Left ventricular (LV) remodeling after myocardial infarction (MI) is the process of complex architectural myocardial alteration and is associated with a poor clinical outcome. Congestive heart failure due to postinfarction LV remodeling remains an unresolved problem, in spite of recent clinical practice. Therefore, better understanding of the molecular and cellular mechanisms involved in this process and the search for alternative therapeutic targets against LV remodeling are matters of great importance.

**Background**—Inflammation and immune responses are integral components in the healing process after myocardial infarction. We previously reported dendritic cell (DC) infiltration in the infarcted heart; however, the precise contribution of DC in postinfarction healing is unclear.

**Methods and Results**—Bone marrow cells from CD11c-diphtheria toxin receptor/green fluorescent protein transgenic mice were transplanted into lethally irradiated wild-type recipient mice. After reconstitution of bone marrow–derived cells, the recipient mice were treated with either diphtheria toxin (DC ablation) or vehicle (control), and myocardial infarction was created by left coronary ligation. CD11c<sup>+</sup> green fluorescent protein–positive DCs expressing CD11b and major histocompatibility complex class II were recruited into the heart, peaking on day 7 after myocardial infarction in the control group. Mice with DC ablation for 7 days showed deteriorated left ventricular function and remodeling. The DC-ablated group demonstrated enhanced and sustained expression of inflammatory cytokines such as interleukin-1β, interleukin-18, and tumor necrosis factor-α, prolonged extracellular matrix degradation associated with a high level of matrix metalloproteinase-9 activity, and diminished expression level of interleukin-10 and endothelial cell proliferation after myocardial infarction compared with the control group. In vivo analyses revealed that DC-ablated infarcts had enhanced monocyte/macrophage recruitment. Among these cells, marked infiltration of proinflammatory Ly6C<sup>high</sup> monocytes and F4/80<sup>+</sup> CD206<sup>−</sup> M1 macrophages and, conversely, impaired recruitment of anti-inflammatory Ly6C<sup>low</sup> monocytes and F4/80<sup>+</sup> CD206<sup>+</sup> M2 macrophages in the infarcted myocardium were identified in the DC-ablated group compared with the control group.

**Conclusions**—These results suggest that the DC is a potent immunoprotective regulator during the postinfarction healing process via its control of monocyte/macrophage homeostasis.

**Key Words:** myocardial infarction ■ remodeling ■ inflammation ■ immune system ■ heart failure

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**Clinical Perspective on p 1245**

Inflammation and immune responses are integral components of the host reaction to myocardial injury and play a crucial role in infarct healing and subsequent LV remodeling. We have previously reported that elevated serum C-reactive protein concentration and peripheral monocytecytosis predict a poor clinical outcome after MI. Moreover, our experimental study demonstrated that the enhanced infiltration of monocytes and macrophages into the infarcted myo-
cardiac induced by granulocyte-macrophage colony-stimulating factor leads to aggravated infarct expansion and LV dysfunction.6 These findings indicate that excessive immune-mediated inflammatory reactions have a deleterious effect on postinfarction LV remodeling. On the other hand, immunosuppressive therapy with corticosteroids resulted in increased catastrophic incidents such as cardiac rupture in clinical practice,7,8 and recent experimental data showed that macrophage depletion with clodronate-containing liposomes (Clo-Lip) impaired infarct healing in a murine model,9 which suggests that controlled inflammation and immune response are prerequisites for appropriate cardiac repair after MI. However, the regulatory mechanism that controls these reactions during the postinfarction healing process remains to be determined.

The dendritic cell (DC) is a potent central immunoregulator that orchestrates various types of inflammatory cells in innate and adaptive immunity.10–12 After microbial infection or tissue injury, bone marrow (BM) and splenic precursors and circulating monocytes are reported to differentiate into DCs and exert various influences on the immune system at the inflammatory site, such as priming of antigen-specific immune responses, induction of tolerance, and chronic inflammation.13–15 Zhang et al16 first demonstrated infiltration of DCs into the infarcted heart in experimental MI. We have reported that an increased number of mature DCs in the infarcted heart is associated with deterioration of LV remodeling in a rat MI model.17 However, the causative effect of infiltrating DCs on LV remodeling and their origin in the postinfarction healing process are unclear.

The primary aim of the present study was to clarify the role of DCs in tissue repair and LV remodeling after MI and the origin of DCs involved in the process. We used transgenic mice expressing an integrin, CD11c, we used a BMT model as described in Methods. The repopulation efficiency of hematopoietic cells after BMT was determined with B6.SJL mice (CD45.1+) as recipients. After reconstitution with donor CD11c-DTR/GFP transgenic marrow cells (CD45.2+), flow cytometric analysis of splenocytes revealed that the reconstitution rate of our BMT method was approximately 96% (online-only Data Supplement Figure IA). Moreover, almost all the CD11c+ DCs were CD45.2+ (CD45.2+ CD11c+ recipient-derived DCs were absent), and more than 97% of these DCs were also positive for green fluorescent protein GFP (online-only Data Supplement Figure IB). These data indicated that cells such as DCs of BMT mice were almost totally replaced with donor BM-derived cells in our BMT mice.

We assessed the effect of DT inoculation in BMT mice by flow cytometry. Flow cytometric analysis of splenocytes revealed that CD11c+ GFP+ and CD11c+ major histocompatibility complex class II (MHCII)-positive DCs were completely depleted by DT administration (online-only Data Supplement Figure IIA), whereas other immune cells (CD4+ T cells, CD8+ T cells, CD19+ B cells, and CD11b+ monocytes/macrophages) were barely affected (online-only Data Supplement Figure IIB). These data were compatible with other previously published studies that used this transgenic mouse system.18,19

Recruitment of DCs Into the Infarcted Heart
Peripheral circulating CD11c+ GFP+ DCs that expressed MHCII increased rapidly after MI in control mice and were almost completely abolished by DT administration in DC-ablated mice. Immunofluorescent staining showed that resident DCs were...
Control MI
DC-ablation MI

DC infiltration after MI in control and DC-ablated mice. Scale bars indicate 30 μm. B, High-magnification view of rectangle in A. White arrows indicate CD11c\(^{+}\) GFP\(^{+}\) heart-infiltrating DCs (yellow). Scale bar indicates 30 μm. C, Time course of CD11c\(^{+}\) GFP\(^{+}\) DC infiltration after MI in control and DC-ablated mice analyzed by immunofluorescent staining. Data were obtained from 5 independent experiments at each time point. D, Representative flow cytometric dot plots of heart-infiltrating CD45\(^{+}\) leukocytes for CD11c-allophycocyanin and CD11b-phycocerythrin or major histocompatibility complex (MHC) class II-phycocerythrin in our BMT model. IL-18, tumor necrosis factor-α, IL-10, CCL2, and CX3CL1 expression after MI. In control mice, the expression of IL-1\(\beta\), IL-18, tumor necrosis factor-α, IL-10, CCL2, and CX3CL1 expression after MI. In control mice, the expression of IL-1\(\beta\), IL-18, tumor necrosis factor-α, and CCL2, which are proinflammatory, increased 3 days after MI and gradually decreased thereafter (Figures 3A–3D). In contrast, DC-ablated mice had sustained elevation of IL-1\(\beta\), IL-18, tumor necrosis factor-α, and CCL2 for 7 days after MI (Figure 3A). We found that the incidence of death due to heart failure tended to be higher in DC-ablated mice than in control mice after MI (21.7% versus 9.4%, \(P=0.086\)), although the occurrence of cardiac rupture was similar in the 2 groups (13.0% versus 9.4%, \(P=0.53\)). Masson’s trichrome staining revealed that there was no difference in the gross morphology in sham-operated hearts between control and DC-ablated mice (Figure 2B). Twenty-eight days after MI, DC-ablated mice demonstrated enhanced infarct expansion and wall thinning compared with control mice, although the infarct size was similar in the 2 groups (Figures 2B–2D). Collagen volume fraction detected by picrosirius red staining in the noninfarcted area was significantly higher in DC-ablated mice than in control mice after MI (21.7% versus 9.4%, \(P=0.01\)).

DC Ablation Aggravates LV Dysfunction and Remodeling After MI
Cardiac function was analyzed 28 days after MI (Table). Echocardiographic measurements revealed acceleration of cardiac dilatation and deterioration of LV function in DC-ablated mice compared with control mice. Hemodynamic data also indicated that the maximum rate of isovolumic pressure development after MI was significantly lower in DC-ablated mice than in control mice. Moreover, heart and lung weights adjusted for body weight were similar in nontreated and DT-treated wild-type mice (online-only Data Supplement Figure III).

Acceleration of MI-Induced Death, Wall Thinning, and Myocardial Fibrosis in DC-Ablated Mice
The survival rate tended to be lower in DC-ablated mice than in control mice 28 days after MI, whereas all sham-operated animals in both groups survived throughout the study (Figure 2A). We found that the incidence of death due to heart failure tended to be higher in DC-ablated mice than in control mice after MI (21.7% versus 9.4%, \(P=0.086\)), although the occurrence of cardiac rupture was similar in the 2 groups (13.0% versus 9.4%, \(P=0.53\)). Masson’s trichrome staining revealed that there was no difference in the gross morphology in sham-operated hearts between control and DC-ablated mice (Figure 2B). Twenty-eight days after MI, DC-ablated mice demonstrated enhanced infarct expansion and wall thinning compared with control mice, although the infarct size was similar in the 2 groups (Figures 2B–2D). Collagen volume fraction detected by picrosirius red staining in the noninfarcted area was significantly higher in DC-ablated mice than in control mice 28 days after MI (21.7% versus 9.4%, \(P=0.01\)).

Absence of DCs Enhances Inflammatory Cytokine But Suppresses Anti-Inflammatory Cytokine Expression After MI
Figure 3 demonstrates the temporal changes of interleukin (IL)-1\(\beta\), IL-18, tumor necrosis factor-α, IL-10, CCL2, and CX3CL1 expression after MI. In control mice, the expression of IL-1\(\beta\), IL-18, tumor necrosis factor-α, and CCL2, which are proinflammatory, increased 3 days after MI and gradually decreased thereafter (Figures 3A–3D). In contrast, DC-ablated mice had sustained elevation of IL-1\(\beta\), IL-18, tumor necrosis factor-α, and CCL2 for 7 days after MI.
MI compared with control mice (Figures 3A–3D). On the other hand, the expression of IL-10 and CX3CL1, which are anti-inflammatory, 7 days after MI was lower in DC-ablated mice than in control mice (Figures 3E and 3F). Western blotting analysis confirmed that expression of IL-10 at the protein level was significantly lower in DC-ablated mice than in control mice 7 days after MI (Figures 3G and 3H).

**DC Ablation Accentuates Matrix Metalloproteinase-9 Activity and Inducible Nitric Oxide Synthase Expression After MI**

As shown in Figure 4, matrix metalloproteinase (MMP)-9 activity was increased in the infarcted heart 3 days after MI and decreased thereafter in control mice, whereas its activity in DC-ablated mice was persistently upregulated for 28 days after MI (Figures 4A and 4B). The activity of MMP-2 tended to be higher in DC-ablated mice than in control mice, although there was no statistical significance between the 2 groups (Figures 4A and 4C). Furthermore, inducible nitric oxide synthase was also increased by MI, and DC ablation further enhanced the induction of inducible nitric oxide synthase 7 days after MI (Figures 4D and 4E).

**Postinfarction Myocardial Angiogenesis Is Impaired by DC Depletion**

To assess the influence of DC depletion on cardiac angiogenesis, we performed immunoblot and histological analyses. As shown in Figures 5A and 5B, the expression of vascular endothelial growth factor at the protein level, which was upregulated in control infarcts, was significantly suppressed by DC ablation 7 days after MI. Moreover, immunohistochemical staining revealed that the number of blood vessels in the infarcted myocardium was smaller and the fraction of CD31* Ki67* proliferating endothelial cells was significantly lower in DC-ablated mice than in control mice 7 days after MI (Figures 5C and 5D), which suggests that DC depletion might affect cardiac neoangiogenesis during the postinfarction healing process.

**Enhanced Infiltration of Inflammatory M1 Macrophages and Diminished Recruitment of Anti-Inflammatory M2 Macrophages Into the Infarcted Myocardium in Response to DC Ablation**

We evaluated the degree of infiltration by various inflammatory cells after MI in control and DC-ablated mice. The number of total CD45* leukocytes in the infarcted heart was significantly higher in DC-ablated mice than in control mice 7 days after MI (online-only Data Supplement Figure IVA).
Immunofluorescent staining also indicated that infiltration of CD45+ leukocytes into the infarcted myocardium 7 days after MI was greater in DC-ablated mice than in control mice (online-only Data Supplement Figure IVB). Immunohistochemical staining for Mac3 showed that the number of differentiated macrophages that infiltrated into the infarcted myocardium peaked on day 7 after MI and was significantly higher in DC-ablated mice 7 and 14 days after MI than in control mice (Figures 6A and 6B). Flow cytometric analysis also demonstrated that the percentage of Mac3+ macrophages in the heart 7 days after MI in DC-ablated mice was significantly higher than in control mice (online-only Data Supplement Figures IVC and IVD). Furthermore, among tissue-infiltrating F4/80+ macrophages, DC-ablated infarcts had enhanced infiltration of F4/80+CD206+ inflammatory MI macrophages and diminished recruitment of F4/80+CD206+ anti-inflammatory M2 macrophages compared with control mice 7 days after MI, on the basis of flow cytometry (Figures 6C and 6D). The number of myeloperoxidase-positive neutrophils in the infarcted heart 7 days after MI tended to be higher in DC-ablated mice than in control mice (Figure 6E); however, the number of CD3+ T cells that
Figure 3. Ablation of dendritic cells (DCs) enhances proinflammatory cytokine but suppresses anti-inflammatory cytokine expression in the infarcted myocardium. A through F, Time course of mRNA expression of interleukin-1β (IL-1β; A), IL-18 (B), tumor necrosis factor (TNF)-α (C), CCL2 (D), IL-10 (E), and CX3CL1 (F) in the infarcted heart after myocardial infarction (MI) in control and DC-ablated mice. Data were obtained from 8 to 10 independent experiments at each time point. G, Representative immunoblotting image for IL-10 in the infarcted heart of control and DC-ablated mice 7 days after MI or sham operation. H, Quantitative analysis of IL-10 protein expression in control and DC-ablated mice 7 days after MI or sham operation (n=5 per group). *P<0.05 and **P<0.01 vs sham of same group; †P<0.05 vs control MI.
infiltrated the infarcted myocardium was comparable in the 2 groups (Figure 6F).

**DC Ablation Causes Increased Ly6C\textsuperscript{high} Monocyte But Decreased Ly6C\textsuperscript{low} Monocyte Infiltration Into the Infarcted Heart**

Monocytes, which play important roles in tissue repair after MI, were evaluated in peripheral blood and heart tissue. Flow cytometric analysis showed that the total number of peripheral blood monocytes was higher in DC-ablated mice than in control mice (online-only Data Supplement Figure VA). In addition, peripheral blood Ly6C\textsuperscript{high} monocytes (monocytes with high levels of lymphocyte antigen 6 complex, locus C [Ly6C]) were persistently increased whereas Ly6C\textsuperscript{low} monocytes were decreased in DC-ablated mice compared with control mice, especially 7 days after MI (Figures 7A and 7B). Moreover, among the inflammatory leukocytes isolated from the infarcted heart, the number of total monocytes and inflammatory Ly6C\textsuperscript{high} monocytes was markedly higher and the number of reparative Ly6C\textsuperscript{low} monocytes lower in DC-ablated mice 7 days after MI than in control mice (Figures 7C and 7D, online-only Data Supplement Figure VB).

**Discussion**

We demonstrated in the present study that mature, activated CD11c\textsuperscript{+} CD11b\textsuperscript{+} DCs that originate from the BM infiltrate the infarcted heart during the healing process after MI. Selective depletion of DCs exacerbated postinfarction LV remodeling in association with enhanced inflammatory cytokine expression, inducible nitric oxide synthase production,
and MMP-9 activation, likely via marked infiltration of proinflammatory Ly6C^{high} monocytes and F4/80^{+} CD206^{+} M1 macrophages into the infarcted myocardium. Meanwhile, the number of anti-inflammatory Ly6C^{low} monocytes and F4/80^{+} CD206^{+} M2 macrophages, myocardial expression of IL-10, and cardiac angiogenesis after MI were suppressed by selective ablation of DCs. These results indicate the protective role of DCs in postinfarction inflammation and subsequent LV remodeling by regulation of cellular employment in the heart.

Leukocyte influx is a highly sophisticated system that contributes to cardiac repair after MI. Inflammatory signals recruit neutrophils to the ischemic heart immediately after MI, and monocytes/macrophages shortly thereafter. A large number of reports have indicated that excessive cardiac macrophage infiltration is associated with facilitated infarct expansion and adverse LV remodeling with augmented inflammation and MMP activity.6,21,22 Macrophages can be divided into 2 populations, which have a classically (M1) and alternatively (M2) activated phenotype.23 In addition to macrophages, growing evidence has emerged that monocytes, classically recognized as the precursor of tissue-infiltrating differentiated macrophages, per se have an important role in the postinfarction healing process. In the mouse, there are 2 distinctive subsets of monocytes: CD11b^{high} Ly6C^{high} proinflammatory monocytes and CD11b^{high} Ly6C^{low} reparative monocytes.24,25 Nahrendorf et al26 reported that the postinfarction repair process consists of biphasic reactions, in which the early phase is dominated by Ly6C^{high} monocytes that are recruited in a CCL2-CCR2–dependent manner and exhibit phagocytic, proteolytic, and inflammatory properties, and the late phase is dominated by Ly6C^{low} monocytes that are recruited in a CX3CL1-CX3CR1–dependent manner and promote angiogenesis and resolution of inflammation. Moreover, they also demonstrated that Ly6C^{high} monocytosis observed in apolipoprotein E knockout atherosclerotic mice was associated with exacerbated postinfarction LV remodeling through increased inflammatory reaction and MMP activity, whereas in vivo depletion of neutrophils did not improve LV dysfunction after MI.27 On the other hand, infiltrating macrophage depletion by intravenous injection of Clo-Lip markedly impaired wound healing and accelerated LV remodeling and mortality after myocardial injury.9,26 Furthermore, depletion of circulating Ly6C^{high} monocytes by administration of Clo-Lip during the early phase of MI caused larger areas of debris and necrotic tissue, whereas depletion of Ly6C^{low} monocytes by late-phase administration of Clo-Lip resulted in decreased collagen deposition and reduced neoangiogenesis,9,26 which suggests that successful cardiac repair depends on well-coordinated recruitment of monocytes/macrophages in the injured heart. However, the molecular or cellular mechanisms upstream of this critical framework remain to be elucidated.

As the conductor of immune-mediated inflammatory reactions, since their discovery in 1973, DCs have attracted a great deal of attention because of their distinctive and wide variety of functions.28 DCs are of hematopoietic origin and are widely distributed in organs and tissues. They are characterized by their capability for antigen capture, migration, antigen presentation, and activation of other immune cells, and they control immunity and toler-
ance in various conditions, such as infection, autoimmune disease, tumor, and allergy. However, there are few reports regarding the role of DCs in cardiovascular disease, especially in LV remodeling after MI. Maekawa et al., using mice deficient in IL-1 receptor–associated kinase (IRAK)-4, demonstrated that activation of IRAK-4, which is a downstream molecule of IL-1 and Toll-like receptor family members, in DCs adversely affected postinfarction inflammation and LV remodeling. However, the IRAK-4 signaling pathway is also operative in cardiomyocytes and other immune cells in addition to DCs. Because the initial inflammatory signals after MI are known to involve

Figure 6. Enhanced infiltration of inflammatory M1 macrophages and diminished recruitment of anti-inflammatory M2 macrophages by dendritic cell (DC) ablation after myocardial infarction (MI). A, Representative photographs of immunohistochemical staining for Mac3+ macrophages in infarcted and border areas 7 days after MI. Scale bars indicate 100 μm. B, Time course of Mac3+ macrophage infiltration in control and DC-ablated mice after MI. Data were obtained from 5 independent experiments at each time point. C, Representative flow cytometric dot plots of CD45+ leukocytes in the infarcted hearts for CD206-Alexa 647 and F4/80-phycoerythrin in control and DC-ablated mice 7 days after MI or sham operation. D, The percentage of heart-infiltrating F4/80+ CD206+ M1 (upper) and F4/80+ CD206+ M2 (lower) macrophages in total F4/80+ macrophages of control and DC-ablated hearts 7 days after MI or sham operation (n=4 per group). E and F, Time course of myeloperoxidase-positive (MPO+) neutrophil (E) and CD3+ T-cell (F) recruitment after MI in control and DC-ablated mice analyzed by immunohistochemical staining. Data were obtained from 5 independent experiments at each time point. *P<0.05 and **P<0.01 vs sham of same group; †P<0.05 vs control MI.
Cardiomyocytes and are further strengthened by neutrophils and monocytes/macrophages that express IRAK-4, their results might reflect the involvement of these cells in the development of postinfarction LV dysfunction. Therefore, the precise contribution of DCs in the infarcted heart remains unclear.

In the present study, we used CD11c-DTR/GFP transgenic mice to examine the effect of DC depletion on postinfarction myocardial remodeling. Conditional DC depletion for 7 days after MI resulted in dysregulation of organized monocyte/macrophage employment, critical for the postinfarction repairing process. In vivo analyses revealed that the number of peripheral monocytes and tissue monocytes/macrophages was higher in DC-ablated mice than in control mice. Among these monocytes/macrophages, marked infiltration of inflammatory Ly6Chigh monocytes and F4/80+ CD206+ M1 macrophages and suppressed recruitment of anti-inflammatory Ly6Clow monocytes and F4/80+ CD206+ M2 macrophages were identified in DC-ablated mice, and this was associated with increased expression of CCL2 and decreased expression of CX3CL1. Prolonged expression of inflammatory cytokines and inducible nitric oxide synthase and enhanced MMP-9 activation were also observed in DC-ablated mice and were associated with increased peripheral blood and tissue inflammatory monocytes/macrophages. Consistent with a previously published report that demonstrated that thick collagen fibers were degraded by augmented MMPs and replaced by premature collagen fibers in the infarcted heart, the present study based on picrosirius polarized microscopy showed that DC-ablated infarcts had disorganized collagen fibers, which may lead to subsequent infarct expansion and heart failure. Moreover, cardiac angiogenesis, which has a favorable effect on tissue repair after MI, was inhibited in DC-ablated mice compared with control mice. As discussed above, Ly6Clow monocytes selectively express higher levels of vascular endothelial growth factor, and M2 macrophages also have proangiogenic properties. Taken together, these findings indicate that the absence of DCs leads to activation of inflammatory monocytes/macrophages and suppression of reparative monocytes/macrophages, inducing enhanced inflammation, extracellular matrix degradation, and impaired neoangiogenesis in the postinfarction healing process.

DCs, macrophages, and monocytes are members of the mononuclear phagocyte system, defined as nongranulocytic myeloid cells that play important roles in tissue remodeling and homeostasis, as well as regulatory and stimulatory aspects of innate and adaptive immunity. Their origins are presumed to be the same BM precursor cells, termed macrophage DC progenitors. Moreover, several reports have shown that peripheral monocytes transmigrate into inflamed tissue and differentiate into...
macrophages or DCs.37–39 On the basis of such tight interaction in the mononuclear phagocyte system, it is possible that selective DC depletion may change the subpopulations of the system and that DCs contribute to the maintenance of disciplined monocyte/macrophage organization after MI. The precise mechanisms of the anti-inflammatory effect of DCs through their control of monocyte/macrophage subsets are not clear, and it may be very difficult to address the mechanism because immune cells communicate through the release of many secreted factors. Further studies are needed to investigate how DCs interact with monocytes/macrophages in the healing myocardium.

In summary, the present study demonstrated that DCs are likely to play an immunoprotective role, especially in monocyte/macrophage homeostasis, during the postinfarction healing process, and suggests that modulation of DCs could be a new therapeutic target in LV remodeling after MI.

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Disclosures
Dr Koyasu is a consultant for Medical and Biological Laboratories, Co Ltd. The other authors report no conflicts.

References
CLINICAL PERSPECTIVE

Left ventricular (LV) remodeling after myocardial infarction is a process of complex architectural myocardial alteration and is associated with a poor clinical outcome. Congestive heart failure due to postinfarction LV remodeling remains an open question for clinicians throughout the world, in spite of recent aggressive revascularization and pharmacological therapy in clinical practice. Inflammation and immune responses are integral components of the host reaction to myocardial injury and play a pivotal role in infarct healing and subsequent LV dysfunction. The dendritic cell (DC) is like a maestro conducting various types of inflammatory cells in innate and adaptive immunity. We found that temporal and spatial characteristics of heart-infiltrating DCs and conditional depletion of DCs resulted in adverse infarct expansion and LV dysfunction. This was accompanied by a sustained enhanced inflammatory response and extracellular matrix degradation and suppressed myocardial neoangiogenesis. This impaired tissue repair was associated with augmented infiltration of inflammatory Ly6Chigh monocytes and F4/80+/CD206+ M1 macrophages and, conversely, reduced recruitment of anti-inflammatory Ly6Clow monocytes and F4/80−/CD206+ M2 macrophages in the infarcted heart. These data indicate that DC is a potent immunoprotective regulator that acts via control of monocyte/macrophage homeostasis during the postinfarction healing process. Modulation of DCs could be a novel therapeutic target in LV remodeling after myocardial infarction.
Regulatory Role of Dendritic Cells in Postinfarction Healing and Left Ventricular Remodeling

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Bone-marrow (BM) Cell Preparation and BM Transplantation (BMT)

A BMT model was used to evaluate the role of dendritic cell (DC) by depleting CD11c+ diphtheria toxin receptor (DTR)+ cells by diphtheria toxin (DT) administration. To harvest BM cells, femurs and tibias were taken from 8-week-old male CD11c-DTR/GFP transgenic mice. BM was flushed out with a 24-gauge syringe, and a single cell suspension in phosphate buffered saline (PBS) was made through a 100-µm nylon mesh after red blood cell lysis using ACK lysis buffer solution (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA [pH 7.2]). Five million BM cells were transferred intravenously into lethally irradiated (10 Gy) 6- to 8-week-old male wild-type (WT) C57BL/6 mice. The mice were allowed to rest for 8 weeks, and the success of BMT was checked by polymerase chain reaction (PCR) to confirm the presence of transgene in peripheral blood cells of the recipient mice before use. To evaluate the BM chimerism after BMT, lethally irradiated (10Gy) CD45.1+ B6.SJL recipient mice were reconstituted with BM cells of CD11c-DTR/GFP donor mice (CD45.2+) under the same protocols described above.

Creation of Myocardial Infarction (MI)

Experimental MI was induced as previously described. In brief, mice were anesthetized
with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), then
intubated and connected to a rodent ventilator. The chest cavity was opened via left
thoracotomy to expose the heart, and the left coronary artery was 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 died within 24 hours after surgery were excluded from the experiment. Sham-operated
animals in both groups underwent the same procedure without coronary artery ligation. The
deceased mice were performed an autopsy to determine the cause of death: cardiac rupture
was confirmed by the presence of blood coagulation around the pericardial sac and in the
chest cavity, and heart failure was diagnosed by lung congestion with chest fluid
accumulation.

**Echocardiography and Hemodynamics**

Echocardiographic and hemodynamic evaluations were performed on day 28 after the
operation. For echocardiography, mice were anesthetized with 1-2% of isoflurane. M-mode
tracings were recorded through the anterior and posterior left ventricular (LV) walls at the
papillary muscle level to measure anterior wall thickness (AWT), posterior wall thickness
(PWT), LV end-diastolic diameter (LVEDD), and end-systolic diameter (LVESD), using an
echocardiographic system (12-MHz linear transducer; EnVisor C, Philips Medical Systems,
Andover, MA). The following formulas were used to calculate LV fractional shortening (FS) and LV mass: 

\[
FS(\%) = \left[ \frac{(LVEDD-LVESD)}{LVEDD} \right] \times 100 \quad \text{and} \quad LV\ mass = 1.055 \times \left[ (AWT+PWT+LVEDD)^3 - LVEDD^3 \right] , \text{respectively.}
\]

Cardiac catheterization studies were performed using a 1.4 French microtip catheter (SPR-671, Miller Instruments, Houston, TX) under sedation with intraperitoneal injection of pentobarbital sodium (40 mg/kg) with spontaneous respiration. LV end-systolic pressure, maximum rate of isovolumic pressure development and minimum rate of isovolumic pressure decay were measured using analysis software (PowerLab, AD Instruments, Bella Vista, NSW, Australia). Ten sequential beats were averaged for each measurement.

**Morphometric Analysis**

Heart tissue was fixed in formalin, embedded in paraffin, and cut into 5-µm-thick sections. Sections were stained with hematoxylin and eosin, Masson’s trichrome and picrosirius red to determine the infarct size and cardiac fibrosis, and identify their morphology. To evaluate the quality of collagen fibers, picrosirius red stained-sections were studied with polarized microscopy. The infarct size was assessed as total infarct circumference divided by total LV circumference times 100, as described previously.\(^4\) The wall thickness of the scar at the papillary muscle level was also measured. The fraction of collagen volume was assessed in 10 randomly chosen high-power fields (×200) in each section. These data were analyzed
using ImageJ software (version 1.38×, National Institutes of Health).

**Real-Time Quantitative PCR**

Total RNA was isolated by the acid-phenol extraction method in the presence of chaotropic salts (Trizol, Invitrogen, Carlsbad, CA) and subsequent isopropanol-ethanol precipitation as described previously.\(^5\) Reverse transcription was performed using a Super-Script First-Strand Synthesis System (Invitrogen) in accordance with the manufacturer’s protocol. The sequences of primer pairs were designed using Primer Express III software (Applied Biosystems, Foster City, CA) and are described in online-only Data Supplement Table 1. Real-time quantitative PCR was performed using an ABI Prism 7500 sequence detection system (Applied Biosystems). The obtained data were normalized using the expression levels of mouse 18S rRNA.

**Western Blotting**

Frozen tissue was homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL) with protease inhibitors (Thermo Scientific). After centrifugation, the supernatant was collected and protein concentration measured using a Bradford assay system. Equal amounts of protein samples (30 µg) were electrophoresed on 4-12% NuPAGE Bis-Tris gels (Invitrogen), and then proteins were electroblotted onto polyvinylidene
difluoride membranes. After blocking with 5% nonfat dried skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 at room temperature (RT) for 1 hour, the membranes were incubated with the primary antibodies anti-interleukin (IL)-10 (Abcam, Cambridge, MA), anti-inducible nitric oxide synthase (iNOS) (BD Bioscience, San Jose, NJ), anti-vascular endothelial growth factor (VEGF) (Santa Cruz Biotech., Santa Cruz, CA) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotech.) at 4°C overnight or for 1 hour at RT, followed by exposure to the secondary horseradish peroxidase (HRP)-conjugated antibody for 1 hour at RT. The immunoblots were developed by enhanced chemiluminofluorescence method. The signals were scanned with MF-ChemiBIS (DNR Bio-Imaging Systems, Jerusalem, Israel) and analyzed using Quantity One software (Bio-Rad, Hercules, CA).

**Gelatin Zymography**

To evaluate the activity of gelatinase, matrix metalloproteinase (MMP)-9 and MMP-2, gelatin zymography was performed. Equal volumes of 30 µg protein were loaded onto each lane of 10% gelatin zymogram gels (Novex, Invitrogen). After running at 80V for 2 hours, the gels were incubated in 2.5% Triton-X 100 for 30 min at RT, washed and then further incubated in 50 mM Tris-HCl, pH 7.4, containing 200 mM NaCl and 5 mM CaCl₂ at 37°C overnight with gentle agitation. After washing with deionized water, the gels were stained with Coomassie
blue for 90 min followed by destaining with a solution containing 30% ethanol and 10% acetic acid. The presence of different MMPs was identified on the basis of their molecular weight. The gels were photographed using MF-ChemiBIS (DNR Bio-Imaging Systems) and analyzed using Quantity One software (Bio-Rad).

**Immunohistochemical Staining**

Immunohistochemical studies were performed by immunoperoxidase methods using paraffin-embedded tissue sections. After inhibiting endogenous peroxidase activity, the sections were incubated with primary anti-Mac3 (BD Bioscience), anti-MPO (Dako, Glostrup, Denmark) or anti-CD3 (Dako) antibodies at 4°C overnight. After incubation, a Vectastain ABC elite Kit (Vector Laboratories, Burlingame, CA) was used according to the manufacturer’s instructions. Following visualization with 3,3’-diaminobenzidine, the sections were finally counterstained with hematoxylin. The same methods were performed without the primary antibodies, as negative controls. The numbers of Mac3+ macrophages, MPO+ neutrophils, and CD3+ T lymphocytes were assessed by counting the total cell numbers in the infarcted and border areas in 20 randomly chosen fields in each section. For the assessment of endothelial proliferation in the infarcted heart, double immnohistochemical staining for CD31 (Santa Cruz Biotech.) and Ki67 (Dako) was performed. After antigen retrieval, tissue sections were incubated with primary antibodies followed by the reaction with secondary
antibodies and labeling with alkaline phosphatase (for CD31) and 3,3’-diaminobenzidine (for Ki67). Finally, sections were counterstained with hematoxylin. The number of blood vessels and CD31+ Ki67+ proliferating endothelial cells were counted per unit area.

**Immunofluorescent Staining**

For immunofluorescent staining, frozen tissue sections were used. The excised hearts were embedded in OCT compound, snap frozen in liquid nitrogen, and then cut into 6-µm-thick sections. After fixing in acetone at RT for 10 min, cryosections were blocked using Block Ace (DS Pharma Biomedical Co. Ltd., Osaka, Japan) at 37°C for 30 min, and then incubated with anti-CD11c-APC (BD Bioscience) and purified anti-GFP (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) antibodies at 4°C overnight to identify BM-derived DCs. Alexa-Fluor 488-conjugated secondary antibody (Molecular Probes, Carlsbad, CA) was added to detect GFP+ cells. To evaluate infiltrating leukocytes, primary purified anti-CD45 (BD Bioscience) and Alexa-Fluor 647-conjugated secondary (Molecular Probes) antibodies were used. Nuclei were identified with DAPI. As negative controls, the same procedures were performed without the primary antibodies. The sections were finally photographed under a confocal laser-scanning microscope (TCS-SP5, Leica).

**Preparation of Splenic and Peripheral Blood Cells**
Spleens were removed, mushed and then passed through a 100-µm nylon mesh in PBS. After addition of ACK lysis buffer solution to exclude erythrocytes, the single cell suspension in PBS was refiltered through a 100-µm nylon mesh to remove connective tissue. Peripheral blood was drawn via cardiac puncture and collected into a heparinized tube. ACK lysis buffer solution was also added to the collected peripheral blood, followed by washing in PBS.

**Isolation of Infiltrating Leukocytes from the Infarcted Heart**

On the indicated days after the operation, mice were deeply anesthetized and intracardially perfused with ice-cold PBS to remove blood cells before euthanasia. Infarcted tissue was dissected, minced with fine scissors, and enzymatically digested with a cocktail of type II collagenase (Worthington Biochemical Corporation, Likewood, NJ), elastase (Worthington Biochemical Corporation) and DNase I (Sigma, St. Louis, MO) for 1 hour at 37°C with gentle agitation. After digestion, the tissue was then triturated and passed through a 100-µm cell strainer. For the separation of leukocytes, cells were incubated with biotin-conjugated anti-CD45 antibody (BioLegend, San Diego, CA) at 4°C for 10 min, followed by incubation with streptoavidin-conjugated microbeads (Miltenyi Biotec, Sunnyvale, CA) at 4°C for 15 min. Then, CD45⁺ leukocytes were positively collected by magnetic sorting with AutoMACS (Miltenyi Biotec). The purity of cells was generally > 97%.
Flow Cytometry

Isolated splenocytes, peripheral blood cells, and leukocytes isolated from the heart were analyzed by flow cytometry. To block nonspecific binding of antibodies to Fcγ receptors, isolated cells were first incubated with anti-CD16/32 antibody (BD Bioscience) at 4°C for 5 min. Subsequently, the cells were stained with a mixture of the following antibodies at 4°C for 20 min: anti-CD11c-PE, anti-CD11c-APC, anti-CD45.1-PerCP-Cy5.5, anti-MHCII-PE, anti-CD11b-PE, anti-Mac3-PE, anti-Ly6C-APC, anti-F4/80-PE, anti-CD4-PE, anti-CD8-PE, anti-CD19-PE (BD Bioscience), CD45.2-APC (BioLegend), and Alexa-Fluor 647-conjugated anti-CD206 antibody (eBioscience, San Diego, CA). Peripheral blood monocytes were defined as SSC\text{low} CD11b\text{high} cells as previously described. After staining with 7-amino-actinomycin D (Sigma) to discriminate dead cells, flow cytometry was performed on a FACSCalibur (BD Bioscience) and the data analyzed with FlowJo software (Tree Star, Ashland, OR).
References


### Supplemental Table 1. Primers for real-time PCR in this study

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<tr>
<th>Gene</th>
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(F), Forward Primer; (R), Reverse Primer.
Supplemental Figure Legends

**Supplemental Figure 1.** Evaluation of BM chimerism of BMT mice. A, Representative flow cytometric dot plots of splenocytes for recipient-type CD45.1-PerCP-Cy5.5 and donor-type CD45.2-APC. B, Representative flow cytometric dot plots of splenocytes for CD11c-PE and CD45.2-APC, and histogram for GFP of CD45.2+ CD11c+ splenocytes.

**Supplemental Figure 2.** Depletion of DCs by DT injection. A, Representative flow cytometric dot plots of splenocytes for CD11c-APC and GFP or MHCII-PE in control and DC-ablated mice. B, Representative flow cytometric histograms of splenocytes for CD4, CD8, CD19 and CD11b in control (solid line) and DC-ablated (dotted line) mice.

**Supplemental Figure 3.** Inefficacy of DT on the WT mice heart. Echocardiographic measurements and heart/lung weight adjusted for body weight in non-treated and DT-treated WT mice are shown.

**Supplemental Figure 4.** Enhanced infiltration of CD45+ leukocytes and Mac3+ macrophages into the infarcted heart by DC-ablation. A, Time course of CD45+ leukocyte infiltration into the heart of control and DC-ablated mice after MI. Data were obtained from 5 independent experiments at each time point. B, Representative immunofluorescent staining patterns for
CD45 in infarcted and border areas 7 days after MI. Scale bars indicate 30 μm. C, Representative flow cytometric dot plots of heart-infiltrating CD45⁺ leukocytes for Mac3-PE in control and DC-ablated mice 7 days after MI. D, Quantification of Mac3⁺ macrophages based on flow cytometry in control (n = 5) and DC-ablated (n = 5) mice 7 days after MI. *P < 0.05 and **P < 0.01 vs sham of same group; †P < 0.05 and ††P < 0.01 vs control MI.

**Supplemental Figure 5.** DC-ablation induces marked monocyte recruitment after MI. A, Temporal changes in number of total peripheral blood monocytes by flow cytometry. B, Number of tissue monocytes in control and DC-ablated mice 7 days after MI or sham operation. *P < 0.05 and **P < 0.01 vs sham of same group; †P < 0.05 vs control MI.
The online data supplement Figure 1.

A

B
The online data supplement Figure 2.

A

![Graph showing the comparison of Control and DC-Ablation for CD11c, GFP, MHC class II, and CD11c labeling.](image)

B

![Graphs showing the counting of CD4, CD8, CD19, and CD11b](image)
The online data supplement Figure 3.
The online data supplement Figure 4.

A

![Bar graph showing CD45+ leukocytes (×10^4) over time for Control and DC-ablation MI groups.](image)

Sham Day 1 Day 3 Day 7 Day 14

B

![Images showing DAPI / CD45 stains for Infarct and Border areas for Control MI and DC-ablation MI groups.](image)

C

![Flow cytometry dot plots showing Mac3+ macrophages (%).](image)

Control MI DC-ablation MI

D

![Bar graph showing Mac3+ macrophages (%) for Control and DC-ablation MI groups.](image)
The online data supplement Figure 5.