Differential Effects of GM-CSF and G-CSF on Infiltration of Dendritic Cells during Early Left Ventricular Remodeling after Myocardial Infarction

Kotaro Naito,* Toshihisa Anzai,2* Yasuo Sugano,* Yuichiro Maekawa,* Takashi Kohno,* Tsutomu Yoshikawa,* Kenjiro Matsuno,‡ and Satoshi Ogawa*

Several lines of evidence suggest that the immune activation after myocardial infarction (MI) induces secondary myocardial injury. Although dendritic cells (DC) are potent regulators of immunity, their role in MI is still undetermined. We investigated the effect of DC modulation by CSF on left ventricular (LV) remodeling after MI. MI was induced by ligation of the left coronary artery in male Wistar rats. G-CSF (20 μg/kg/day, MI-G, n = 33), a GM-CSF inducer (romurtide, 200 μg/kg/day, MI-GM, n = 28), or saline (MI-C, n = 55) was administered for 7 days. On day 14, MI-G animals had higher LV max dP/dt and smaller LV dimensions, whereas MI-GM animals had lower LV max dP/dt and larger LV dimensions than did MI-C animals, despite similar infarct size. In MI-C, OX62+ DC infiltrated the infarcted and border areas, peaking on day 7. Bromodeoxyuridine-positive DC were observed in the border area during convalescence. Infiltration by DC was decreased in MI-G animals and increased in MI-GM compared with MI-C (p < 0.05). In the infarcted area, the heat shock protein 70, TLR2 and TLR4, and IFN-γ expression were reduced in MI-G, but increased in MI-GM in comparison with those in MI-C animals. IL-10 expression was higher in MI-G and lower in MI-GM than in MI-C animals. In conclusion, G-CSF improves and GM-CSF exacerbates early postinfarction LV remodeling in association with modulation of DC infiltration. Suppression of DC-mediated immunity could be a new strategy for the treatment of LV remodeling after MI. The Journal of Immunology, 2008, 181: 5691–5701.

left ventricular (LV) remodeling, an important structural event after myocardial infarction (MI), is characterized by myocardial necrosis, wall thinning, infarct expansion, collagen accumulation, and noninfarcted myocardial hypertrophy, and contributes significantly to a worse clinical outcome. These alterations are caused not only by significant loss of myocardium, but also by myocardial injury secondary to local and systemic factors such as neurohumoral activation, oxidative stress, and inflammatory response. The inflammatory response after MI is integral to the healing process and contributes to LV remodeling (1–3). However, no effective therapeutic strategy against inflammation has been established.

We previously reported that elevated concentrations of serum C-reactive protein (4), plasma IL-6 (5), and peripheral monocyte (6) predict a worse clinical outcome after acute MI, suggesting that an immune-mediated inflammatory response may have some role during infarct healing and ventricular remodeling. Although an excessive inflammatory response after MI is associated with a poor clinical outcome (2), antiinflammatory therapy using corticosteroids (7, 8) or nonsteroidal antiinflammatory drugs (9, 10) leads to catastrophic results such as a higher incidence of infarct expansion and cardiac rupture. These findings suggest that an inflammatory reaction is a prerequisite for the healing process. As the cause of inappropriate activation of the inflammatory response after MI, an autoimmune reaction is a possible mechanism relating to LV remodeling (11–13). Abbate et al. reported infiltration of activated T cells into both infarcted and remote areas of the myocardium in patients with recent MI (1). Moreover, the presence of autoimmunity to cardiac myosin (12), actin (14), and troponin (15) is associated with an adverse clinical outcome after MI. These findings suggest that autoimmune responses may contribute to secondary myocardial injury after MI.

For the activation of autoimmune responses, myocardial Ag presentation is required. Dendritic cells (DC) are potent regulators of immunity by presenting Ag, activating T cells, and by causing differentiation T cells into Th1 and Th2 cells (16–18). After tissue injury, heat shock proteins (HSP) released from necrotic cells can promote activation and maturation of DC through stimulation of TLRs (19, 20). Additionally, Cheng et al. demonstrated that Th1/Th2 imbalance participated in ventricular remodeling after MI (21). However, the role of DC during the healing process after MI has not been determined.

The development of DC from hematopoietic progenitor cells is differentially regulated by various cytokines such as GM-CSF and G-CSF. We previously reported that G-CSF treatment improved (22) and GM-CSF induction aggravated (23) early LV remodeling after MI through modification of the infarct healing process. GM-CSF induces differentiation from immature DC to myeloid DC, with a subsequent increase in Th1 cells, whereas G-CSF induces...
plasmacytoid DC followed by proliferation of Th2 cells (24–26). Therefore, we hypothesized that G-CSF and GM-CSF diversely regulate DC maturation through modulation of the Th1/Th2 balance during infarct healing and affect postinfarction LV remodeling. To test this hypothesis, we examined the dynamics of DC in a rat MI model using OX62 Ab, which is a reliable marker for the

![FIGURE 1. DC in infarcted heart. A. Triple immunofluorescent staining for OX62 (red), OX6 (green), and type IV collagen (blue). B. Triple immunofluorescent staining for OX62 (red), V65 (green), and type IV collagen (blue). C. Triple immunofluorescent staining for OX62 (red), CD4 (green), and type IV collagen (blue). All images in A–C are from myocardium of MI-C group on day 7. D. Immunohistochemical staining of myocardial sections to evaluate the time course changes in OX62⁺ DC (blue) infiltration of infarcted (I), border (B), and noninfarcted (NI) areas (n = 4/group). All specimens were stained for type IV collagen (brown) to show the tissue framework. Values are means ± SEM.](image)

### Table 1. Heart weight, echocardiographic, and hemodynamic data on day 14*

|                     | Sham (n = 9) | MI-C (n = 10) | MI-G (n = 10) | MI-GM (n = 10) |
|---------------------|-------------|--------------|--------------|----------------|
| BW, g               | 252 ± 13    | 254 ± 14     | 242 ± 11     | 242 ± 14       |
| RVW/BW, g/kg        | 0.5 ± 0.03  | 0.8 ± 0.06*  | 0.8 ± 0.05*  | 0.9 ± 0.06*    |
| LVW/BW, g/kg        | 2.0 ± 0.04  | 2.1 ± 0.08   | 2.1 ± 0.08   | 2.1 ± 0.12     |
| LVEDD, mm           | 6.2 ± 0.2   | 8.5 ± 0.2*   | 8.1 ± 0.1†   | 9.0 ± 0.1†     |
| LVEDD, mm           | 3.7 ± 0.1   | 6.9 ± 0.1*†  | 6.1 ± 0.2†   | 7.6 ± 0.1†     |
| FS, %               | 41 ± 1.9    | 19 ± 1.5*‡   | 24 ± 1.1†‡   | 15 ± 0.7†‡     |
| LVSP, mmHg          | 123 ± 3     | 101 ± 4*     | 102 ± 2*     | 110 ± 5*       |
| LVEDP, mmHg         | 3.4 ± 0.4   | 9.7 ± 0.6*   | 8.0 ± 0.5†   | 12.3 ± 1.1†    |
| LV +dP/dtmax, mmHg/s| 9415 ± 441  | 4530 ± 195*  | 5828 ± 227†  | 3608 ± 356*†   |
| LV −dP/dtmin, mmHg/s| -7280 ± 830 | -3536 ± 242‡ | -3595 ± 159‡ | -3675 ± 220*‡  |

*BW, body weight; RVW, right ventricular weight; LVW, left ventricular weight; LVEDD, left ventricular end-diastolic dimension; LVEDD, left ventricular end-systolic dimension; FS, fractional shortening; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV +dP/dtmax, left ventricular maximum rate of isovolumic pressure development; LV −dP/dtmin, left ventricular minimum rate of isovolumic pressure decay. Values are means ± SEM. *p < 0.05 vs. sham; †, p < 0.05 vs. MI-C.
FIGURE 2. MHC class II+ cells in myocardium. A, Immunohistochemical staining of myocardial sections using OX6 (blue) mAb to evaluate the time course changes in MHC class II+ (OX6+) cell infiltration of infarcted (I), border (B), and noninfarcted (NI) areas (n = 4/group). All specimens were stained for type IV collagen (brown) to show the tissue framework. B, Triple immunofluorescent staining for OX6 (green), ED-1 (red), and type IV collagen (blue). C, Triple immunofluorescent staining for OX6 (green), ED-2 (red), and type IV collagen (blue). D, Triple immunofluorescent staining for OX6 (green), ED-3 (red), and type IV collagen (blue). Values are means ± SEM.

Materials and Methods

Animals and surgical procedure

All procedures were performed in accordance with the Keio University animal care guidelines, which conform to the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication 85-23, revised 1996). Left ventricular MI was created in 9-wk-old male Wistar rats (Sankyo Laboratory Service) weighing 220–280 g, by left coronary artery ligation as previously described (22). Coronary ligation was performed in 166 rats. The same procedure was performed for sham-operated control animals (n = 27) except that the ligature was left untied. The MI rats surviving the operation for 3 h (n = 151) were randomly assigned to three groups: 1) recombinant human G-CSF (Kyowa Hakko Kogyo; 20 μg/kg/day) administered s.c. for 7 days (MI-G, n = 45), (2) GM-CSF inducer (romurtide, 200 μg/kg/day) administered i.p. for 7 days (MI-GM, n = 40), and (3) saline-treated controls (MI-C, n = 66). Rats were housed under standardized conditions with free access to standard food and drinking water. A total of 116 rats (MI-G, n = 33; MI-GM, n = 28; MI-C, n = 55) survived and were sacrificed 1, 3, 5, 7, 10, 14, and 28 days after surgery according to the study protocol.

Echocardiographic, hemodynamic, heart weight, and infarct size measurements

Animals (sham-operated rats, n = 9; MI-C, n = 10; MI-G, n = 10; MI-GM, n = 10) were lightly anesthetized by i.p. administration of pentobarbital 2 wk after MI. Transthoracic echocardiography (8.5-MHz linear transducer; EnVisor C, Philips Medical Systems) and hemodynamic measurements using a miniature pressure transducer (SPC-320, Millar Instruments) were performed as previously described (22). The hearts were then excised and divided into left and right ventricles, and each ventricle was weighed separately. The investigators who conducted these procedures were blinded to the treatment of the animals. Infarct size was measured as the percentage of infarcted epicardium and endocardium of the LV, as previously described (22).

Antibodies and reagents

For immunohistochemical analyses, mouse mAbs specific for rat determinants, including Abs against rat DC (OX62), MHC class II RT1B (OX6), CD4 (W3/25), γδ T cells (V65), CD68 (ED-1), CD163 (ED-2), CD3, CD45, CD45R (His24), and CD86 (B7-2) were obtained from Serotec. An anti-BrdU mAb was purchased from Oxford Biotechnology. A rabbit anti-mouse type IV collagen polyclonal Ab to outline the tissue framework was purchased from LSL. As secondary Abs, an alkaline phosphatase (ALP)-labeled goat anti-mouse IgG (Sigma-Aldrich), a HRP-labeled anti-rabbit IgG (Cappel), and an ALP-labeled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) were employed. For immunoblotting analyses, mouse anti-rat HSP70 mAb (StressGen Biotechnologies) and rabbit polyclonal anti-TLR4 (Jackson ImmunoResearch Laboratories) and an HRP-labeled anti-rabbit IgG (Santa Cruz Biotechnology) were employed.

Immunohistochemical analyses

Triple immunoenzymatic staining with mouse mAbs was performed as previously described (28). In brief, fresh cryosections were fixed in acetone for 10 min, air-dried, and rehydrated in PBS. Sections were fixed further with formol calcium solution (4% parafomaldehyde in 1% CaCl2, pH 7.0) for 2 min and washed. As the first step, sections were incubated with the first mAbs for 1 h at room temperature and then with ALP-labeled anti-mouse IgG for 1 h at room temperature. After fixing in 1% glutaraldehyde for 30 s in PBS, the ALP activity was developed as blue with a
Vector blue substrate kit (Vector Laboratories). As the second step, sections were reacted with a second mAb and then with a HRP-labeled anti-mouse IgG and developed as brown with a diaminobenzidine substrate for 5–10 min. For BrdU staining, the above samples were further fixed in 1% glutaraldehyde for 10 min and digested in a pepsin solution (0.006% in 0.01 N HCl) for 10 min at 37°C. Samples were treated with 4 N HCl for 30 min at room temperature and neutralized with borate buffer (0.1 M (pH 8.5)). BrdU was detected with an anti-BrdU mAb and then with an ALP-labeled anti-mouse IgG and developed as red with a New Fuchsin substrate kit (DakoCytomation) for 5–10 min. All rats for immunohistochemical analyses received BrdU i.v. (Sigma-Aldrich; 1 mg/50 g body weight) and were sacrificed 1 h later. Slides were mounted in AquaTex (Merck) after immunostaining. Cells infiltrating the myocardium were evaluated in the infarcted, border, and noninfarcted areas.

Immunofluorescent staining
Immunofluorescent staining was performed for double labeling by each Ab against cell surface Ags. Mouse mAbs as described above were used as the first Abs. We applied Cy5-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) as the secondary Ab for type IV collagen, and a Zenon Alexa Fluor 488 mouse IgG labeling kit and Alexa Fluor 546 mouse IgG labeling kit (Invitrogen/Molecular Probes) were used as secondary reagents for mouse mAbs.

Flow cytometric analyses
The spleens were minced and digested in 2 mg/ml collagenase D (Roche Diagnostics) in RPMI 1640/1% FCS for 30 min at 37°C. EDTA at 10 mM was added for the last 5 min, and the cell suspension was pipetted up and down several times and filtered with a cell strainer. To lyse the RBC, ACK lysis buffer (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA (pH 7.2)) was added to the cell suspension for 5 min at room temperature, and the cells were washed twice. Peripheral blood was collected into a heparinized tube. ACK lysis buffer was also added to peripheral blood for 5 min at room temperature to remove RBC. For cytometric analyses, 2 × 10⁵ splenocytes and peripheral blood cells were incubated with PE-conjugated OX62 mAb and FITC-conjugated OX6 mAb or FITC-conjugated CD86 mAb for 40 min at 4°C. Cells were washed twice and analyzed on a Coulter EpicsXL-MCL flow cytometer (Beckman Coulter).

Real-time quantitative RT-PCR
After the heart had been excised, all tissues were snap frozen in liquid nitrogen and then preserved at −80°C. The LV of sham-operated rats was collected for control studies. Total RNA was isolated by a modification of the acid guanidinium thiocyanate and phenol/chloroform extraction method. After homogenizing the heart tissues with a Polytron homogenizer in TRIzol reagent (Invitrogen), total RNA was extracted with chloroform and samples were centrifuged at 16,600 × g for 15 min at 4°C. RNA was precipitated by addition of isopropanol, and the pellet was dissolved in diethyl pyrocarbonate water. Total RNA concentration was determined by spectrophotometric analysis at 260 nm. Reverse transcription was performed using TaqMan reagents (Applied Biosystems). Real-time quantitative PCR of each sample was conducted with TaqMan Gene Expression Assays and an ABI Prism 7700 sequence detection system (Applied Biosystems), based on methods described previously (22). The TaqMan assays used were TLR2 (Rn02133647_s1), TLR4 (Rn00569848_m1), IL-10 (Rn00563409_m1), and IFN-γ (Rn00594078_m1) from Applied Biosystems. For each sample, CT values were subtracted from that of the housekeeping gene GAPDH to generate the Ct values. Primer pairs and probe for GAPDH were as follows: forward primer, AACTCCCT

FIGURE 3. Proliferative response of DC after MI. Triple immunostaining of myocardium (A) and spleen (B) for OX62 (blue), BrdU (red), and type IV collagen (tissue framework; brown) was performed. Arrows indicate cells positive for both OX62 and BrdU. All figures in A are from border area of myocardium. Sham-operated group and MI-C group at 7 days post-MI were compared. Values are means ± SEM.
CAAGATTGTCAGCAA; reverse primer, GTGGTCAATGAGCCCTTCCA; TaqMan probe: CTGCACCCACCAACCTGCTTAGCCCC.

Immunoblotting analyses

For Western blotting, frozen tissue was homogenized in cell lysis buffer (Cell Signaling Technology) containing 1% Triton X-100 and protease inhibitors. After centrifugation at 16,000 \( \times \) g for 30 min at 4°C, the supernatant liquid was collected. Protein concentrations were measured using Coomassie protein assay reagent (Pierce Biotechnology) based on the Bradford assay. Immunoblotting analysis was conducted as previously described (22). Equal amounts of 50 μg protein were electrophoresed on 10% SDS polyacrylamide gels (150 V, 60 min). Proteins were electrophoresed onto nitrocellulose membranes (Amersham Biosciences) for 60 min at 100 V. After blocking with 5% nonfat dried milk in Tris-buffered saline for 60 min, the membranes were incubated with the first Ab for 60 min, followed by exposure to the second Ab for 45 min. The immunoblots were developed by enhanced chemiluminofluorescence method. The signals were quantified by densitometry (GS-800, Bio-Rad).

Statistical analysis

All data were expressed as mean ± SEM. One-way ANOVA followed by Bonferroni’s multiple-comparison tests was performed for statistical comparisons. All statistical analyses were performed using SPSS 13.0 for Windows. Statistical significance was defined as a \( p \)-value of <0.05.

Results

LV functional analyses

Table I shows heart weight and echocardiographic and hemodynamic data on day 14. Left and right ventricular weight per body weight and LV systolic pressure in the MI-C, MI-G, and MI-GM groups did not differ significantly. Maximum dP/dt was lower and LV end-diastolic pressure (LVEDP) was higher in MI-GM than in MI-C animals. MI-GM animals had significantly larger LV end-diastolic dimension.
(LVEDD) and LV end-systolic dimension (LVESD), and lower fractional shortening (FS) than did MI-C animals. Higher maximum dP/dt and lower LVEDP were observed in MI-G animals than in MI-C animals. LVEDD and LVESD were smaller, and FS was greater in MI-G than in MI-C animals. Infarct size was similar in all groups.

Infiltration of DC and MHC class II-positive cells into myocardium

To evaluate involvement of DC in the myocardium, double immunostaining was performed with OX62, a specific marker of rat DC, and OX6 for MHC class II. Most OX62⁺ cells were positive for OX6 (Fig. 1A). Serial sections were stained with V65 mAb, a marker of γδ T cells, which are known to be recognized by OX62 mAb. No V65⁺ were detected in the myocardium of both sham-operated and MI-created rats (Fig. 1B). Thus, OX62⁺ cells detected in the myocardium after MI were most probably DC. Moreover, we examined the expression of CD4 on OX62⁺ DC that infiltrated the myocardium after MI to analyze DC subsets. Approximately 55% of OX62⁺ cells were also CD4⁺, and the remaining cells (45%) were CD4⁻ (Fig. 1C).

The time course of DC infiltration into the infarcted, border, and noninfarcted areas in MI-C animals is shown in Fig. 1D. A marked increase in DC was noted in the infarcted (90 ± 9 cells/mm²) and border (83 ± 6 cells/mm²) areas, peaking on day 7. DC were rarely observed in the noninfarcted area or in the myocardium of sham-operated rats (2 ± 1 cells/mm²).

Fig. 2A shows the time course of MHC class II (OX6)⁺ cell infiltration into the myocardium in MI-C group. A marked increase of MHC class II⁺ cells was noted in all areas after MI, peaking on day 7 (infarcted area, 751 ± 39 cells/mm²; border area, 718 ± 48 cells/mm²; noninfarcted area, 105 ± 11 cells/mm²). A few MHC class II⁺ cells were observed in the myocardium of sham-operated rats (41 ± 9 cells/mm²). Most of MHC class II⁺ cells were OX62⁺, ED-1⁺ (Fig. 2B), and ED-2⁺ (Fig. 2C), indicating a predominance of recruited infiltrating macrophages in MHC class II⁺ cells. ED-3⁺ cells were rarely observed in the infarcted myocardium (Fig. 2D). Abundant CD45⁺ (pan-leukocyte marker) cells infiltrated the myocardium mainly from epicardial sites after MI. Infiltration of these cells was found in the infarcted heart from the early phase after MI, peaking on day 7, and was considerably decreased on day 14. ED-1⁺ cells also infiltrated similar sites of the myocardium to CD45⁺ cells.

Proliferative responses of DC after MI

Triple immunostaining for OX62, BrdU, and type IV collagen was performed to detect DC with a proliferative response
(OX62+ BrdU+ cells) in the myocardium after MI. OX62+ BrdU+ cells were observed mainly in the border area on days 3, 7, and 14 (Fig. 3A). The ratio of OX62+ BrdU+ cells/OX62+ cells was 16% (day 3), 24% (day 7), and 11% (day 14). Cells positive for OX62 or BrdU were rarely observed in the myocardium of sham-operated rats. A persistent proliferative response of DC was observed for at least 14 days after MI. These findings suggested that activated DC are present in the myocardium after MI.

To evaluate the proliferative response of DC in the spleen, we performed triple immunostaining for OX62, BrdU, and type IV collagen. The number of OX62+ BrdU+ cells increased significantly in the spleen after MI, whereas such cells were uncommon in rats that had undergone sham operation (Fig. 3B). These findings indicated that splenic DC matured after MI. There was no significant difference in the number of OX62+ cells, and both OX62+CD86+OX62+ and OX62+CD86+OX62+ cell ratios in the spleen among MI-C, MI-G, and MI-GM groups. Few OX62+ cells were detected in peripheral blood (Fig. 4B). However, there was no significant difference in the number of OX62+ cells among all groups.

**Effects of G-CSF and GM-CSF inducer on infiltration of MHC class II-positive cells into myocardium**

The effects of G-CSF and GM-CSF inducer on DC infiltration into the infarcted area (Fig. 5, A and D) and border area (Fig. 5, B and E) were examined. The infiltration of DC was suppressed in the MI-G group on day 7 in the infarcted (55 ± 6 vs 90 ± 9 cells/mm2, p = 0.003) and border (51 ± 7 vs 83 ± 6 cells/mm2, p = 0.004)
areas, and on day 14 in the infarcted (24 ± 5 vs 44 ± 5 cells/mm², \( p = 0.008 \)) and border (20 ± 3 vs 43 ± 7 cells/mm², \( p = 0.008 \)) areas compared with that in MI-C group (Fig. 5). The infiltration of DC was increased in the MI-GM group in the infarcted (89 ± 8 vs 54 ± 5 cells/mm², \( p = 0.007 \)) and border (92 ± 8 vs 59 ± 7 cells/mm², \( p = 0.008 \)) areas on day 3 and day 7 (infarcted 119 ± 5 vs 90 ± 9 cells/mm², \( p = 0.011 \); border 114 ± 7 vs 83 ± 6 cells/mm², \( p = 0.004 \)) compared with the MI-C group (Fig. 5).

Fig. 6 demonstrates the effect of G-CSF and GM-CSF inducer on infiltration of MHC class II⁺ cells into the infarcted area (Fig. 6, A and D), border area (Fig. 6, B and E), and noninfarcted area (Fig. 6, C and F) of myocardium. On day 7, positive cells were decreased in the MI-G group in the infarcted (591 ± 40 vs 751 ± 39 cells/mm², \( p = 0.013 \)) and border (524 ± 41 vs 718 ± 48 cells/mm², \( p = 0.013 \)) areas, and the same was found on day 14 (infarcted 332 ± 35 vs 500 ± 44 cells/mm², \( p = 0.007 \); border 350 ± 47 vs 531 ± 46 cells/mm², \( p = 0.036 \), Fig. 6, A, B, D, and E). The infiltration of MHC class II⁺ cells was also suppressed in the noninfarcted area in the MI-G group on day 14 (61 ± 6 vs 105 ± 11 cells/mm², \( p = 0.022 \), Fig. 6, C and F), whereas GM-CSF inducer increased the infiltration of MHC class II⁺ cells on day 3 in both the infarcted (460 ± 39 vs 262 ± 40 cells/mm², \( p = 0.011 \)) and border (550 ± 33 vs 382 ± 49 cells/mm², \( p = 0.016 \)) areas. The same was true on day 7 (infarcted area 917 ± 37 vs 751 ± 39 cells/mm², \( p = 0.010 \); border area 912 ± 52 vs 718 ± 48 cells/mm², \( p = 0.013 \), Fig. 6, A, B, D, and E). There was no significant difference in DC infiltration into the noninfarcted area between the MI-C and MI-GM groups.

**HSP70, TLR2, and TLR4 expression**

Treatment with G-CSF decreased myocardial mRNA expression of TLR2 on day 7 (Fig. 7A), and of TLR4 on days 7 and 14 (Fig. 7B) in the infarcted area compared with that in the MI-C group. In the MI-GM group, TLR2 expression was increased in the infarcted area on days 3 and 7 (Fig. 7A) and TLR4 expression was increased in the infarcted area on days 7 and 14 (Fig. 7B) compared with that in MI-C animals.

Immunoblotting showed that the expression of HSP70 and TLR4 proteins was significantly increased in the infarcted area, peaking on day 7 after MI, similar to the time course of DC infiltration. The expression of HSP70 and TLR4 was lower in MI-G than in MI-C animals on day 7 (Fig. 8). HSP70 and TLR4 in the infarcted area were up-regulated in MI-GM compared with those in MI-C animals (Fig. 8). Expression of HSP70 and TLR4 in the infarcted area on days 3 and 14 did not differ among the groups.

**IFN-γ and IL-10 expression in infarcted myocardium**

To evaluate the expression of both Th1 and Th2 cytokines in the infarcted myocardium after MI, we investigated the mRNA expression of IFN-γ as a representative Th1 cytokine, and of IL-4 and IL-10 as Th2 cytokines. No IL-4 was detected in this study. The expression of IFN-γ was higher in MI-GM and lower in MI-G on day 7 than in MI-C animals (Fig. 9A). On the other hand, the expression of IL-10 was lower in MI-GM on day 7 and higher in MI-G on days 3 and 7 than in MI-C group (Fig. 9B). Neither IFN-γ nor IL-10 was detected in the myocardium of sham-operated rats and MI rats on day 14.
Operated rats or MI rats 14 days after surgery. Values are means ± SEM.

A

B

FIGURE 9. mRNA expression of IFN-γ (A) and IL-10 (B) on days 3 and 7 in infarcted myocardium by RT-PCR (n = 5/group). Ratios of mRNA expression in MI-G and MI-GM groups are shown relative to the MI-C group. IFN-γ and IL-10 were not detected in myocardium of sham-operated rats or MI rats 14 days after surgery. Values are means ± SEM. *p < 0.05 vs. MI-C.

Discussion

We demonstrated activated innate immune responses, including infiltration of OX62+ DC with a proliferative response, up-regulation of HSP70 and TLRs, and increased IFN-γ and decreased IL-10 expression, in the infarcted myocardium of the rat. Our findings also revealed that MI induced proliferation and maturation of DC in the spleen. Induction of GM-CSF by romurtide increased infiltration of DC into the necrotic myocardium and adversely affected LV remodeling, whereas G-CSF administration decreased DC infiltration and improved LV function. Immunoblotting revealed that GM-CSF induction increased, but G-CSF decreased, the expression of HSP70 and TLR4 in the infarcted myocardium. Moreover, mRNA expression of TLR2, TLR4, and IFN-γ in the infarcted area was increased in MI-GM, but decreased in MI-G animals, and IL-10 was decreased in MI-GM and increased in MI-G animals. These findings suggest that CSFs regulate innate immune responses, especially DC mobilization, thereby affecting LV remodeling after MI.

Previously, Zhang et al. reported a marked increase in cells with dendrites in the infarcted and border myocardium in rat experimental MI. These investigators suggested the presence of DC by immunohistochemical staining using OX6 Ab (30). However, OX6 Ab recognizes the MHC class II molecule, which is expressed on several types of APCs as well as DC. The presence of DC in the infarcted heart has not been confirmed since their report. In the present study, using OX62, OX6, and V65 Abs and BrdU staining, we clearly demonstrated that DC infiltrate the infarcted heart in association with a proliferative response. Additionally, we confirmed that MHC class II+ cells were markedly increased in the infarcted and noninfarcted myocardium after MI. Most MHC class II+ cells were OX62+, ED-1+, and ED-2+ cells, suggesting that most of these cells were recruited infiltrating macrophages (31, 32). However, it is possible that some of MHC class II OX62+ cells are also DC, because OX62 is not expressed on all DC (33). Abbate et al. described widespread myocardial inflammation in the peri-infarct and remote regions after recent MI (1). Our findings also indicated the existence of an inflammatory reaction in both infarcted and remote areas of the myocardium. Besides Ag-presenting function, cytotoxic activity has been reported in a subset of DC (CD4+ subsets of OX62+ DC) in the lymph nodes (34) and spleen (35). We observed both CD4+OX62+ and CD4−OX62+ DC in the infarcted myocardium. It is possible that such subsets of DC also play some role in myocardial injury after MI.

DC play critical roles in initiating and modulating immune responses and are characterized by a high capability for Ag capture and processing, migration to lymphoid organs, and expression of various costimulatory molecules. Several recent investigations revealed that endogenous toxins such as molecules released from necrotic cells can activate DC (20, 36). The heart possesses a gene-encoded innate stress response that is activated by different types of injury such as ischemia (37). Varda-Bloom et al. have shown that lymphocytes obtained from the spleen of rats that have suffered MI can injure normal cardiomyocytes (38), and Maisel et al. reported that heart failure was induced by adoptive transfer of splenic lymphocytes from rats after MI (11). Therefore, autoimmune responses against myocardial Ags may be a novel mechanism of postinfarction LV remodeling (13). Although there are few papers examining DC in the heart, a previous study using a rat permanent cerebral artery occlusion model showed that the grade of DC infiltration correlated with the extent of infarction (39). Another study using ischemia/reperfusion of rat kidney revealed infiltration and maturation of DC in infarcted renal tissue (40). Therefore, DC may play some role in secondary injury after tissue necrosis.

DC mature in lymphoid organs such as the lymph nodes and spleen after capturing Ag in peripheral tissues. Maturation of DC is essential for efficient T cell differentiation and is associated with high surface expression of MHC class II and costimulatory molecules. We observed proliferation and maturation of splenic DC after MI, indicating that DC induced intense activation of the innate immune system. The autoimmune response is regarded as one of the mechanisms of unnecessary inflammatory reactions induced secondary to myocardial injury (11–13). Our findings suggest that DC, which are potent regulators of the immune system, have a significant role in the excessive inflammatory response after MI. Moreover, the appearance of OX62+ cells was not altered in peripheral blood after MI, despite dynamic movement of OX62+ DC in the infarcted myocardium. These findings indicate that local expansion of DC occurred in the myocardium after MI.

Considerable evidence indicates that HSPs, especially HSP70, are potent activators of the innate immune system, which can induce production of proinflammatory cytokines by the monocyte-macrophage system and activation and maturation of DC via the TLR2 and TLR4 signal-transduction pathways (41–43). Previous reports also have shown that HSP released from necrotic cells after...
tissue injury activates and matures DC through TLR stimulation (19, 20). In fact, Dybdahl et al. revealed that HSP70 is increased in the serum of MI patients and correlates with the extent of myocardial damage (44). Thus, HSP may serve as a danger signal to the innate immune system at the site of ischemic injury. Frantz et al. reported that TLR4 expression is up-regulated in failing myocardium (45) and that TLR4 plays an important role in the inflammatory response after MI (46, 47). TLR2 also is reported to contribute to ventricular remodeling (48). We found that the expression of HSP70, TLR2, and TLR4 was up-regulated after MI, peaking on day 7, similar to the time course of DC infiltration. We proposed that the increased expression of HSP70 and TLRs triggers activation of DC after MI.

DC are divided into major two subsets, myeloid and plasmacytoid. Several investigators demonstrated that GM-CSF increased myeloid DC, which induce Th1 cell differentiation, and G-CSF increased plasmacytoid DC, which induce Th2 cell differentiation (24, 25). OX62 is known to be a marker of myeloid, but not plasmacytoid, DC (33). In the present study, we revealed that G-CSF decreased and GM-CSF inducer increased the number of OX62+ DC and the expression of IFN-γ in the infarcted myocardium compared with control tissue. These findings suggest that G-CSF could suppress differentiation to myeloid DC (OX62+), with a decreased Th1/Th2 ratio, whereas GM-CSF facilitates differentiation to myeloid DC, with an increased Th1/Th2 ratio. A Th1/Th2 functional imbalance influences various allergic and autoimmune diseases. For example, acute coronary syndrome is associated with a Th1/Th2 balance tending to Th1 cell dominance in the myocardium (21) and peripheral blood lymphocytes (49, 50). It was reported that statins inhibited secretion of Th1 cytokines and induced secretion of Th2 cytokines (51). Statin treatment for patients with MI also resulted in decreased Th1/Th2 ratio in peripheral T cells evaluated by flow cytometry (52, 53). Moreover, Th1-polarized mice are characterized by tissue damage, which is caused by proinflammatory responses and suppression of collagen synthesis, including matrix metalloproteinase (MMP) activation and tissue inhibitor of MMP (TIMP) deactivation (54–56). In contrast, Th2 responses direct wound healing and fibrosis, which accounts for antiinflammatory responses and enhanced collagen deposition (54–56). These findings suggest that Th1/Th2 balance may disturb reparative fibrosis and facilitate infarct expansion. Therefore, the changes in DC subtypes after alteration of the Th1/Th2 balance by CSFs may influence the healing process and ventricular remodeling after MI.

This paper has several limitations. First, numerous studies have revealed that G-CSF improves ventricular remodeling and cardiac function after MI by various mechanisms in mice and rats (22, 57–59). Although previous investigators postulated that G-CSF induces regeneration, angiogenesis, and an antiapoptotic response (58–61), we did not examine such effects in the present study. Instead, we focused on its immunomodulatory effects, especially the DC-mediated innate immune response. The dose of G-CSF used was a tenth lower than that in previously described studies using a murine MI model to mobilize bone marrow stem cells into the peripheral circulation. Moreover, accumulating evidence has questioned whether G-CSF induces transdifferentiation from bone marrow progenitor cells to cardiomyocytes (62–65). Second, we could not exclude direct effects of CSFs on cardiomyocytes. Harada et al. reported G-CSF receptor expression on both adult mouse heart and cultured neonatal murine cardiomyocytes (58). The GM-CSF receptor is also reported to be expressed on human cardiomyocytes (66). Additionally, GM-CSF induction in cancer patients aggravated LV dysfunction (67). Their findings suggest that G-CSF and GM-CSF directly affect LV function. Alternatively, CSFs might affect the postinfarction healing process by modulation of other cytokines, although our previous study showed that the mRNA expression of IL-6 and TNF-α was not altered by G-CSF treatment. Third, the ratio of DC subsets in the rat spleen depends on strain (68) and OX62 is not expressed in all rat DC (33). The precise roles of all the DC subsets during LV remodeling are still undetermined. Fourth, CD45+ and CD68+ cells were also observed in the infarcted myocardium. We could not clarify which cell mostly operates in the healing process in this study. Further study will be required to clarify the specific role of DC during LV remodeling after MI.

In conclusion, experimental MI activates innate immunity, such as DC infiltration, HSP70 and TLR4 expression, and elevation of IFN-γ and reduction of IL-10 expression in the infarcted heart. G-CSF improves postinfarction LV remodeling in association with an attenuated innate immune reaction, whereas GM-CSF exacerbates LV remodeling with enhanced innate immunity. Suppression of DC-mediated immunity could be a new strategy to treat LV remodeling after MI.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Abbate, A., E. Bonanno, A. Maurillo, R. Bussani, G. G. Biondi-Zoccai, G. Luzzo, A. M. Leone, F. Silvestri, A. Dobrina, F. Baldi, et al. 2004. Wide-spread myocardial inflammation and infarct-related artery patency. *Circulation* 110: 46–50.
2. Nian, M., P. Lee, N. Kasper, and P. Liu. 2004. Inflammatory cytokines and postmyocardial infarction remodeling. *Circ. Res.* 94: 1543–1553.
3. Frangogiannis, N. G., G. C. Smith, and M. L. Entman. 2002. The inflammatory response in myocardial infarction. *Cardiovasc. Res.* 53: 31–47.
4. Anzai, T., T. Yoshikawa, H. Shiraki, Y. Asakura, M. Akaishi, H. Mitamura, and S. Ogawa. 1997. C-reactive protein as a predictor of infarct expansion and cardiac rupture after a first Q-wave acute myocardial infarction. *Circulation* 96: 778–784.
5. Takahashi, T., T. Anzai, T. Yoshikawa, Y. Maekawa, Y. Asakura, T. Satoh, H. Mitamura, and S. Ogawa. 2003. Serum C-reactive protein level in left ventricular remodeling after acute myocardial infarction: role of neurohormones and cytokines. *Int. J. Cardiol.* 88: 257–265.
6. Maekawa, Y., T. Anzai, T. Yoshikawa, Y. Asakura, T. Takahashi, S. Ishikawa, H. Mitamura, and S. Ogawa. 2002. Prognostic significance of peripheral mononuclear cytokines after reperfused acute myocardial infarction: a possible role for left ventricular remodeling. *J. Am. Coll. Cardiol.* 39: 241–246.
7. Roberts, R., V. DeMello, and B. E. Sobel. 1976. Deleterious effects of methylprednisolone in patients with myocardial infarction. *Circulation* 53 (Suppl. 3): 1204–1206.
8. Hammerman, H., R. A. Kloner, F. Hafe, F. J. Schoen, and E. Braunwald. 1983. Dose-dependent effects of short-term methylprednisolone on myocardial infarct extent, scar formation and ventricular function. *Circulation* 68: 446–453.
9. Brown, E. J., Jr., R. A. Kloner, F. J. Schoen, H. Hammerman, S. Hale, and E. Braunwald. 1983. Scar thinning due to ibuprofen administration after experimental myocardial infarction. *Am. J. Cardiol.* 51: 877–883.
10. Jugdutt, B. I. 1985. Delayed effects of early infarct-limiting therapies on healing after myocardial infarction. *Circulation* 72: 907–914.
11. Maisel, A., D. Cesarino, S. Baird, J. Rehman, P. Haghibi, and S. Carter. 1998. Experimental autoimmune myocarditis produced by adoptive transfer of splenocyte after myocardial infarction. *Circ. Res.* 82: 458–463.
12. Moraru, M., A. Roth, G. Keren, and J. George. 2006. Cellular autointImmunity to cardiac myosin in patients with recent myocardial infarction. *Int. J. Cardiol.* 107: 61–66.
13. Liao, Y. H., and X. Cheng. 2006. Autoimmunity in myocardial infarction. *Int. J. Cardiol.* 112: 21–26.
14. De Scheerder, I., J. Vandekerckhove, J. Robbrecht, L. Algoord, M. De Buyzere, J. De Langhe, G. De Schrijver, and D. Clement. 1985. Post-cardiac injury syndrome and an increased humoral immune response against the major contractile proteins (actin and myosin). *Am. J. Cardiol.* 56: 631–633.
15. Eriksson, S., J. Hellman, and K. Pettersson. 2005. Autoantibodies against cardiac troponins. *N. Engl. J. Med.* 353: 98–100.
16. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106: 255–258.
17. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenecity. *Annu. Rev. Immunol.* 9: 271–296.
28. Saiki, T., T. Ezaki, M. Ogawa, and K. Matsuno. 2001. Trafficking of host- and 
27. Brenan, M., and M. Puklavec. 1992. The MRC OX-62 antigen: a useful marker 
26. Shaughnessy, P. J., C. Bachier, C. F. Lemaistre, C. Akay, B. H. Pollock, and 
41. Asea, A., S. K. Kraeft, E. A. Kurt-Jones, M. A. Stevenson, L. B. Chen, 
42. Vabulas, R. M., P. Ahmad-Nejad, S. Ghose, C. J. Kirschning, R. D. Issels, and 
43. Wallin, R. P., A. Lundqvist, S. H. More, A. von Bonin, R. Kiessling, and 
25. Arpinati, M., C. L. Green, S. Heimfeld, J. E. Heuser, and C. Anasetti. 2000. 
23. Maekawa, Y., T. Anzai, T. Yoshikawa, Y. Sugano, K. Mahara, T. Kohno, 
22. Sugano, Y., T. Anzai, T. Yoshikawa, Y. Maekawa, T. Kohno, K. Mahara, 
21. Cheng, X., Y. H. Liao, H. Ge, B. Li, J. Zhang, J. Yuan, M. Wang, Y. Liu, Z. Guo, 
19. Millar, D. G., K. M. Garza, B. Odermatt, A. R. Elford, N. Ono, Z. Li, and 
18. Arpinati, M., L. Kobzik, Y. D. Kim, R. Fukazawa, R. Medzhitov, R. T. Lee, and 
17. Frantz, S., L. Kobzik, Y. D. Kim, R. Fukazawa, R. Medzhitov, R. T. Lee, and 
16. Sandler, N. G., M. M. Mentink-Kane, A. W. Cheever, and T. A. Wynn. 2003. 
15. Shimada, K., K. Miyaiyama, and H. Huida. 2004. Early intervention with atorva-
14. Millar, D. G., K. M. Garza, B. Odermatt, A. R. Elford, N. Ono, Z. Li, and 
13. Frantz, S., L. Kobzik, Y. D. Kim, R. Fukazawa, R. Medzhitov, R. T. Lee, and 
12. Varda-Bloom, N., J. Leor, D. G. Ohad, Y. Hasin, M. Amar, R. Fixler, A. Battler, 
11. Kostulas, N., H. L. Li, G. G. MacPherson. 1997. Dendritic cell 
10. Fox, C. B., C. Voisin, C. A. H. A. Stassen, J. D. W. F. E. Hoogerbrugge, P. J. Kamp, 
9. Millar, D. G., K. M. Garza, B. Odermatt, A. R. Elford, N. Ono, Z. Li, and 
8. Arpinati, M., L. Kobzik, Y. D. Kim, R. Fukazawa, R. Medzhitov, R. T. Lee, and 
7. Kilian, M., K. Naito, and S. Ogawa. 2005. Granulocyte colony-stimulating factor 
6. Youssef, S., O. Stuve, J. C. Patraro, P. J. Ruiz, J. L. Radosovich, E. M. Hur, 
5. Cheng, X., Y. H. Liao, H. Ge, B. Li, J. Zhang, J. Yuan, M. Wang, Y. Liu, Z. Guo, 
4. Asea, A., S. K. Kraeft, E. A. Kurt-Jones, M. A. Stevenson, L. B. Chen, 
3. Frantz, S., L. Kobzik, Y. D. Kim, R. Fukazawa, R. Medzhitov, R. T. Lee, and 
2. Asea, A., S. K. Kraeft, E. A. Kurt-Jones, M. A. Stevenson, L. B. Chen, 
1. Arpinati, M., L. Kobzik, Y. D. Kim, R. Fukazawa, R. Medzhitov, R. T. Lee, and 

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