipopolysaccharide-induced CD80 and CD86 expression in MDSCs. However, rapamycin had no effect on MDSC proliferation.

Figure 4. Improvement of isoproterenol (ISO)–induced pressure-overloaded heart failure by adoptive transfer of myeloid-derived suppressor cells (MDSCs).

A total of 1×10^7 purified MDSCs from the spleen of mice 2 weeks for ISO infusion were transferred through the tail vein into counterpart recipient mice 3 days after ISO treatment (n=6 each group). A, Representative macrograph of hearts (left) and quantitative data of heart weight to body weight ratio (HW/BW; right).

B, Hematoxylin and eosin–stained myocardial sections (×400 magnification, left) and quantitative data of myocyte area (right).

C and D, Quantitative real-time polymerase chain reaction analysis of mRNA transcription for α–major histocompatibility complex (MHC), β-MHC, and ANP (atrial natriuretic peptide) in myocardial tissues. E, Ejection fraction (EF) detected by echocardiography. F and G, Plasma levels of interleukin (IL)–6 and IL-10 determined by ELISA. H, Western blot analysis of IL-6 and IL-10 in myocardial tissues. I, CD3+ lymphocytes accumulated in the heart tissues detected by immunohistochemistry (×200 magnification, left) and quantitatively analyzed in 10 fields per section (n=6 mice each group) as the rates of T lymphocytes in the heart (right). Quantitative analysis data represent mean±SEM. *P<0.05, **P<0.01, ***P<0.001.
Figure 5. Rapamycin treatment increases myeloid-derived suppressor cells (MDSCs) through suppressing their differentiation.

Three days after isoproterenol (ISO) infusion, rapamycin (2 mg/kg) was intraperitoneally injected every day (n=5 each group). A through D, CD11b+ GR1+ murine MDSCs detected by flow cytometry in the bone marrow, peripheral blood, spleen, and heart. Cytograms are shown on the right, and quantitative data of fluorescence-activated cell sorting are shown on the left. E, Quantitative data of the percentage of CD11b+LY6G+LY6C+ granulocytic (G)-MDSCs or CD11b+LY6G−LY6C+ monocytic (M)-MDSCs in the above sites. F through H, Purified MDSCs from spleens of mice with ISO-induced heart failure were treated with rapamycin (100 μmol/L) or vehicle for 30 minutes before lipopolysaccharide (LPS; 1 μg/mL) stimulation for 24 hours. The expression of CD80, CD86, and major histocompatibility complex (MHC) II was detected by flow cytometry. All quantitative data represent mean±SEM. *P<0.05. **P<0.01. ***P<0.001.
accumulated evidence has demonstrated that rapamycin attenuates pressure overload–induced cardiac hypertrophy. To address whether the cardioprotective role of rapamycin is related to MDSCs, 5FU (50 mg/kg) was used to deplete endogenous MDSCs before rapamycin treatment. Indeed, 5FU treatment completely blocked rapamycin-induced increases in MDSCs in the peripheral blood, spleen, and heart, but not in the bone marrow (Figure 6A through 6D). Consistent with the effects of adoptive transfer of MDSCs, administration of rapamycin reduced isoproterenol-induced cardiac dysfunction, as indicated by decreased heart size, heart weight to body weight ratio, myocyte area, and β-MHC/α-MHC transcript ratio (Figure 6E through 6G). Isoproterenol-induced cardiac dysfunction was also improved by rapamycin treatment, according to increases in ejection fraction, dP/dtmax and dP/dtmin (Figure 6H through 6J). However, MDSC depletion by 5FU almost completely blocked the above--mentioned beneficial effects of rapamycin on cardiac hypertrophy and function. These data suggest that rapamycin-induced MDSC accumulation is associated with its cardioprotection.

MDSCs Mediate Anti-Inflammatory and Antihypertrophic Effects on Isoproterenol-Stimulated Cardiomyocytes Through Release of IL-10 and NO

To investigate the mechanisms that mediate MDSC-specific cardioprotective function, we cocultured H9C2 myocardial cells with MDSCs isolated from mice with isoproterenol-induced HF either with direct cell contact or with separation across a Transwell membrane. For the cell-size assay, we cultured myocardial cells with conditioned media from MDSCs instead of using the Transwell system. MDSCs, under both culture conditions, significantly suppressed the isoproterenol-induced hypertrophic response of myocardial cells, as shown by reduced cell size, ANP and BNP expression, and β-MHC/α-MHC transcript ratio (Figure 7A through 7C). Moreover, MDSCs significantly suppressed isoproterenol-induced mRNA upregulation of the proinflammatory cytokines IL-1β and IL-6 under both conditions (Figure 7D and 7E). These phenomena were also observed in primary cardiomyocytes from neonatal rats (Figure 7F through 7J).

To explore the different effects of both subtypes of MDSCs, we isolated M-MDSCs or G-MDSCs from isoproterenol-treated mice. The purity of both subtypes was >90% (Figure VIIA in the online-only Data Supplement); M-MDSCs expressed mainly arginase 1 and iNOS, whereas G-MDSCs expressed nicotinamide adenine dinucleotide phosphate-oxidase 2, which is associated with reactive oxygen species production (Figure VIIJ in the online-only Data Supplement). Both subtypes of MDSCs displayed anti-inflammatory and antihypertrophic activities under both direct contact and separated culture conditions, as evidenced by decreased isoproterenol-induced mRNA levels of IL-1β and IL-6, BNP expression, and β-MHC/α-MHC transcript ratios in cardiomyocytes (Figure VII, parts D through I, in the online-only Data Supplement).

These data indicate that the soluble mediators released by both subtypes of MDSCs chiefly exert cardioprotective effects on myocardial cells. Because IL-10 is an anti-inflammatory cytokine released by both subtypes of MDSCs (Figure VIIJ in the online-only Data Supplement), we hypothesized that IL-10 is responsible for suppressing proinflammatory cytokine production by cardiomyocytes. To test this hypothesis, we used an antibody to neutralize IL-10 produced by MDSCs and then cocultured them with H9C2 cells under separated conditions. As expected, neutralization of IL-10 partially blocked the inhibitory effect of MDSCs (Figure 7K and 7L) and either subtype of MDSCs (Figure VIIK through VIIJ, VIIN, and VIIO in the online-only Data Supplement) on IL-1β and IL-6 mRNA upregulation in isoproterenol-treated cardiomyocytes. Moreover, the suppression of isoproterenol-induced increases in BNP expression and β-MHC/α-MHC transcript ratios by both subtypes of MDSCs was also partially reversed by IL-10 neutralization (Figure VIIIM and VIIP in the online-only Data Supplement).

Adoptive transfer of MDSCs from green fluorescent protein–transgenic mice into wild-type mice 3 days after isoproterenol infusion resulted in peaks of green fluorescent protein–positive MDSCs on day 1 in the peripheral blood, day 3 in the spleen, and day 9 in the heart tissues after transfer (Figure 8A). Notably, on day 9 after MDSC transfer, cardiac NO levels in recipient mice increased significantly compared with those in mice with isoproterenol treatment alone (Figure 8B), indicating that MDSCs might exert a protective effect through NO production. An increased release of NO was confirmed in vitro in supernatants from cocultures of MDSCs and H9C2 cells with or without isoproterenol, whereas treatment with the NO inhibitor l-NMMA almost completely blocked NO release from MDSCs (Figure 8C). Moreover, inhibition of NO production partially blocked the suppressive effects of MDSCs on isoproterenol-induced increases in cell size, ANP and BNP expression, and β-MHC/α-MHC transcript ratios (Figure 8D through 8F); l-NMMA blocked M-MDSC–mediated, but not G-MDSC–mediated, suppression of the cardiac hypertrophic phenotype (Figure 8G and 8H). However, L-NMMA did not affect MDSC–mediated inhi-
bition of isoproterenol-induced mRNA upregulation of IL-1β and IL-6 (Figure 8I and 8J).

DISCUSSION

In this study, we demonstrated that MDSC numbers were significantly elevated in patients with HF and in pressure overload–induced HF mouse models. MDSC depletion aggravated isoproterenol- and TAC-induced cardiac hypertrophy and dysfunction and inflammation, but adoptive transfer of MDSCs alleviated the disease. IL-10 mediated the anti-inflammatory and antihypertrophic effects of G-MDSCs and M-MDSCs, whereas NO mediated the antihypertrophic effect of M-MDSCs. The beneficial effects of rapamycin treatment by increasing MDSCs in mice with HF identified MDSCs as a potent target for HF treatment.

In contrast to Treg cells, which are markedly decreased in HF,9 our data verified that the HLA-DR−CD33+CD11b+ MDSCs were increased in the peripheral blood of patients with HF and positively correlated with disease severity. A similar phenomenon was observed in TAC- and isoproterenol-induced HF mouse models. Although a recent report showed that isoproterenol treatment for
Figure 7. Myeloid-derived suppressor cells (MDSCs) play a cardioprotective role through secretory mediators.

A total of $10^6$ purified MDSCs from isoproterenol (ISO)-treated mice were cocultured with $2 \times 10^5$ H9C2 cells or neonatal rat primary cardiomyocytes (CMCs) in the presence of ISO (10 μmol/L) for 24 hours at a ratio of 5:1 in either direct-contact or separated condition with a Transwell membrane. Vehicle served as a control. A and F. Cardiac cells were cultured with supernatants of 12-hour–cultured MDSCs instead of the Transwell system. Representative images showing cardiac cells stained with Actin-Tracker Green (×400 magnification, left), and quantitative data of cell surface area analyzed by Image-Pro Plus 6.0 software (right). B and G. Western blot analysis of ANP (atrial natriuretic peptide) and BNP (B-type natriuretic peptide) expression in cardiac cells. (Continued)
2 weeks induced cardiac hypertrophy but not HF, in our hands, isoproterenol induced both cardiac hypertrophy and HF. The discrepancy in the response to isoproterenol is probably the result of different strains of mice being used. We found that only M-MDSCs were significantly increased in patients with HF, but both MDSC subsets were enhanced in isoproterenol- and TAC-induced HF mice. The former finding is consistent with a study by Zhang et al, who reported elevated circulating M-MDSC proportions in patients with dilated cardiomyopathy, as well as their positive correlation with NT-proBNP levels. However, the patterns of increased MDSC subtypes between human and mouse HF models are different, which may have resulted from more complex conditions in patients with HF, accompanied by hypertension, coronary heart disease, or diabetes mellitus (Table II in the online-only Data Supplement). The fact that MDSCs from either patients with HF or mice with HF could suppress ex vivo T-cell proliferation and interferon-γ production demonstrated their suppressive activities. Previous studies have shown that circulating levels of inflammatory factors exhibit prognostic importance in the setting of HF. Proinflammatory cytokines such as IL-1β and IL-6 can recruit and activate MDSCs; therefore, it is unsurprising that the MDSC proportion is positively correlated with levels of these proinflammatory cytokines in patients with HF, given their role in a compensatory mechanism involved in resolving inflammation.

Because MDSCs include immature macrophages, granulocytes, and dendritic cells, it is possible that, in the environment associated with HF, MDSCs differentiate into mature versions of these cells in vivo, facilitating inflammation and aggravating the HF process. To clarify the role of MDSCs in HF in vivo, we depleted or adoptively transferred MDSCs in mice and found that MDSC depletion exacerbated isoproterenol- and TAC-induced cardiac hypertrophy, cardiac dysfunction, and inflammation, in addition to increasing T-lymphocyte accumulation in the heart. However, adoptive transfer of M-MDSCs alleviated these pathological and pathophysiological changes, suggesting a protective role for MDSCs in HF. A previous study reported that MDSC transfer facilitated immune tolerance and prevented type 1 diabetes mellitus. It is possible that MDSCs not only promote the resolution of inflammation but also strengthen heart functions by unknown mechanisms in the context of HF.

Because we demonstrated that MDSCs can infiltrate the heart tissue, MDSCs might directly interact with myocardial cells and exert their nonimmune functions. In addition, MDSC-induced suppression requires cell-to-cell contact through interactions of cell surface markers and secretion of transitory mediators in a paracrine manner. To verify the effect of MDSCs on myocardial cells, we cocultured these 2 cell types under direct-contact or separated conditions. Our results showed that MDSCs and either subtype of MDSCs significantly suppressed isoproterenol-induced hypertrophy (ANP or BNP expression and the β-MHC/α-MHC transcript ratio) and mRNA expression of inflammatory cytokines (IL-1β and IL-6) in cardiomyocytes under both culture conditions, suggesting that MDSC-mediated cardioprotection is associated with MDSC-secreted mediators. Our results showed that both subtypes of MDSCs released IL-10 and that neutralization of IL-10 markedly blocked anti hypertrophic and anti-inflammatory effects of both subtypes of MDSCs on H9C2 cells. These findings are consistent with a previous report showing that isoproterenol- or TAC-induced pressure-overloaded cardiac hypertrophy and cardiac dysfunction were further exaggerated in IL-10 knockout mice but significantly improved by systemic treatment with IL-10.

NO plays an important role in the protection against the onset and progression of cardiovascular disease. Here, we found that adoptive transfer of MDSCs markedly increased cardiac NO production in mice with isoproterenol-induced HF. This led us to test the effect of MDSC-derived NO on isoproterenol-induced cardiac hypertrophy in vitro. Our results revealed that treatment of MDSCs with the NO inhibitor L-NMMA partially blocked their antihypertrophic effect on H9C2 cells. This finding agreed with a previous report showing that suppression of iNOS activity by simvastatin attenuates isoproterenol-induced cardiac hypertrophy in rats. Because only M-MDSCs express iNOS, it is not surprising that L-NMMA attenuated the antihypertrophic effect of only M-MDSCs, not of G-MDSCs, indicating that the 2 subtypes of MDSCs can mediate cardioprotection via shared or distinct mechanisms.

Several factors influence MDSC expansion and recruitment, however, no clinically effective measures targeting MDSCs have been developed yet. Rapamycin is a specific inhibitor of the mammalian target of rapamycin and promotes MDSC recruitment and induces iNOS expression, thereby protecting against concanavalin A–induced immunological hepatic injury or prolonging cardiac allograft survival in mouse models. Our results demonstrated that rapamycin treatment increases MDSCs, including M-MDSCs and G-MDSCs, in the peripheral blood, spleen, and heart and significantly improved
isoproterenol- or TAC-induced cardiac hypertrophy and dysfunction, similar to the cardioprotective effects observed after adoptive transfer of MDSCs. Increasing evidence demonstrates that rapamycin improves pressure-overloaded hypertrophy through different mechanisms, including inhibition of global protein synthesis, immunoproteasome induction, nuclear factor-κB activation, inflammation, and prevention of fibrosis. In this
study, we found that MDSC depletion almost completely blocked the beneficial effects of rapamycin treatment for HF, indicating that rapamycin-induced accumulation of MDSCs is associated with its cardioprotection. The evidence that the proportion of MDSCs was unaffected in the bone marrow but was increased in the periphery by rapamycin treatment for HF led us to hypothesize that rapamycin affects differentiation or proliferation rather than production of MDSCs. Indeed, rapamycin significantly suppressed lipopolysaccharide-induced MDSC differentiation into mature myeloid cells without affecting their proliferation, leading to an increase in MDSCs. This is one of the mechanisms by which rapamycin plays a cardioprotective role in pressure overload–induced HF.

CONCLUSIONS

Our results suggest that MDSCs exert a cardioprotective effect in the context of HF by antihypertrophy and anti-inflammation through release of IL-10 and NO in addition to immune suppression. Pharmacological targeting of MDSCs (eg, by rapamycin) might represent an effective method for the development of new therapeutic strategies for HF treatment.

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Disclosures

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REFERENCES


