Dectin-2 Deficiency Modulates Th1 Differentiation and Improves Wound Healing After Myocardial Infarction

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Rationale: Macrophages are involved in wound healing after myocardial infarction (MI). The role of Dectin-2, a pattern recognition receptor mainly expressed on myeloid cells, in the infarct healing remains unknown.

Objective: The aim of this study is to determine whether Dectin-2 signaling is involved in the healing process and cardiac remodeling after MI and to elucidate the underlying molecular mechanisms.

Methods and Results: In a mouse model of permanent coronary ligation, Dectin-2, mainly expressed in macrophages, was shown to be increased in the early phase after MI. Dectin-2 knockout mice showed an improvement in the infarct healing and cardiac remodeling, compared with wild-type mice, which was demonstrated by significantly lower mortality because of cardiac rupture, increased wall thickness, and better cardiac function. Increased expression of α-smooth muscle actin and collagen I/III was observed, whereas the levels of matrix metalloproteinase-2 and matrix metalloproteinase-9 were decreased in the hearts of Dectin-2 knockout mice after MI. Dectin-2 deficiency inhibited the rate of apoptotic and necrotic cell death. However, Dectin-2 did not affect immune cell infiltration and macrophage polarization, but it led to a stronger activation of the Th1/interferon-γ immune reaction, through the enhancement of interleukin-12 production in the heart. Interferon-γ was shown to downregulate transforming growth factor-β–induced expression of α-smooth muscle actin and collagen I/III in isolated cardiac fibroblasts, leading to a decrease in migration and myofibroblast differentiation. Finally, Dectin-2 knockout improved myocardial ischemia–reperfusion injury and infarct healing.

Conclusions: Dectin-2 leads to an increase in cardiac rupture, impairs wound healing, and aggravates cardiac remodeling after MI through the modulation of Th1 differentiation. (Circ Res. 2017;120:1116-1129. DOI: 10.1161/CIRCRESAHA.116.310260.)

Key Words: collagen ■ flow cytometry ■ myocardial infarction ■ Th1 cells ■ wound healing

The extent of postinfarction remodeling depends on the infarct size and on the quality of cardiac repair.1 Postinfarct healing and stable scar formation are important for the strengthening of the infarct tissue and prevention of adverse remodeling and increased cardiac rupture.2 Accumulating evidence has shown that immune response plays a central role in the healing process that follows acute myocardial infarction (MI). Immune cells integrate and cooperate with cardiac innate cells to perform the clearance of cell debris and angiogenesis and to coordinate reparative mechanisms.1,2

Excessive early inflammation and prolonged inflammatory reaction may augment matrix degradation, causing cardiac rupture, and impair collagen deposition, which may lead to the formation of scar tissue with reduced tensile strength, increasing chamber dilation.1 However, immunosuppressive therapy, with the application of corticosteroids, was shown to result in an increased rate of serious side effects in clinical practice, such as cardiac rupture,3 and recent experimental data showed that macrophage depletion impairs wound healing and increases left ventricular (LV) remodeling after MI.4 This suggests that controlled inflammation and immune response represent the prerequisites for appropriate cardiac repair after MI. Therefore, a better understanding of the molecular and cellular mechanisms underlying the inflammatory response after MI and the development of alternative therapies against LV remodeling are urgently required.

Myocardial necrosis causes sterile inflammation, which is characterized by the recruitment and activation of immune...
These immune cells may perform cell-specific functions during the process of ischemia injury and cardiac repair. Recently, Yan et al.5 and Nahrendorf et al.7 have delineated the biphasic role of Dectin-2, a member of the C-type lectin receptor family, participates in various aspects of innate immune responses. In the early post-MI phase, Dectin-2 increases Th1 activity and IFN-γ production, leading to the suppression of myofibroblast differentiation and migration in the infarcted area.

Nonstandard Abbreviations and Acronyms

- D2KO: Dectin-2 knockout
- IFN-γ: interferon-γ
- IL: interleukin
- IR: ischemia reperfusion
- LV: left ventricular
- MI: myocardial infarction
- MMP: matrix metalloproteinase
- α-SMA: α-smooth muscle actin
- TGF-β: transforming growth factor-β
- Treg: regulatory T cell
- WT: wild type

What is Known?

- Postinfarct healing and stable scar formation prevent adverse remodeling and reduce the incidence of cardiac rupture.
- Immune responses contribute to the healing process after acute myocardial infarction (MI).
- Dectin-2, a member of the C-type lectin receptor family, participates in a variety of immune responses.

What New Information Does This Article Contribute?

- Dectin-2 aggravates adverse myocardial remodeling and in turn increases ventricular rupture and mortality. In contrast, Dectin-2 deficiency improves wound healing and cardiac remodeling.
- In the early post-MI phase, Dectin-2 increases Th1 activity and IFN-γ production, leading to the suppression of myofibroblast differentiation and migration in the infarcted area.

Novelty and Significance

The treatment of adverse remodeling and cardiac rupture after MI is a difficult clinical problem; early intervention is important. In this study, we show that Dectin-2 deficiency improves wound healing and cardiac remodeling after MI and ischemia–reperfusion injury. Moreover, we discovered that Dectin-2 aggravates infarct healing through the specific modulation of Th1 differentiation, without affecting Th2, Th17, or Tregs. Th1 activation increases the production of IFN-γ, which leads to more matrix degradation and cell death. It also affects the differentiation and migration of fibroblasts in the infarcted area. However, the effect of Dectin-2 is not related to myeloid cells infiltration and macrophage polarization after MI and ischemia–reperfusion injury. These findings suggest that Dectin-2 may serve as a potential target for early intervention to improve the prognosis of patients with MI.

cells associated with innate and adaptive immune system.5,6 These immune cells may perform cell-specific functions during the process of ischemia injury and cardiac repair. Recently, Yan et al.5 and Nahrendorf et al.7 have delineated the biphasic infiltration of innate and adaptive immune cells after MI by flow cytometry. Neutrophils accumulated immediately, and their number peaked at day 3 post-MI, whereas macrophages were the predominant cell type in the first week post-MI. Macrophages are functionally heterogeneous, and the proinflammatory M1 macrophages were shown to be predominant at day 1 to 3 post-MI, which was associated with excessive inflammation and ischemic injury, whereas the reparative M2 macrophages represented the predominant macrophage subset after 5 days.

To identify the potential effective therapeutic target against M1-associated macrophage inflammation in MI setting, we analyzed macrophages obtained from the infarcted heart at different time points after MI. Our analysis revealed that Dectin-2 (Clec4n) is highly expressed in the early phase of macrophage infiltration, suggesting that Dectin-2 may be involved in M1 macrophage activation, which contributes to cardiac ischemic injury and wound-healing impairment. C-type lectin receptors, one of the 4 families of pattern recognition receptors, have central roles in the immune response to fungal pathogens.8 The expression of Dectin-2, which belongs to C-type lectin receptors, has been observed in myeloid cells, inducing cytokine production through an FcRγ (Fc receptor γ chain and Syk (spleen tyrosine kinase)-CARD9 (capsase recruitment domain-containing protein 9)-NF (nuclear factor)-κB-dependent signaling pathway, without the involvement of MAP (mitogen-activated protein) kinases, to protect the organisms against fungal and mycobacterium infections.9 Additionally, Dectin-2 mediates pulmonary graft-versus-host disease development after allogeneic hematopoietic stem cell transplantation.10 However, little is known about the precise role of Dectin-2—signaling pathway in the remodeling of the myocardium after MI. Therefore, we examined the effects of Dectin-2 in myocardial inflammation, wound healing, and survival in Dectin-2 knockout (D2KO) mouse model of MI.

Methods

An expanded Methods section is available in the Online Data Supplement.

Mice

Clec4n−/− (D2KO) mice in a C57BL/6 background were generated as described previously.11 Wild-type (WT) C57BL6/J mice (20–25 g, male, 10–16 weeks old; SLRC Laboratory Animal, Shanghai, China) were used as controls in this study. This study and all animal procedures conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996), and were approved by the Animal Care Committee of Shanghai Jiaotong University School of Medicine.

Induction of MI

Permanent ligations of the left anterior descending artery (MI) or sham operations without ligation were performed on the experimental animals, as described previously.12 Briefly, mice were lightly anesthetized with diethyl ether, intubated, and fully anesthetized with 1.0 to 1.5% isoflurane gas, whereas the mechanical ventilation was performed with a rodent respirator. The chest cavity was opened via left thoracotomy, in order to expose the heart so that the left anterior descending coronary could be visualized using a microscope and permanently ligated with a 7-0 silk suture at the site of its emergence from the left atrium. Complete occlusion of the vessel was confirmed by the presence of myocardial blanching in the perfusion bed. Mice that did not survive the recovery from anesthesia were excluded from...
the analysis. Sham-operated animals underwent the same procedure without coronary artery ligation. To induce ischemia–reperfusion (IR) injury, a slipknot was made around the left anterior descending coronary artery against PE10 tubing with a 7-0 silk suture. After 45-minute ligation, the ligature was released to allow reperfusion.

Additional Methods
The expanded Methods section in the Online Data Supplement contains information on the cell preparation for flow cytometry; the description of flow cytometric analysis; intracellular cytokine staining; quantitative real-time polymerase chain reaction; immunohistochemistry; pressure–volume loop analysis; echocardiography; infant size and wall thickness determination; Western blotting; cDNA microarray analysis; and the culturing and stimulation of murine cardiac fibroblasts. All antibodies used in this study are listed in Online Table I.

Statistical Analysis
All values are presented as mean±SEM. Comparisons between groups were made using Mann–Whitney U test, whereas the data obtained from multiple groups were compared using Kruskal–Wallis test with Dunn multiple comparison test or Bonferroni post hoc analysis. P values <0.05 were considered statistically significant. Statistical analyses were performed with GraphPad Prism 5.0 (Graph Pad Prism Software Inc, San Diego, CA) and SPSS 15.0 for Windows (SPSS, Inc, Chicago, IL).

Results
Dectin-2 Levels Were Shown to Be Increased in Cardiac Macrophages After MI
M1 macrophages are involved in the ischemia injury and impaired wound healing.13 Therefore, to identify the potential therapeutic targets for the treatment of M1 macrophage inflammation, macrophages from the infarcted heart were obtained at different time points after MI and analyzed using gene microarray. We found that 884 genes were upregulated in the early phase and downregulated after that, and this cluster of genes was shown to be the most abundant (Online Figure I), suggesting that the expression of these genes may be induced during M1 macrophage polarization. We have demonstrated that Dectin-2, a pattern recognition receptor, is highly expressed in the early phase of macrophage infiltration (Online Figure II). The results of quantitative polymerase chain reaction and Western blotting showed that the kinetics of Dectin-2 expression in sorted macrophages and heart tissue at different time points post-MI was consistent with the microarray data (Figure 1A through 1C). Furthermore, the expression of Dectin-2 was shown to be much higher in the infarct area than in the noninfarcted area. Flow cytometric analysis showed that Dectin-2 is mainly expressed in myeloid cells, where 40% of macrophages express Dectin-2, whereas <10% of neutrophils were shown to express this molecule (Figure 1D; Online Figure III). A detailed temporal analysis of cellular infiltrate dynamics for Dectin-2+ macrophages and Dectin-2+ neutrophils demonstrated that both of them peaked at day 1 after MI, and Dectin-2+ macrophages predominated compared with Dectin-2+ neutrophils in the post-MI heart. However, Dectin-2 was not expressed in T cells or B cells in the post-MI heart tissue (Figure 1E).

Dectin-2 Knockout Led to an Improvement of Cardiac Function After MI
Next, we examined the cardiac remodeling and dysfunction in WT and D2KO mice (Online Figure IX). Echocardiographic parameters measured in D2KO mice were comparable to those determined in the WT mice at baseline (Online Table II). Cardiac function was analyzed at day 28 post-MI, and echocardiographic analyses demonstrated that the acceleration of cardiac dilatation (LV end-diastolic diameter: 5.62±0.17 versus 5.01±0.13 mm; P<0.01) and deterioration of LV function (ejection fraction: 22.33±1.70% versus 33.75±2.03%; P<0.001) were inhibited in D2KO mice, in comparison with those in the WT mice (Figure 2A; Online Table III). Pressure and volume parameter values, derived from pressure–volume loop data, demonstrated that systolic and diastolic functions were greatly improved in the D2KO mice compared with those in the WT mice (Figure 2B; Online Table IV). Furthermore, the weight of hearts and lungs, adjusted for body weight at day 28 post-MI, were significantly lower in D2KO mice than in WT mice (Online Table III).

Dectin-2 Knockout Led to an Improvement in MI-Induced Cardiac Rupture, Wall Thinning, and Healing
We showed that the survival rate was higher in D2KO mice than in the WT mice at day 28 after MI (69.57% versus 49.01%, respectively; log-rank test P=0.0562), although all sham-operated animals in both groups survived to the end of the study (Figure 3A). The incidence of cardiac rupture–induced death was significantly higher in WT mice than in D2KO mice (35.8% versus 17.4%, respectively; P=0.04), although the heart failure rates were similar in both groups (15.09% versus 13.04%, respectively; P=0.87; Figure 3B). Masson trichrome staining revealed no difference in the gross morphology of the sham-operated hearts between WT and D2KO mice. Twenty-eight days after MI, D2KO mice had a much higher wall thickness at the infarct area compared with that observed in the WT mouse, although the infarct size was similar in both groups (Figure 3C). Collagen volume fraction detected by picrosirius red staining in the infarct and border areas was significantly higher in D2KO mice than in the WT mice 28 days after MI (Figure 3D). However, myocardial fibrosis in the noninfarct area did not differ significantly between WT and D2KO mice (Online Figure V). Moreover, picrosirius red polarized microscopy analyses of collagen fibers in the infarcted area demonstrated the predominance of loosely assembled green or yellow fibers in WT mice, whereas well-aligned thick fibers were observed in D2KO mice at day 28 after MI (Figure 3E).

Dectin-2 Deficiency Was Shown to Promote Wound Healing and Scar Formation After MI
We analyzed myocardial wound-healing parameters and myocardial fibroblast differentiation in the early phase after MI, in order to understand the mechanisms underlying the cardiac rupture. The expression of α-smooth muscle actin (α-SMA), collagen I, and collagen III in the infarcted heart was determined to be much higher in D2KO mice than in WT mice at both day 4 and day 14 (Figure 4A). The protein levels of α-SMA and collagen I were significantly higher in D2KO mice than in WT mice, at day 4 post-MI (Figure 4B). Additionally, immunohistochemical analyses were performed to assess the expression of these proteins at day 28 post-MI, which demonstrated that the
Figure 1. Dectin-2 levels were increased in the heart after myocardial infarction (MI). A, Macrophages were isolated from the mouse hearts at different time points after MI, and Dectin-2 mRNA expression was examined (n=4–6). B, Dectin-2 expression levels were analyzed in both infarct and noninfarct area at different time points after MI (n=4–5). C, Dectin-2 protein expression in (Continued)
expression levels of these proteins were significantly higher in D2KO mice than in the WT mice (Figure 4C through 4E).

**Deficiency of Dectin-2 Did Not Affect MI-Induced Angiogenesis but Inhibited Cardiomyocyte Apoptosis and Necrotic Cell Death**

Myocardial angiogenesis was reported to be crucial for the wound healing and cardiac remodeling, and therefore, we examined the levels of angiogenesis by isolectin IB4 staining 28 days post-MI. Vascular density was slightly increased at day 1 post-MI and later decreased, but we did not observe any differences in this parameter between WT and D2KO mice at any time point after MI (Figure 4F). Furthermore, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling) staining and propidium iodide staining were performed to detect cardiomyocyte apoptosis and necrotic cell death at various time points after MI. We showed that both the apoptosis and necrotic cell death rates peaked at day 1 post-MI and decreased thereafter. Further analyses revealed that the number of apoptotic cells at the border areas was significantly lower in D2KO mice compared with that in the WT mice, although no significant differences in the infarct area were observed at day 1 after MI. However, the rates of apoptosis at both infarct and border areas were significantly decreased in D2KO mice compared with those determined in the WT mice at day 3 post-MI. As for the necrotic cell death rate, we demonstrated that the number of propidium iodide-positive cells considerably decreased in D2KO mice in comparison with that in WT mice, in both infarct and border areas at day 3 post-MI, but no significant difference was observed at day 1 and day 7 post-MI (Online Figure VI).

**Dectin-2 Knockout Led to the Inhibition of Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 Expression After MI**

Next, we assessed matrix metalloproteinase (MMP)-2 and MMP9 expression levels in the heart at different time points after MI, and this showed that MI stress leads to the upregulation of both MMP2 and MMP9 expression. However, their expression levels were shown to be significantly suppressed in the D2KO mice in both infarct and border areas at days 1, 3, and 7 after MI (Figure 5; Online Figure VII).

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**Figure 1 Continued.** Heart was determined at different time points (n=4–6). **D. Left:** The representative flow cytometric analysis of Dectin-2 expression on gated CD11b+ myeloid cells, CD11b+F4/80+ macrophages, and CD11b+Ly-6G+ neutrophils in the heart at day 1 post-MI. **Right:** The temporal dynamics of Dectin-2+ macrophages and Dectin-2+ neutrophils were calculated as cell number per microgram of the heart tissue (n=4–6). **E.** Dectin-2 expression on gated T and B cells in heart was measured at day 1 post-MI in both wild-type (WT) and Dectin-2 knockout (D2KO) mice (n=3). Data are expressed as mean±SEM. NS indicates not significant. *P<0.05; **P<0.01; ***P<0.001. Data in (A, C, and D) were analyzed using Kruskal–Wallis tests with Dunn multiple comparison; data in (B and E) were analyzed using Mann–Whitney U tests.

**Figure 2.** Cardiac function comparison between wild-type (WT) and Dectin-2 knockout (D2KO) mice after myocardial infarction (MI). **A.** Echocardiographic analysis of left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), fractional shortening (FS), left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), and ejection fraction (EF) at day 28 after the MI or sham operation (n=8–9). **B.** Pressure-volume loop analysis was performed in both WT and D2KO mice at day 28 post-MI. LVESV, LVEDV, LV end-systolic pressure (ESP), and LV end-diastolic pressure (EDP) were calculated from the pressure-volume loop data (n=7–9). Data are expressed as mean±SEM. NS indicates not significant. *P<0.05; **P<0.01; ***P<0.001. Data were analyzed using Kruskal–Wallis tests followed by Bonferroni post hoc analysis.
Dectin-2 Knockout Did Not Affect the Immune Cell Infiltration and Macrophage Polarization in the Heart After MI

Flow cytometric analysis was applied to evaluate the degree of infiltration by various inflammatory cells after MI in both mice strains. The number of total CD45+ leukocytes in the infarcted heart was significantly higher compared with the sham-operated heart, but Dectin-2 deficiency did not affect the degree of the infiltration. Furthermore, the numbers of CD11b+ myeloid cells, CD11b+F4/80+ macrophages, CD11b+Ly-6G+ neutrophils, and CD45+CD3+ T cells did not significantly differ between WT and D2KO mice, although MI itself induced a robust infiltration of myeloid cells (Online Figure VIII A). Because proinflammatory M1 macrophages and reparative anti-inflammatory M2 macrophages play opposing roles in tissue repair after MI, we assessed M1 and M2 macrophage levels in the heart (Online Figure VIII B). The percentage of CD11b+F4/80+CD206− M1 macrophages was significantly increased, and the percentage of CD11b+F4/80+CD206− M2 macrophages decreased in response to MI stress; however, there was no significant difference in the percentage of M1 and M2 macrophages between the 2 investigated mouse strains after MI.

Dectin-2 Deficiency Suppressed Th1 Differentiation in the Heart After MI

We measured the levels of transcriptional factors associated with T-cell differentiation, and we observed no significant differences in Gata3, RORc, and Foxp3 expression, which represent the major transcriptional factors regulating Th2, Th17, and regulatory T-cell (Treg) polarization, respectively, in the infarcted heart. However, we found that the expression...
of T-bet, a transcriptional factor responsible for Th1 differentiation, was significantly suppressed in D2KO mice, compared with that in the WT group (Figure 6A). After this, we examined the myocardial expression of signature cytokines, interferon (IFN-γ) for the Th1 cells, and interleukin (IL)-17A for Th17 cells. In line with our previous findings, MI led to an increase in the levels of IFN-γ and IL-17A, but the increase in IFN-γ production was significantly inhibited in D2KO mice, whereas IL-17A expression did not significantly differ between the 2 strains (Figure 6B). Additionally, gene expression levels of IL-12p35 and IL-12p40, crucial factors for the induction of Th1 differentiation in the myocardium, were shown to be lower in D2KO mice after MI (Figure 6C). Furthermore, the expression of these genes was significantly reduced in macrophages isolated from D2KO mice compared with those measured in the WT mice (Figure 6D).

The cell source of IFN-γ and IL-17A in the heart after MI was analyzed, and the obtained results showed that >85% of CD3+IFN-γ+ cells were CD4+ T cells (Th1), whereas <10% were CD8+ T cells or γδT cells. Additionally, we found that almost all of IL-17A+ T cells were γδT cells (Figure 6E). Flow cytometric analysis confirmed that the percentage of IFN-γ+ T cells was

Figure 4. Dectin-2 deficiency improved wound healing and scar formation after myocardial infarction (MI). A, Gene expression of collagen I, collagen III, and α-smooth muscle actin (α-SMA) at different time points after MI (n=4–5). B, Collagen I and α-SMA protein expression at day 4 post-MI, in the scar tissue isolated from wild-type (WT) and Dectin-2 knockout (D2KO) mice (n=4). Immunohistochemical analyses of α-SMA (C), collagen I (D), collagen III (E; bars: white=50 μm and yellow=20 μm), and isolecitin B4 (B4; bar=20 μm; F) in the heart tissue at day 28 post-MI (n=4–6). Data are expressed as mean±SEM. NS indicates not significant. *P<0.05; **P<0.01; ***P<0.001. Data in (A and C–F) were analyzed using Mann–Whitney U tests. Data in (B) were analyzed using Kruskal–Wallis test followed by Bonferroni post hoc analysis.
greatly reduced, but IL-17A+ T-cell number was not altered in D2KO mice after MI (Figure 6F). The neutralization of IL-12 was shown to lead to a considerable reduction in IFN-γ production by T cells (Figure 6G), indicating that Dectin-2–dependent IL-12 expression is required for Th1 differentiation in the heart after MI.

Dectin-2 Deficiency Was Shown to Improve the Wound-Healing Process Through the Regulation of IFN-γ Production

We examined the effects of IFN-γ on the migratory capacity of cardiac fibroblasts using an in vitro wound-healing model. Stimulation of cardiac fibroblasts with 10 ng/mL of IFN-γ decreased their migratory capacity in comparison with that of the control untreated fibroblasts, as assessed by the covered area 36 hours after the initial scratch in the culture well (Figure 7A). Fibroblasts were isolated from the hearts of WT mice, and they were stimulated with transforming growth factor-β (TGF-β) for 24 hours in the presence or absence of IFN-γ. Stimulation with IFN-γ led to a significant downregulation of TGF-β–induced α-SMA and collagen I expression (Figure 7B). Immunofluorescence staining confirmed that IFN-γ suppresses TGF-β–induced α-SMA expression in the isolated cardiac fibroblasts (Figure 7C). To further investigate the effect of Dectin-2 deficiency on wound healing, mononuclear cells were isolated from the infarcted heart of WT and D2KO mice at day 4 post-MI, treated with PMA (phorbol-12-myristate-13-acetate) and ionomycin, and the resulting conditioned media were used to stimulate cardiac fibroblasts in the presence of TGF-β. MI triggered IFN-γ production from the mononuclear cells of WT mice, whereas Dectin-2 deficiency led to a suppression of this induction (1.85±0.26 versus 0.56±0.18 ng/mL, respectively; *P<0.01; Online Figure IX). The obtained results demonstrate that the stimulation of cardiac fibroblasts with the conditioned medium from D2KO mice led to a significantly higher migratory capacity of these cells compared with the cells isolated from WT mice (Figure 7D). Additionally, the conditioned medium obtained using the cells isolated from D2KO mice failed to inhibit TGF-β–induced fibroblast activation (Figure 7E). We showed that the inhibitory effects of the conditioned medium from WT heart on fibroblast activation and migratory capacity can be reversed by using the IFN-γ–neutralizing antibody. These results clearly indicate that Dectin-2 deficiency can improve wound healing and fail to inhibit TGF-β–induced fibroblast activation through the regulation of IFN-γ expression.

Dectin-2 Deficiency Improved Myocardial IR Injury

Finally, we investigated the role of Dectin-2 in myocardial IR injury because this model represents a less severe injury compared with the irreversible ischemic model, and in clinical practice, most of the acute MI patients are able to reach the hospital in time for the reperfusion therapy. First, we demonstrated that the protein expression levels of Dectin-2 peaked at day 1 after IR and begun to decrease thereafter (Figure 8A). To examine the functional role of Dectin-2 in myocardial IR injury, mice were subjected to ischemia for 45 minutes and reperfusion for 48 hours, and afterward, infarct size was determined by TTC (2,3,5-triphenyltetrazolium chloride) staining and area

Figure 5. Effects of Dectin-2 deficiency on matrix metalloproteinase (MMP)-2 and MMP9 expression after myocardial infarction (MI). Expression levels of MMP2 (A) and MMP9 (B) in the heart were examined by immunohistochemistry at days 0, 1, 3, 7, and 14 post-MI (n=4–6; bar=20 μm). Data are expressed as mean±SEM. *P<0.01; **P<0.01; ***P<0.001, for comparisons between wild-type (WT)-infarct and Dectin-2 knockout (D2KO)-infarct areas. 4P<0.01; **P<0.01; ***P<0.001, for comparisons between WT-border and D2KO-border areas. Data were analyzed by Kruskal–Wallis test followed by Bonferroni post hoc analysis.
Figure 6. Effects of Dectin-2 deficiency on CD4+ T-cell differentiation. A, T-bet (Th1), Gata3 (Th2), RORc (Th17), and Foxp3 (Treg) gene expression levels at different time points after myocardial infarction (MI) were determined (n=4–6). B, Th1- and Th17-related cytokines, interferon-γ (IFN-γ) and interleukin (IL)-17A, were detected in the heart after MI (n=4–6). C, IL-12p35 and IL-12p40 mRNA levels in heart were determined (n=4–6). D, CD11b+F4/80+ macrophages were isolated from the heart of wild-type (WT) and Dectin-2 knockout (D2KO) mice at day 1 post-MI, and gene expression levels of IL-12p35 and IL-12p40 were examined (n=3). E, IFN-γ and IL-17A expression on gated CD3+, CD4+, and CD8+ T cells and γδT cells was analyzed in the heart tissue samples at day 5 post-MI (n=3). F, CD3+IFN-γ+ and CD3+IL-17A+ T-cells in the heart at day 5 post-MI were analyzed in WT and D2KO mice (n=4). G, Percentage of CD3+IFN-γ+ T cells in the heart was assessed at day 5 post-MI after the neutralization of IL-12p35 in vivo (n=4). Data are expressed as mean±SEM. NS indicates not significant. *P<0.05; **P<0.01; ***P<0.001. Data in (A–D and G) were analyzed by Mann–Whitney U tests. Data in (F) were analyzed using Kruskal–Wallis test followed by Bonferroni post hoc analysis.
at risk by Evans blue. Compared with that in the WT mice, the infarct/area at risk ratio was considerably decreased in D2KO mice (44.3±1.8% versus 31.2±1.2%, respectively; *P*<0.001; Figure 8B). In parallel, a decreased number of apoptotic and necrotic cells was observed in both infarct and border areas of the injured myocardium at different time points after IR injury (Figure 8C and 8D; Online Figure X). However, the numbers of CD11b+F4/80+ macrophages, CD11b+Ly-6G+ neutrophils, and M1 and M2 macrophages in the ischemic heart did not significantly differ between WT and D2KO mice at day 1 after IR injury (Online Figure XI). After this, mice were examined for 14 days to determine the morphology, myocardial fibrosis, and cardiac function. We demonstrated that infarct size assessed by Masson staining at 14 days after MI was considerably decreased in D2KO mice compared with that in the WT group (15.6±1.0% versus 32.2±1.5%, respectively; *P*<0.01; Figure 8E), which was accompanied with an improved systolic function (ejection fraction: 50.9±2.9% versus 41.6±1.6%; *P*<0.01; Figure 8F). However, collagen volume fraction detected by picrosirius red staining in the infarct areas was significantly higher in D2KO mice than in WT mice at day 14 after IR, while the examination of collagen fibers in the infarct area by polarized microscopy predominantly showed loosely assembled green or yellow fibers in WT mice, whereas well-aligned orange thick fibers were observed in D2KO mice at day 14 after IR (Figure 8G). Immunohistochemical analyses showed significantly increased α-SMA, collagen I, and collagen III expression levels in D2KO mice, in comparison with those in the WT mice at day 14 after IR (Figure 8H; Online Figure XIIIB), whereas the levels of angiogenesis detected by isolectin IB4 staining did not differ between the 2 strains (Online Figure XIIIC). In addition, MMP2 and MMP9 expression levels in the heart were shown to be significantly suppressed in the D2KO mice than in WT ones in the early phase after IR (Online Figure XIII). Therefore, Dectin-2 may have a significant role in myocardial IR injury and healing process.
Figure 8. Dectin-2 deficiency improved myocardial ischemia–reperfusion (IR) injury and wound healing. A, Dectin-2 expression levels were analyzed by Western blot in the heart at different time points after myocardial IR injury (n=4). B, Representative Evans blue/TTC (2,3,5-triphenyltetrazolium chloride) staining of the heart tissue sections obtained from the wild-type (WT) and Dectin-2 knockout (D2KO) mice at day 2 after myocardial IR injury (left). Quantitative analysis of infarct size (right) at day 2 post-IR in WT and D2KO mice (n=7–8). The ratios of area at risk/left ventricular (AAR/LV) and infarct area/area at risk (infarct area/AAR) were determined. C and D, Representative TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling; C) and propidium iodide (PI; D) staining of the heart tissue sections obtained from WT and D2KO mice at day 1 post-IR injury. TUNEL-positive (Continued)
Dectin-2 Deficiency Improves Wound Healing and Cardiac Remodeling

We demonstrated that Dectin-2 is highly expressed in macrophages at the early post-MI phase, suggesting that this molecule may be involved in M1 macrophage activation. Further analyses revealed that Dectin-2 promotes cardiac rupture and remodeling by increasing the expression of MMP2 and MMP9, inhibiting myofibroblast activation and collagen deposition. The protein and mRNA levels of collagen I and III and α-SMA were increased in the D2KO mice, indicating a higher level of myofibroblast differentiation in the D2KO mice. Activated myofibroblasts migrate into the infarct area, express α-SMA, and can produce collagen I and III in abundance, which leads to the formation of a collagenous scar, crucial for the prevention of infarct expansion and LV rupture.14 However, Dectin-2 deficiency did not affect angiogenesis, although it is important for the granulation tissue formation. These results suggest that Dectin-2-signaling pathway specifically affects the healing processes by modulating the transition of fibroblasts to myofibroblasts. Additionally, we found that the beneficial effect of Dectin-2 inhibition on infarct size was modest in the chronic irreversible injury model, although in a less severe injury (IR model), Dectin-2 deficiency considerably improved myocardial IR injury and healing process. This indicates that Dectin-2 shows a more powerful effect on ischemic stress in the less severe injury model. This was observed in previous studies as well, where it was demonstrated that the inhibition of angiotensin-converting enzyme15 or the activation of natural killer T cells16,17 can improve infarct healing but did not affect the infarct size in a permanent MI model. However, they were shown to lead to decrease in the infarct size in a transient myocardial IR model.

Dectin-2 Deficiency Effects on Wound Healing Are Not Related to Myeloid Cells Infiltration and Macrophage Polarization After MI

Local inflammatory environment orchestrates macrophage infiltration and polarization.52 Two main subsets of monocyte/macrophages have been identified in mice: Ly6C(high) proinflammatory monocyte/macrophages (M1 macrophages) migrate into the injured tissues and express high levels of CC chemokine receptor CCR2, and they are responsible for the scavenging of debris and secretion of proinflammatory cytokines and matrix-degrading proteases, which contribute to the myocardial injury. The alternatively activated Ly6(ch) monocyte/macrophages (M2 macrophages) show increased phagocytic activity and are able to produce and secrete inhibitory mediators, such as IL-10 and TGF-β, and these cells represent the negative regulators of inflammation, producing angiogenic mediators and contributing to the infarct healing. In this study, we demonstrated that Dectin-2, mainly expressed in macrophages, and to a lesser extent in neutrophils, play a crucial role in post-MI healing. Previous studies reported that mice with Dectin-2 deficiency have reduced levels of inflammatory cytokines (tumor necrosis factor-α, IL-6, and others) and chemokines (CXCL1 and CXCL2), which leads to the attenuated recruitment of macrophages and neutrophils in response to inflammatory insults and fungi infection.9,11 Our results showed that Dectin-2 deficiency does not alter the rate of cell infiltration and macrophage polarity after MI, indicating the existence of a different underlying mechanism. However, the classification of macrophages into M1 and M2 groups represents an oversimplification. It is increasingly recognized that the inflamed tissues contain a wide spectrum of macrophage subpopulations with distinct properties and functional characteristics.18 In the complex environment of the injured heart, the spatially and temporally regulated expression of a variety of cytokines, chemokines, and growth factors is likely to affect macrophage phenotype leading to sequential generation of many distinct subsets. Therefore, further studies are needed to explore the effects of Dectin-2 on the macrophage phenotypic characteristics, molecular profiles, and their functional roles in the healing process after MI.

Dectin-2 Aggravates Infarct Healing by Enhancing Th1 Activation

The activation of innate immune response in the early stages after MI is followed by the migration of the cells that are involved in the adaptive immune responses, especially CD4⁺ T cells,3 which is important for the orchestration of the wound-healing process in the heart.219 CD4⁺ T cells were shown to contribute to IR injury, especially by secreting IFN-γ.20 In contrast to this, the activation of CD4⁺ T cells, most likely through the recognition of autoantigens, improves cardiac wound healing by modulating local innate immune activation, especially monocyte infiltration and polarization.19 CD4⁺ T cells may be differentiated into Th1, Th2, Th17, and Tregs, depending on the cytokine stimulation or variable local environments.21 In
our previous study, we showed that conventional effector CD4+ T cells, mainly with a Th1 cytokine profile, and Tregs infiltrate the myocardium within a few days after MI. Individual subsets of T cells have their specific functional roles in the myocardial healing, and for example, Foxp3+ Tregs assist post-MI wound healing through the modulation of monocyte/macrophage differentiation toward a prohealing M2-like phenotype. A growing body of evidence shows that Dectin-2, expressed on antigen-presenting cells, recognizes carbohydrate structures on the fungal cell wall and is responsible for the tailoring of adaptive responses through CD4+ T-helper cell differentiation. Dectin-2-dependent IL-12 production contributes to Th1 differentiation and IFN-γ production after Streptococcus pneumoniae infection, and this molecule was shown to induce IL-23, IL-1β, and IL-12p70 expression, leading to Th17 cell differentiation and preventing Candida albicans infection. However, a different study reported that Dectin-2, expressed on CD11b+ dendritic cells, promotes house dust mite–induced Th2 and Th17 cell differentiation and allergic airway inflammation. This molecule, coupled with FcRγ-Syk-CARD9 innate signaling pathway, is able to activate macrophage/dendritic cells and regulate adaptive immune responses, involving Th1, Th2, or Th17 cells, to different pathogens. Here, we demonstrated that Dectin-2 aggravates infarct healing through the specific modulation of Th1 differentiation, without affecting Th2, Th17, or Tregs. Furthermore, we showed that IL-12 levels were reduced in macrophages isolated from D2KO mice after MI, and neutralization of IL-12 effectively suppressed IFN-γ production in the heart, indicating that Dectin-2–dependent IL-12 production directs Th1 polarization in response to MI injury, impairing the wound healing and leading to LV ruptures. Additionally, we demonstrated that cardiomycyte apoptotic and necrotic cell death rates were reduced by Dectin-2 deficiency. Previous studies have delineated the key roles of NOr as the initiator and JNK (c-Jun N-terminal kinase), ROS (reactive oxygen species), and peroxynitrite as the effectors during IFN-γ production. IFN-γ mediated cell death, implying that IFN-γ may be partially involved in Dectin-2–induced cell death in heart after MI.

We demonstrated, in a previous study, that γδ T cells, the major source of IL-17A, are recruited to the myocardium within days after MI, and they play a significant role in myocardial remodeling. Toll-like receptor and IL-23/IL-1β stimulation are crucial for γδT-cell activation, indicating that they contribute to post-MI innate immune response. Additionally, IL-17A secreted by γδT cells promotes the infiltration of neutrophils and monocytes, stimulating the production of proinflammatory cytokines, inducing cardiomyocyte death and enhancing fibroblast proliferation and profibrotic gene expression. However, we showed that the protein and mRNA levels of IL-17A did not significantly differ between WT and D2KO mice, indicating that γδT cells and IL-17A do not mediate Dectin-2 effects on wound healing.

Further investigations are needed to elucidate how Dectin-2 molecules are able to recognize damage-associated molecular patterns, released by necrotic cells and matrix fragments, and activate CD4+ T cells or whether a different mechanism of Dectin-2 signal pathway activation exist. Dectin-2 was reported to bind endogenous ligands, and further studies are needed for the characterization of the antigens that can activate Dectin-2/Th1 immune axis in experimental models of ischemic tissue injury. Furthermore, although we revealed that Dectin-2 is mainly expressed on macrophages and less on neutrophils, and the present study focused on macrophages, we cannot completely exclude the possibility that the alterations in neutrophil responses contribute to the development of phenotype observed in D2KO mice. Therefore, these mechanisms, and the cell-specific effects of Dectin-2, should be further examined in the ischemic injury.

Th1-Related Cytokine, IFN-γ,Suppresses Myofibroblast Activation

The interaction between immune cells and cardiac fibroblasts during wound healing has been gaining a lot of attention recently. Th1 cells and IFN-γ production are considered to have deleterious effects in several immune-mediated diseases, atherosclerosis, and vascular inflammation. In a recent study conducted by Savvatis et al., it was determined that IL-23 deficiency leads to the aggravated myocardial inflammation and impaired wound healing in the ischemic heart, which was associated with high LV rupture rate and adverse remodeling, and these effects were mediated by the increased levels of Th1 differentiation and IFN-γ production. IFN-γ stimulation led to a significant downregulation of α-SMA and collagen I and III in healthy cardiac fibroblasts and in the fibroblasts isolated from the scar tissue. Furthermore, IFN-γ–stimulated fibroblasts showed a decreased migratory ability in the wound-healing assay. In line with these findings, we showed that TGF-β induces α-SMA, collagen I, and collagen III expression in the isolated cardiac fibroblasts, whereas IFN-γ inhibited the expression of these molecules and prevented the fibroblast–myofibroblast switch induced by TGF-β. Furthermore, conditioned medium obtained by the incubation of culture medium with mononuclear cells isolated from the heart of WT mice at day 3 post-MI suppressed TGF-β–induced α-SMA expression in the cultured cardiac fibroblasts, whereas the conditioned medium obtained using D2KO cells did not have the same effects. Additionally, we showed that the observed effects can be reversed in the presence of IFN-γ–neutralizing antibody. These results suggest that Dectin-2 contributes to the impairment of infarct area healing through the increase in IFN-γ production, but the involvement of other signaling pathway or cytokines, which can mediate the effects of Dectin-2 on wound healing, cannot be excluded.

In conclusion, we demonstrated that Dectin-2 can impair the scar formation in the ischemic heart. Dectin-2 regulates myocardial immune response in the early post-MI phase, increases Th1 activity in the scar, and leads to an increase in the production of IFN-γ. Through the indirect effects on myofibroblast differentiation and migration in the infarcted area, Dectin-2 can enhance the adverse myocardial remodeling and ventricular rupture and increase mortality rate.

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Disclosures

None.

References


Dectin-2 Deficiency Modulates Th1 Differentiation and Improves Wound Healing After Myocardial Infarction
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell preparation for flow cytometry

Single cell suspensions were prepared as described previously, with some modifications. Briefly, mice were deeply anesthetized and intracardially perfused with 40 mL of ice-cold phosphate-buffered saline (PBS), in order to eliminate blood cells. The hearts were dissected, minced with fine scissors, and enzymatically digested with a cocktail of type II collagenase (Worthington Biochemical Corporation, Likewood, NJ, USA), elastase (Worthington Biochemical Corporation) and DNase I (Sigma-Aldrich, St. Louis, MO, USA) for 1.5 h at 37°C with gentle agitation. After the digestion, the tissue samples were triturated and passed through a 70-μm cell strainer. The obtained cells were washed with RPMI-1640 cell culture medium for further analysis. Spleens were removed, homogenized, and passed through a 70-μm nylon mesh in PBS. Following the addition of red blood cell lysis buffer (eBioscience) in order to exclude erythrocytes, single cell suspension in PBS was refiltered through a 70-μm nylon mesh to remove connective tissue.

Flow cytometric analysis

To block the nonspecific binding of antibodies to Fcy receptors, isolated cells were incubated first with anti-CD16/32 antibody (2.4G2; BD Bioscience) at 4°C for 5 min. Subsequently, the cells were incubated with a mixture of antibodies at 4°C for 20 min. Anti-CD45-FITC (30F11.1, eBioscience), anti-CD11b-PerCP-Cy5.5, anti-CD11b-FITC (M1/70, eBioscience), anti-CD3e-FITC, anti-CD3e-APC (145-2C11, eBioscience), anti-CD4-PE (GK1.5, eBioscience), anti-CD4-APC-H7 (GK1.5, BD Pharmingen), anti-CD19-PE (1D3; BD Biosciences), anti-CD8a-PE (53-6.7; eBioscience), anti-CD4-APC (GK1.5, BD Pharmingen), anti-CD19-PE (1D3; BD Biosciences), anti-CD8a-PE (53-6.7; eBioscience), anti-CD4-APC-H7 (GK1.5, BD Pharmingen), anti-CD206 (MMR)-AlexaFluor647 (MR5D3; Biolegend) antibodies were used for flow cytometric analysis. The obtained results were expressed as the percent or cell number per microgram of tissue. Flow cytometric analysis and sorting were performed on a FACSAria I and III instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star).

The detailed antibody combinations for flow cytometry were described as follows: CD11b-Perdp-cy5.5, F4/80-FITC, Ly-6G-PE, and Dectin-2-PAC (for the analyses presented in Figure 1D); CD45-FITC, CD3-PE, and Dectin-2-APC mixture and CD45-FITC, CD19-PE, and Dectin-2-APC (Figure 1E); CD3-FITC, CD11b-Perdp-cy5.5, CD4-PE, and IFN-γ-APC or CD3-FITC, CD8-PE, and IFN-γ-APC, or CD3-FITC, TCRγδ-PE, IFN-γ-APC, or CD3-FITC, CD11b-Perdp-cy5.5, TCRγδ-PE, IL-17A-APC (Figure 6E); CD3-FITC, CD4-PE, and IFN-γ-APC, or CD3-FITC, TCRγδ-PE, and IL-17A-APC (Figures 6F and 6G); CD3-FITC, CD4-PE, and IFN-γ-APC (Figure 6G); DAPI, CD11b-Perdp-cy5.5, F4/80-FITC, and Ly-6G-PE (Online Figure I); CD45-FITC, CD11b-Perdp-cy5.5, and CD3-PE, or CD11b-Perdp-cy5.5, F4/80-FITC, Ly-6G-PE, and CD206-AlexaFluor647 (Online Figure VII).

Intracellular cytokine staining

For intracellular cytokine staining, single cells prepared from spleen and heart samples were re-stimulated for 4.5 h with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 1 μg/mL of ionomycin (Sigma-Aldrich) in the presence of Golgistop.
(Cytofix/Cytoperm Plus Kit with Golgistop, BD Biosciences). Surface staining was performed for 20 min with the corresponding mixture of fluorescently labeled antibodies. After fixation and permeabilization, the cells were incubated for 30 min at 4°C with anti-IL-17A-APC (eBio17B7; eBioscience) and anti-IFN-γ-APC (XMG1.2, eBioscience) antibodies.

**Quantitative real-time PCR**

Total RNA samples from the isolated, sorted cells, cultured cells, and tissues were prepared using RNeasy Mini Kit (Qiagen) or Trizol reagent (Invitrogen), according to the manufacturers’ instructions. First-strand cDNA synthesis kit (Invitrogen) was used for cDNA synthesis. Quantitative real-time PCR was performed using ABI Prism 7700 sequence detection system (Applied Biosystems). Predesigned gene-specific primer and probe sets (Taqman Gene Expression Assays, Applied Biosystems) were used. The 18S ribosomal RNA levels were used as an internal control.

**Western blotting**

Heart tissue samples or cultured cells were homogenized in lysis buffer containing proteinase and phosphatase inhibitor cocktail. Dectin-2 (Clone: IMG3D1, Abcam), α-SMA (Clone: Alpha Sr-1, Abcam) and collagen I (Clone: C18, Santa Cruz) were detected with specific antibodies, as previously described. 2 α-tubulin (Clone: DM1A, Sigma) levels were used as an internal control.

**Infarct size and infarct wall thickness determination**

Heart tissue samples were fixed in formalin, embedded in paraffin, and cut into 5-μm-thick sections. Hematoxylin and eosin (H&E), picrosirius red, and Masson's trichrome staining of the paraffin-embedded sections were performed to determine the morphological effects and infarct size, with the latter calculated as total infarct circumference divided by total LV circumference × 100, as described previously. 3 The wall thickness of the scars at the papillary and apical levels was measured as well. The fraction of collagen volume was assessed in 10 randomly chosen high-power fields (200×) in each section. These data were analyzed using Image J software (version 1.38×, National Institutes of Health).

**Determination of infarct size after myocardial ischemia reperfusion (IR) injury**

Infarct size after IR injury was determined as previously described. 1 Briefly, 2 days after IR injury, mice were anesthetized, the LAD was re-occluded at the previous ligation, and 1 ml of 1.0% Evans blue (Sigma-Aldrich) was injected into the left ventricular (LV) cavity. The heart was quickly excised, washed twice, immediately frozen, and sliced into 1-mm thick sections. These samples were then incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) solution and digitally photographed. LV area, area at risk (AAR), and infarct area were determined by computerized planimetry using ImageJ software (version 1.38×, National Institutes of Health).

**Echocardiography**

Transthoracic echocardiography was performed with a Vevo 2100 instrument (VisualSonics) equipped with an MS-400 imaging transducer. Mice were kept awake, without applying anesthesia, during the echocardiographic examination to minimize data deviation, and heart rate was maintained at approximately 550-650 bpm in all mice. M-mode tracings were recorded through the anterior and posterior LV walls at the papillary muscle level to measure LV end-diastolic dimension (LVEDD) and LV end-systolic dimension (LVESD). LV fractional shortening (FS) was calculated according
to the following formula: \( FS = \left( \frac{LVEDD - LVESD}{LVEDD} \right) \times 100 \). End-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), and ejection fraction (EF) were calculated by using the spherical formula.

**Pressure-volume (PV) loop analysis**

At the end of the study (week 4), invasive hemodynamic measurements were performed under general anesthesia (1.5% isoflurane v/v in 100% O2). A 1.2-Fr pressure-volume conductance catheter (Transonic Scisense Inc, London, ON, Canada) was inserted into the right carotid artery and advanced into the left ventricle. Pressure-volume data were recorded with iWorx Systems and analyzed off-line using LabScribe3 (Dover, NH 03820, USA) data analysis software. At the end of the experiments, anaesthetized animals were sacrificed by cervical dislocation. Hearts were rapidly excised and prepared for histological analysis, and protein and RNA extraction.

**Immunohistochemistry**

Immunofluorescence analyses were performed on the paraffin-embedded sections of the heart tissue or on the cultured cells fixed with 4% paraformaldehyde as described previously.4 The sections were incubated with primary rabbit polyclonal anti-collagen I (ab34710, Abcam), rabbit polyclonal anti-collagen III (ab7778, Abcam), mouse monoclonal anti-\( \alpha \)-SMA (Clone: Alpha Sr-1, ab28052, Abcam), rabbit polyclonal anti-MMP2 (ab37150, Abcam), rabbit polyclonal anti-MMP9 (ab38898, Abcam), isolectin B4 (B-1205, Vector), and mouse monoclonal anti-\( \alpha \)-actinin (Clone: EA-53, A7811, Sigma-Aldrich) antibodies overnight at 4°C. Following this, the sections were incubated with the appropriate Alexa-Fluor-coupled secondary antibodies for 1 h at room temperature, and counterstained with DAPI. Images were acquired with Olympus BX61 microscope and analyzed using ImageJ software (version 1.38\( \times \), National Institutes of Health).

**Propidium iodide (PI) and TUNEL staining**

PI (10 mg/kg; Sigma-Aldrich) was injected into the mice belonging to all groups, in order to label the necrotic cells at 1 h before their sacrifice. Afterward, their hearts were rapidly removed, washed in ice-cold PBS, embedded in OCT, frozen in liquid nitrogen, and cut into 8-\( \mu \)m sections. TUNEL staining was performed with In Situ Cell Death Detection Kit-Fluorescein (Cat. 11684795910, Roche), according to the manufacturer's instructions. Sections were counterstained with DAPI. Images were acquired with Olympus BX61 microscope and analyzed using ImageJ (version 1.38\( \times \), National Institutes of Health).

**cDNA microarray**

Total RNA obtained from the macrophages isolated from the infarcted hearts at different time points after MI were subjected to microarray analysis using 3D-Gene microarray (TORAY Industries, Japan), according to the standard protocol. Gene expression was considered significantly different when the false discovery rate was less than 0.05 and the fold change was more than two, compared with the sham control group. Trend analysis was performed as follows. Based on a different trend of signal density change under different conditions, we identified a set of unique model expression tendencies. The raw expression values were converted into log2 ratio. Using a strategy for the clustering of short time-series gene expression data, we defined several unique profiles. The expression model profiles are related to the actual or the expected number of genes assigned to each model profile. The \( P \)-values obtained for the profiles showing statistical significance are higher than expected by Fisher's exact test and multiple comparison test.5, 6 Cluster analyses were performed using Cluster 3.0 software and the result were displayed using the TreeView program (http://rana.lbl.gov/eisen/). Full datasets
available online at NCBI GEO (accession number: GSE88924).

**Culture and stimulation of murine cardiac fibroblasts**

Cardiac fibroblasts were isolated from the healthy mouse hearts. The obtained tissue samples were cut into small pieces, which were fixed in 10-cm culture dishes. The outgrown primary fibroblasts were cultured in Dulbecco’s Modified Eagle’s medium (DMEM; Gibco), containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) at 37°C in the atmosphere containing 5% CO2. Cardiac fibroblasts at passage 3 to 6 were used for IFN-γ and TGF-β stimulation experiments, and their wound healing capacity was assessed. Before the initiation of the stimulation, the cells were washed once with PBS and starved in DMEM containing 1% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) for 24 h. Afterward, they were treated with 20 ng/mL of TGF-β (R&D Systems) in the presence or absence of 10 ng/mL of IFN-γ (R&D Systems) for 24 h, and these cells were harvested for further western blot analysis or immunostaining with anti-α-SMA antibody.

The healing capacity of cardiac fibroblasts was assessed by scratch assay. Fibroblasts from sham-operated mouse hearts, untreated or stimulated with IFN-γ were further treated as described previously. A wound area was obtained by scratching across the bottom of each well with a 1000 μL pipette tip. Healing capacity of the fibroblasts was calculated as the percentage of the initial scratch area at 0 h that was repopulated by fibroblasts after 36 h.

Mononuclear cells were isolated from the infarcted hearts of WT and D2KO mice at day 4 post-MI, treated with PMA (50 ng/mL) and ionomycin (1 μg/mL) for 1 h, and the resulting conditioned media were used to stimulate cardiac fibroblasts for 24 h in the presence of 20 ng/mL of TGF-β. Afterward, the cells were immunostained for α-SMA expression. In the wound healing assay, the conditioned media were used to stimulate cardiac fibroblasts for 36 h. IFN-γ neutralizing antibody was used as well. Additionally, IFN-γ concentration in the conditioned media was measured with an ELISA kit (R&D Systems), according to the manufacturer’s instructions.
REFERENCES
Online Figure I. Macrophage sorting strategy for gene chip analysis and the analysis of differential gene expression

(A) CD11b⁺ cells were isolated from the mouse hearts at different time points after MI using magnetic-activated cell sorting (MACS) with CD11b microbeads, and afterward, CD11b⁺F4/80⁻Ly-6G⁻ macrophages were separated by flow cytometry. The obtained cells were analyzed using DNA chip, in order to obtain mRNA expression profile. Sham sample was pooled from 11 mice; samples obtained at day 1, 3, 7, and 14 were pooled from 3, 3, 2, and 2 mice, respectively. (B) Differential gene expression was defined as two-fold change, in comparison with that in the sham control, and the trend analysis was performed. Data are presented based on the number of genes assigned or the P-value of the number of genes assigned versus the expected. Trend designated as No. 42 was shown to include the highest number of genes and the highest significance.
Online Figure II. Identification of Dectin-2 expression in sorted cardiac macrophages

Cluster analysis of differential gene expression in cardiac macrophages. Down&NC means the gene is first downregulated, which is followed by the recovery of gene expression levels. UP&NC indicates that the gene expression is upregulated, and then its expression returns to the control level. Dectin-2 (Clec4n) expression was shown to be induced at first, and then inhibited.
Online Figure III. Flow cytometric analysis of Dectin-2 expression on cardiac macrophages and neutrophils. Related to Figure 1D

The representative flow cytometric dot plots of Dectin-2 expression on gated CD11b^+ F4/80^+ macrophages and CD11b^+ Ly-6G^- neutrophils in the heart at different time points after MI.
Online Figure IV. Dectin-2 knockout (D2KO) mouse genotype

(A) Primer sequences and the results obtained by wild-type (WT) and D2KO mouse-tail DNA genotyping. (B) Dectin-2 mRNA expression levels in hearts obtained from WT and D2KO mice were examined, and no Dectin-2 expression was observed in D2KO mice. The experiments were repeated twice.
Online Figure V. Dectin-2 deficiency did not affect myocardial fibrosis in the remote areas after MI

Representative images of picrosirius red-stained sections of the remote areas of WT and D2KO mouse heart obtained at day 28 post-MI (left; scale bar, 20 μm). Quantification of fibrotic areas in the remote area of WT and D2KO mouse tissue at day 28 post-MI is presented (n = 4).
Online Figure VI. Dectin-2 deficiency decreased cardiomyocyte apoptosis and
necrotic cell death rates after MI

(A) Cardiomyocyte apoptosis rate was measured in the heart tissue sections at day 1, 3, 7, and 14 post-MI, and sham hearts by staining with TUNEL (green), while the nuclei were stained with DAPI (n = 4-6) (Scale bar, 20 μm). (B) PI staining was used to determine the necrotic cell death rate in the heart sections at different time points after MI. Nuclei were stained with DAPI (n = 4-6) (Scale bar, 20 μm). Data are expressed as mean ± SEM. *P < 0.01; **P < 0.01, between WT-infarct and D2KO-infarct sections. #P < 0.01; ##P < 0.01, between WT-border and D2KO-border areas. Data in (A and B) were analyzed using Kruskall-Wallis test, followed by Bonferroni post hoc analysis.
Online Figure VII. Effects of Dectin-2 deficiency on MMP2 and MMP9 after MI. Related to Figure 5

Representative images showing the results of immunofluorescence analyses of MMP2 (A) and MMP9 (B) expression in the heart tissues at different time points after MI (Scale bar, 20 μm).
Online Figure VIII. Dectin-2 deficiency did not alter immune cells infiltration and macrophage polarization

(A) CD45⁺ leukocyte, CD11b⁺ myeloid cell, CD11b⁺F4/80⁺ macrophage, CD11b⁺Ly-6G⁺ neutrophil, and T-cell infiltration in the heart at day 1 post-MI was examined (n = 5). (B) The representative flow cytometric dot plots of M1 (CD11b⁺F4/80⁻CD206⁻) and M2 (CD11b⁺F4/80⁺CD206⁺) macrophages in the heart tissue at day 1 post-MI (n = 5). Data are expressed as mean ± SEM. NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001. Data were analyzed by Kruskall-Wallis test followed by Bonferroni post hoc analysis.
Online Figure IX. Dectin-2 knockout decreased IFN-γ production from mononuclear cells isolated from the post-MI hearts

Mononuclear cells were isolated from the hearts at day 3 after MI, and stimulated with PMA and ionomycin. The resulting conditioned media were used to measure IFN-γ production. Data are expressed as mean ± SEM. NS, not significant; **P < 0.01. Data were analyzed by Kruskall-Wallis test followed by Bonferroni post hoc analysis.
Online Figure X. Effects of Dectin-2 deficiency on cardiomyocyte apoptosis and necrotic cell death rates after myocardial ischemia reperfusion injury. Related to Figure 8B and C

Representative TUNEL (A) and PI (B) staining of the heart tissue sections isolated from WT and D2KO mice at different time points after IR (Scale bar, 20 μm).
Online Figure XI. Dectin-2 did not affect macrophage, neutrophil infiltration and macrophage polarization in the heart after myocardial ischemia reperfusion injury

The cell number of CD11b⁺F4/80⁺ macrophage (Mac), M1, M2 macrophages (M1 Mac, M2 Mac) and CD11b⁺Ly-6G⁺ neutrophil (Neu) in the heart at day 1 post-IR was examined by flow cytometry (n = 4-5). Data are expressed as mean ± SEM. NS, not significant. Data were analyzed by Mann-Whitney U tests.
Online Figure XII. Effect of Dectin-2 deficiency on wound healing after myocardial ischemia reperfusion injury

Immunohistochemical analyses of collagen I (A), collagen III (B) (Scale bars: white, 50 μm; yellow, 20 μm), and isolectin B4 (IB4) (Scale bar, 20 μm) (C) in the heart tissue at day 14 post-IR (n = 4-5). Data are expressed as mean ± SEM. NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001. Data in (A-C) were analyzed using Mann-Whitney U tests.
Online Figure XIII. Deficiency of Dectin-2 suppressed MMP2 and MMP9 expression after myocardial ischemia reperfusion injury

Expression levels of MMP2 (A) and MMP9 (B) in the heart were examined by immunohistochemistry at day 0, 1, 3, 7 and 14 post-IR (n = 4-5) (Scale bar, 20 μm). Data are expressed as mean ± SEM. *P < 0.01; **P < 0.01; ***P < 0.001 for comparisons between WT and D2KO groups. Data were analyzed by Mann-Whitney U tests.
Online Table I. List of the antibodies used in this study.

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</tr>
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<td>IFN-γ</td>
<td>Bio-X- Cell</td>
<td>BE0054</td>
<td>Neutralization</td>
<td>20μg/ml</td>
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</table>
Online Table II. Echocardiographic analysis of WT and D2KO mice at baseline.

<table>
<thead>
<tr>
<th>Group</th>
<th>WT</th>
<th>D2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Weeks)</td>
<td>10-11</td>
<td>10-11</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.23 ± 0.08</td>
<td>3.32 ± 0.08</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.56 ± 0.07</td>
<td>1.65 ± 0.08</td>
</tr>
<tr>
<td>FS (%)</td>
<td>52.72 ± 1.31</td>
<td>50.38 ± 2.15</td>
</tr>
<tr>
<td>LVEDV (μL)</td>
<td>43.91 ± 2.48</td>
<td>45.10 ± 2.36</td>
</tr>
<tr>
<td>LVESV (μL)</td>
<td>6.86 ± 0.80</td>
<td>7.96 ± 1.04</td>
</tr>
<tr>
<td>EF (%)</td>
<td>84.60 ± 1.18</td>
<td>82.27 ± 2.03</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>584.9 ± 9.1</td>
<td>579.5 ± 13.1</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.  
WT = wild-type; D2KO = Dectin-2 knockout; LVEDD = left ventricular end-diastolic dimension; LVESD = left ventricular end-systolic dimension; FS = fractional shortening; LVEDV = left ventricular end-diastolic volume; LVESV = left ventricular end-systolic volume; EF = ejection fraction; HR = heart rate.
Online Table III. The weight of organs and echocardiographic analysis of WT and D2KO mice after MI.

<table>
<thead>
<tr>
<th>Group</th>
<th>WT Sham</th>
<th>D2KO Sham</th>
<th>WT MI</th>
<th>D2KO MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>BW (g)</td>
<td>23.71 ± 0.89</td>
<td>24.13 ± 0.86</td>
<td>23.98 ± 0.86</td>
<td>23.69 ± 0.74</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.36 ± 0.12</td>
<td>4.34 ± 0.15</td>
<td>6.60 ± 0.16***</td>
<td>5.85 ± 0.17##</td>
</tr>
<tr>
<td>LW/BW (mg/g)</td>
<td>4.75 ± 0.18</td>
<td>4.67 ± 0.17</td>
<td>6.77 ± 0.16**</td>
<td>6.00 ± 0.12###</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.25 ± 0.09</td>
<td>3.32 ± 0.07</td>
<td>5.62 ± 0.17**</td>
<td>5.01 ± 0.13###</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.57 ± 0.08</td>
<td>1.63 ± 0.07</td>
<td>5.17 ± 0.18###</td>
<td>4.37 ± 0.15###</td>
</tr>
<tr>
<td>FS (%)</td>
<td>51.91 ± 1.37</td>
<td>50.78 ± 1.72</td>
<td>8.10 ± 0.70**</td>
<td>12.90 ± 0.90###</td>
</tr>
<tr>
<td>LVEDV (μL)</td>
<td>42.73 ± 2.73</td>
<td>44.86 ± 2.08</td>
<td>181.44 ± 16.98***</td>
<td>127.50 ± 9.70###</td>
</tr>
<tr>
<td>LVESV (μL)</td>
<td>7.04 ± 0.95</td>
<td>7.72 ± 0.81</td>
<td>141.94 ± 14.74***</td>
<td>85.69 ± 8.52###</td>
</tr>
<tr>
<td>EF (%)</td>
<td>83.98 ± 1.25</td>
<td>82.80 ± 1.54</td>
<td>22.33 ± 1.70###</td>
<td>33.75 ± 2.03###</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>587.6 ± 10.2</td>
<td>578.1 ± 13.4</td>
<td>580.7 ± 9.2</td>
<td>574.1 ± 12.6</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT sham; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. WT MI.

WT = wild-type; D2KO = Dectin-2 knockout; LVEDD = left ventricular end-diastolic dimension; LVESD = left ventricular end-systolic dimension; FS = fractional shortening; LVEDV = left ventricular end-diastolic volume; LVESV = left ventricular end-systolic volume; EF = ejection fraction; HR = heart rate.
Online Table IV. Pressure-volume loop data obtained in WT and D2KO mice after MI.

<table>
<thead>
<tr>
<th>Group</th>
<th>WT Sham</th>
<th>D2KO Sham</th>
<th>WT MI</th>
<th>D2KO MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>EDV (µL)</td>
<td>46.2 ± 1.4</td>
<td>44.0 ± 1.8</td>
<td>94.9 ± 1.5***</td>
<td>84.8 ± 1.7#</td>
</tr>
<tr>
<td>ESV (µL)</td>
<td>31.2 ± 1.6</td>
<td>30.5 ± 1.8</td>
<td>85.2 ± 1.8</td>
<td>70.4 ± 2.4###</td>
</tr>
<tr>
<td>SV (µL)</td>
<td>15.0 ± 2.2</td>
<td>13.5 ± 0.9</td>
<td>9.7 ± 0.85</td>
<td>14.4 ± 2.2#</td>
</tr>
<tr>
<td>EF (%)</td>
<td>32.1 ± 4.4</td>
<td>30.8 ± 2.2</td>
<td>10.2 ± 0.9</td>
<td>16.9 ± 2.5#</td>
</tr>
<tr>
<td>ESP (mmHg)</td>
<td>83.9 ± 1.3</td>
<td>84.2 ± 1.5</td>
<td>70.4 ± 1.4</td>
<td>78.0 ± 1.6#</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>7.9 ± 0.7</td>
<td>6.9 ± 1.0</td>
<td>18.2 ± 1.2***</td>
<td>14.9 ± 0.6#</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>378.3 ± 13.2</td>
<td>381.3 ± 15.6</td>
<td>379.7 ± 13.1</td>
<td>386.7 ± 18.5</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT sham; #P < 0.05, ##P < 0.01 vs. WT MI.

WT = wild-type; D2KO = Dectin-2 knockout; EDV = end-diastolic volume; ESV = end-systolic volume; SV = stroke volume; EF = ejection fraction; EDP = end-diastolic pressure; ESP = end-systolic pressure; HR = heart rate.