Deletion of CD28 Co-stimulatory Signals Exacerbates Left Ventricular Remodeling and Increases Cardiac Rupture After Myocardial Infarction

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Background: Inflammatory responses, especially by CD4+ T cells activated by dendritic cells, are known to be important in the pathophysiology of cardiac repair after myocardial infarction (MI). Although co-stimulatory signals through B7 (CD80/86) and CD28 are necessary for CD4+ T cell activation and survival, the roles of these signals in cardiac repair after MI are still unclear.

Methods and Results: C57BL/6 (Control) mice and CD28 knockout (CD28KO) mice were subjected to left coronary artery permanent ligation. The ratio of death by cardiac rupture within 5 days after MI was significantly higher in CD28KO mice compared with Control mice. Although there were no significant differences in the infarct size between the 2 groups, left ventricular end-diastolic and end-systolic diameters were significantly increased, and fractional shortening was significantly decreased in CD28KO mice compared with Control mice. Electron microscopic observation revealed that the extent of extracellular collagen fiber was significantly decreased in CD28KO mice compared with Control mice. The number of α-smooth muscle actin-positive myofibroblasts was significantly decreased, and matrix metalloproteinase-9 activity and the mRNA expression of interleukin-1β were significantly increased in CD28KO mice compared with Control mice.

Conclusions: Deletion of CD28 co-stimulatory signals exacerbates left ventricular remodeling and increases cardiac rupture after MI through prolongation of the inflammatory period and reduction of collagen fiber in the infarct scars. (Circ J 2016; 80: 1971–1979)

Key Words: Cardiac fibrosis; CD28; Heart failure; Inflammatory cell; Myocardial infarction

Heart failure is the major cause of death after myocardial infarction (MI). After MI, left ventricular remodeling, which includes dilatation of the left ventricle (LV) and an increase in interstitial fibrosis with histopathological changes in LV, is a key factor inducing cardiac pump dysfunction after MI with poor clinical outcomes. Cardiac rupture is another cause of fatal complications after MI. Although recent medical advances in pharmacological and interventional therapies, including emergent coronary artery reperfusion therapies, fortunately reduce LV remodeling and cardiac rupture, these complications are not fully prevented. Therefore, it is important to elucidate a novel approach to prevent LV remodeling and cardiac rupture after MI.

Recently, it has been reported that monocytes and lymphocytes infiltrated into ischemic heart tissue are major factors in the pathophysiology of tissue repair after MI. When the myocardium is damaged after MI, dendritic cells (DCs) are activated and become capable of productively activating cardiac myosin-specific naive T cells, leading to differentiation of CD4+ T cells. Anzai et al demonstrated that deletion of CD11c+ DCs impaired cardiac function and tissue repair. In contrast, Hofmann et al reported that CD4+ T cells were activated and facilitated wound healing of the injured myocardium after MI. They also demonstrated by using OT-II mice (C57BL/6-Tg(TcraTcrb)425Cbn/Crl) that T cell receptor (TCR) signaling is important to activate CD4+ T cells after MI. Consequently,

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antigen presentation from DCs to CD4+ T cells is important in the pathology of MI. Meanwhile, regulatory T cells (Tregs), which are a subpopulation of T cells and suppress many types of immune cells, also have beneficial effects on wound healing and immune response. Therefore, the role of the CD28 molecule in tissue repair after MI is of interest.

The CD28 molecule, which is the protein expressed on the cell membrane of T cells, is the receptor of B7 (CD80 [B7.1] and CD86 [B7.2]) and provides co-stimulatory signals required for T cell activation and survival. To activate T cells, it is necessary to stimulate simultaneously both TCR and co-stimulatory signals from antigen presenting cells (APCs) such as DCs. In contrast, the CD28 molecule is involved in the development and maintenance of Tregs. Tai et al reported that CD28 co-stimulatory signals directly activate thymocytes to express Foxp3 and to initiate a Tregs differentiation program. However, the role of the CD28 molecule is unclear in the pathophysiology of tissue repair after MI.

Therefore, the purpose of the present study was to elucidate the role of the CD28 molecule in tissue repair and LV remodeling after MI. In this study, we used CD28 knockout (CD28KO) mice to demonstrate the effects of the lack of the CD28 molecule on LV remodeling and cardiac rupture after MI in mice.

Methods

Animals

Wild-type male C57BL/6 mice (8-weeks-old) were purchased from Japan SLC (Shizuoka, Japan) and used in the present study. CD28KO mice, whose construction and characterization has been described previously, were kindly provided by Ryo Abe (Research Institute for Biological Sciences, Science University of Tokyo, Chiba, Japan). These mice were bred and kept under specific pathogen-free conditions. All experimental procedures were performed according to the guidelines established by Chiba University for experiments with animals, which conform to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

Induction of MI

Mice were anesthetized by inhalation of isoflurane (2.5%) and artificially ventilated with a respirator. Mice were subjected to ligation of the left anterior descending coronary artery (LAD) or to a sham operation, as described previously.9,10 The heart was exposed through a thoracotomy, and LAD was permanently ligated with an 8-0 prolene suture to stimulate MI. A sham operation was performed by cutting only the pericardium. After examination by transthoracic echocardiography, the mice were sacrificed by cervical dislocation and the hearts were extirpated.

Echocardiography

Transthoracic echocardiography was performed on 3 and 5 days after MI with a VisualSonics High-Resolution In Vivo Micro-Imaging System (Vevo 770; FujiFilm VisualSonics, Inc, Seattle, WA, USA) equipped with a 30-MHz imaging transducer. Mice were anesthetized by inhalation of isoflurane (0.5–1.0%) during the echocardiographic examination, and the heart rate was approximately 400–550 beats/min in both group mice. Measurements were performed by the same operator, blinded to the experimental groups, before and after MI in each mouse.

Histological Analysis

Mice hearts fixed in 10% formalin overnight were embedded in paraffin and sectioned at 5-µm thickness for Masson’s trichrome staining and picrosirius red staining to determine infarct size and cardiac fibrosis. Immunohistochemical staining was performed with anti α-smooth muscle actin (SMA) antibody (Dako, Glostrup, Denmark).

Gelatin Zymography

To assess the activity of gelatinase, matrix metalloproteinase (MMP)-2 and MMP-9, gelatin zymography was performed, as described previously.4,11,12 The gels were stained with Quick-Coomassie Brilliant Blue (Wako Pure Chemical Industries, Inc, Osaka, Japan). The presence of different MMPs was identified on the basis of their molecular weights. Finally, the gellatinolytic bands were measured with ChemiDoc MP (Bio-Rad, Bio-Rad, 1972, KUBOTA A et al.)

Table 1. Primers for Real-Time Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>(F) CCCTCACACTCAGATCTTTCT</td>
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<tr>
<td></td>
<td>(R) GCTAGACCGGGTCAGG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>(F) GCAACTTCTGAGAATCTACT</td>
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<tr>
<td></td>
<td>(R) ATCTTGGGCTGCCATCA</td>
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<tr>
<td>IFN-γ</td>
<td>(F) ATGAAAGCTAAGACTGCACT</td>
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<td></td>
<td>(R) CATCCTTTGAGCGTTCCCTC</td>
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<tr>
<td>TGF-β</td>
<td>(F) CTCCGGTGCTTTGATAGC</td>
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<td></td>
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<td>(R) GTGAGGGGTGATGTCAC</td>
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<td>Arginase-1</td>
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<td></td>
<td>(R) AGGACGGTCTAGGAGACATC</td>
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<td>Foxp3</td>
<td>(F) CCCATCCCAAGAGTTGCTTG</td>
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<td></td>
<td>(R) ACCATGCTAGGGGACCTGA</td>
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<td>procollagen α-1 (I)</td>
<td>(F) GCTCTCTTAGGAGGCCACT</td>
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<tr>
<td></td>
<td>(R) CCACGTCTCACCATTGGGG</td>
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<td>procollagen α-1 (III)</td>
<td>(F) CTGTAAGTGGGAACCTGGAATAA</td>
</tr>
<tr>
<td></td>
<td>(R) CCATAAGCTGAACTGAAAAACCCACC</td>
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F, forward; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; R, reverse; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.
Role of CD28 in the Pathophysiology of MI

Circulation Journal Vol.80, September 2016

P. 1973

Hercules, CA, USA) and analyzed by Image J.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction
Mice hearts were incubated in RNA later solution (Invitrogen) overnight at 4°C to allow thorough penetration of the tissue, then transferred to −20°C for storage. Then, samples were thawed at room temperature, and RNA was extracted using ISOGEN II (NIPPON GENE, Tokyo, Japan). cDNA was synthesized from 1 μg RNA with an iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative real-time reverse transcriptase-polymerase chain reaction (PCR) was performed by using Step One Plus™ with Fast SYBR® Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The sequences of primer pairs are described in Table 1.

Cell Isolation
At 5 days after MI, the mice were sacrificed by cervical dislocation and intracardially perfused with 25 ml of ice-cold phosphate-buffered saline to wash out blood cells. Whole hearts were soaked in 2.5 ml of RPMI 1640-medium (Thermo Scientific) containing 0.5 mg of Liberase TM (Roche, Basel, Switzerland) and 0.25 mg of DNase I (Roche) and enzymatically digested by using a gentleMACSTM Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). After digestion, the tissue was passed through a 50-μm cell strainer. Leukocyte-enriched fractions were isolated by density gradient centrifugation using Lympholyte (CEDARLANE, Burlington, NC, USA). Cells were removed from the interface and washed with RPMI 1640-medium for further analysis.

Flow Cytometric Analysis
Cells isolated from leukocyte-enriched fractions were incubated by using a Zombie Aqua™ Fixable Viability Kit (Biolegend, San Diego, CA, USA) at room temperature for 15 min, and then anti-CD16/32 antibody (Abcam, United Kingdom) was added at room temperature for 15 min to block Fc receptors. After staining with a mixture of antibodies (Table 2) at 4°C for 20 min, cells were analysed with flow cytometric analysis (Canto II; BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Intracellular Cytokine Analysis
Collected cells from the mice hearts were stimulated with phorbol 12-myristate 13-acetate (PMA) (Merck Millipore, Darmstadt, Germany), ionomycin (Merck Millipore) and BD golgiplug (BD Biosciences) for 1 h. After the surface staining, cells were fixed and permeablized with Perm/Wash buffer (eBiosciences, Santa Clara, CA, USA), and then incubated with each antibody (Table 2) at 4°C for 30 min.

Statistical Analysis
All data are shown as mean±SEM. An unpaired Student’s t-test was used to detect significant differences when 2 groups were compared. The parameters of echocardiography were analyzed for differences by 2-way ANOVA with repeated measures, followed by the Bonferroni post-hoc test to assess individual differences. Values of P<0.05 were considered statistically significant.

Results
Deletion of CD28 Impairs Survival Rate and Cardiac Function
To identify the effects of CD28 deletion after MI, we assessed cumulative survival, cardiac function, and infarct size using CD28KO mice. The survival rate at 5 days after MI was significantly lower in CD28KO mice than in Control mice (Control: 86.21%, CD28KO: 69.84%, P<0.05) (Figure 1A). The rate of cardiac rupture was higher in CD28KO mice than in Control mice (Control: 10.34%, CD28KO: 30.16%, P<0.01) (Figures 1B,C). There were no significant differences in physiological parameters, such as body weight and heart rate, and echocardiographic parameters between Control mice and CD28KO mice before MI (Table 3). At 5 days after MI, LV end-diastolic diameter and LV end-systolic diameter were significantly increased, and the percentage fractional shortening (FS) was significantly decreased in CD28KO mice compared with Control mice. We performed Masson trichrome staining by using heart tissue at 5 days after MI to evaluate infarct size. There were no significant differences in infarct size between Control mice and CD28KO mice (Control: 61.59±3.37%, CD28KO: 64.01±3.13%; NS) (Figure 1D). These results suggest that the deletion of CD28 induces LV dilatation and increases cardiac rupture death independent of

Figure 1. Effects of CD28 deletion on survival rate and infarct size. (A) Kaplan-Meier survival analysis in Control mice (n=58) and CD28 knockout (CD28KO) mice (n=63). (B) Representative macroscopic and microscopic photographs of myocardial infarction (MI) with left ventricular (LV) myocardial rupture in CD28KO mice on day 3 after MI. (C) Analysis of cardiac rupture rate as determined by using the log-rank test. (D) Representative Masson trichrome staining of heart tissue in Control mice and CD28KO mice at 5 days after MI. Quantitative analysis of infarct size at 5 days after MI. *P<0.05 as determined by the 2-tailed Student’s t-test (n=8 per group). Scale bar=1 mm.
Figure 2. Effects of CD28 deletion on cardiac fibrosis after myocardial infarction (MI). (A) Representative picrosirius red staining of the infarct area in Control mice and CD28 knockout (CD28KO) mice at 5 days after MI. Quantitative analysis of the fibrotic area in the infarct area at 5 days after MI. *P<0.05 as determined by the 2-tailed Student’s t-test (n=5 per group). Scale bar=100 μm. (B, C) Representative electron micrographs of the fibrotic area in Control mice and CD28KO mice at 5 days after MI. (D, E) Higher magnification of the boxed area in B, C. Scale bar=1 μm. *Collagen fibrils. (F-I) Representative picrosirius red staining of the infarct area in Control mice (F) and CD28KO mice (G), and polarized microscopic view of a corresponding picrosirius red-stained section (H, I). Scale bar=50 μm.

Table 3. Echocardiographic Data

<table>
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<tr>
<th></th>
<th>Control</th>
<th>CD28KO</th>
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<tbody>
<tr>
<td></td>
<td>Pre-operation 3 days 5 days</td>
<td>Pre-operation 3 days 5 days</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>479±12</td>
<td>419±16*</td>
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<tr>
<td></td>
<td>428±15</td>
<td>441±12*</td>
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<tr>
<td></td>
<td>419±16*</td>
<td>441±12*</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.41±0.01</td>
<td>3.40±0.01</td>
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<td>4.51±0.05*</td>
<td>4.64±0.06*</td>
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<tr>
<td></td>
<td>5.29±0.08*</td>
<td>6.04±0.20*</td>
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<td>LVESD (mm)</td>
<td>1.86±0.01</td>
<td>1.84±0.01</td>
</tr>
<tr>
<td></td>
<td>3.92±0.05*</td>
<td>4.07±0.06*</td>
</tr>
<tr>
<td></td>
<td>4.77±0.08*</td>
<td>5.60±0.20*</td>
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<tr>
<td>FS (%)</td>
<td>45.55±0.28</td>
<td>45.79±0.28</td>
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<tr>
<td></td>
<td>13.09±0.35*</td>
<td>12.16±0.55*</td>
</tr>
<tr>
<td></td>
<td>9.87±0.44*</td>
<td>7.32±0.37*</td>
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<tr>
<td>AWT (mm)</td>
<td>0.86±0.01</td>
<td>0.86±0.01</td>
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<tr>
<td></td>
<td>0.65±0.01</td>
<td>0.66±0.02*</td>
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<tr>
<td></td>
<td>0.58±0.01*</td>
<td>0.53±0.02*</td>
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<tr>
<td>PWT (mm)</td>
<td>0.86±0.01</td>
<td>0.87±0.01</td>
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<tr>
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<td>0.71±0.03*</td>
<td>0.76±0.02*</td>
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<td></td>
<td>0.67±0.03*</td>
<td>0.60±0.02*</td>
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</tbody>
</table>

*P<0.05 vs. corresponding Pre-operation group. †P<0.05 vs. Control group mice at 5 days after MI. AWT, anterior wall thickness; FS, fractional shortening; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; MI, myocardial infarction; PWT, posterior wall thickness.

Figure 3. Effects of CD28 deletion of collagen fiber production and degradation. (A) Representative photograph of immunohistochemical staining for α-smooth muscle actin (α-SMA; brown) in the infarcted heart of Control mice and CD28 knockout (CD28KO) mice at 5 days after myocardial infarction (MI). Quantitative analysis of the number of α-SMA-positive myofibroblasts in the infarct area in Control and CD28KO mice. **P<0.01 as determined by the 2-tailed Student’s t-test (n=5 per group). Scale bar=50 μm. (B) Representative photograph of a zymographic gel demonstrating the time-course of matrix metalloproteinase (MMP)-2 and MMP-9 activities in the infarcted heart in Control mice and CD28KO mice. Quantitative analysis of MMP-9 and MMP-2 activities in the infarcted heart. **P<0.01 vs. corresponding sham group. ††P<0.01 vs. Control group mice at 5 days after MI as determined by 2-way ANOVA followed by the Bonferroni post-hoc test (n=4 per group).
Role of CD28 in the Pathophysiology of MI

These results suggest that there was a delay of the wound healing in the myocardium of CD28KO mice compared with Control mice.

Deletion of CD28 Abolishes Scar Formation After MI

We suspected that the composition of the infarct area may be involved in the increases of LV dilatation and cardiac rupture death in CD28KO mice after MI. To confirm our hypothesis, we performed picrosirius red staining to assess collagen composition in the infarct area. At 5 days after MI, the extent of extracellular collagen in the infarct area was significantly decreased in CD28KO mice compared with Control mice (Control: 29.42±1.84%, CD28KO: 20.34±2.05%, P<0.05) (Figure 2A). There were no significant differences in cardiomyocyte size and myocardial fibrosis in the non-ischemic area between Control mice and CD28KO mice. Electron microscopic analysis demonstrated that there were few collagen fibrils in the myocardium of CD28KO mice compared with Control mice (Figures 2B–E). Furthermore, picrosirius red polarized microscopy of collagen fibers in the infarct area showed that yellow fibers, which are composed by collagen type I, the amount of yellow fibers low, and green fiber, which are composed by collagen type III, tended to be high in CD28KO mice compared with Control mice (Figures 2F–I).

Deletion of CD28 Reduces the Production of Collagen Fibers and Accelerates the Degradation of Collagen Fibers

To investigate the reduction of collagen fibers in the infarct area in CD28KO mice, we evaluated the mRNA expression of procollagen α-1 (I) and procollagen α-1 (III). However, at 5 days after MI, there were no significant differences in the mRNA expression of procollagen α-1 (I) and procollagen α-1 (III) between Control mice and CD28KO mice (procollagen α-1 (I)/HPRT: Control: 1.51±0.19; CD28KO: 2.09±0.30; procollagen α-1 (III)/HPRT: Control: 29.58±4.34; CD28KO: 35.21±2.99). Thus, we also performed immunohistochemical staining and assessed the number of α-SMA-positive spindle-shaped myofibroblasts in the infarct area. At 5 days after MI as determined by 2-way ANOVA followed by the Bonferroni post-hoc test (n=8 per group).

Figure 4. Quantitative analysis of gene expression in the infarct area. Time-course of mRNA expression of tumor necrosis factor (TNF)-α (A), interleukin (IL)-1β (B), interferon (IFN)-γ (C), IL-10 (D), and transforming growth factor (TGF)-β (E) in the infarct area of Control mice and CD28KO mice. *P<0.05 as determined by the 2-tailed Student’s t-test (n=8 per group). Quantitative analysis of mRNA expression of inducible nitric oxide synthase (iNOS) (F), Arginase-1 (G), and Foxp3 (H) in the infarct area at 5 days after myocardial infarction (MI). *P<0.05, **P<0.01 vs. the corresponding sham group. †P<0.05, ††P<0.01 vs. the Control group mice at 3 or 5 days after MI as determined by 2-way ANOVA followed by the Bonferroni post-hoc test (n=8 per group).
Deletion of CD28 Enhances Inflammatory Cytokines

To determine the involvement of inflammation, we evaluated the mRNA expressions of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, interferon (IFN)-γ, IL-10, and transforming growth factor (TGF)-β. There were no significant differences in the mRNA expressions of IL-10 and TGF-β, which in the scar area of CD28KO mice after MI.

MMP-2 and MMP-9 in the hearts after MI. There were no significant differences in the activities of MMP-2 and MMP-9 between Control mice and CD28KO mice at 3 days after MI. But at 5 days after MI, MMP-9 activity was significantly higher in CD28KO mice compared with Control mice (Control: 22.97±7.98; CD28KO: 99.41±17.05, P<0.01) (Figure 3B). These results suggest that the degradation of collagen fiber by MMP-9 is the main reason for the reduction of collagen fiber in the scar area of CD28KO mice after MI.

**Deletion of CD28 Enhances Inflammatory Cytokines**
are associated with suppressing inflammation and wound healing, between Control mice and CD28KO mice at 5 days after MI. But the mRNA expressions of TNF-α, IL-1β, and IFN-γ, which are pro-inflammatory cytokines, were significantly higher in CD28KO mice than in Control mice at 5 days after MI (Figures 4A-E). These results suggest that deletion of CD28 extends the inflammatory period after MI and delays the wound healing in the infarct area.

Deletion of CD28 Extends Inflammatory Cell Infiltration

Next, we determined immune cell infiltration in the myocardium at 5 days after MI by flow-cytometric analysis. There were no significant differences in the total number of infiltrating CD45+ immune cells between Control mice and CD28KO mice. However, the percentage of infiltrating Ly6C+ neutrophils in CD11b+ myeloid cells was significantly higher in CD28KO mice than in Control mice (Control: 10.77±3.30%; CD28KO: 27.80±5.71%; P<0.05) (Figures 5A,B). Furthermore, we focused on CD11b+Ly6G-CD64+ myeloid-epithelial-reproductive tyrosine kinase (MerTK)+ macrophage subsets. The percentage of Ly6C+CD16+M1 macrophages (classically activated macrophages) from the total macrophages was significantly higher (Control: 7.30±1.91%; CD28KO: 16.62±1.43%; P<0.05), and the percentage of Ly6C+CD16+ M2 macrophages (alternatively activated macrophages) from the total macrophages was significantly lower in CD28KO mice than in Control mice (Control: 92.70±1.91%; CD28KO: 83.38±1.43%; P<0.05) (Figures 5A,B). The mRNA expression of inducible nitric oxide synthase (iNOS) was significantly higher in CD28KO mice than in Control mice, but there were no significant differences in the mRNA expression of arginase-1 between the 2 groups (Figures 4F,G). These results suggest that deletion of CD28 extends the persistence of proinflammatory immune cells in the infarct area.

To further investigate the mechanisms of the prolongation of the inflammatory period in the infarct area after MI in CD28KO mice, we analyzed lymphocytes infiltrated into the infarct area. Although there were no significant differences in the percentages of infiltrating CD4+ T cells, CD8+ T cells, γδT cells, and B cells between Control mice and CD28KO mice (Figure 5C), the percentage of Foxp3+ Tregs in total CD4+ T cells was significantly lower in CD28KO mice than in Control mice (Figure 5D). The mRNA expression of Foxp3 was also lower in CD28KO mice than in Control mice (Figure 4H). These results suggest that insufficient recruitment of Tregs in the infarct area may induce prolongation of the inflammatory period and delay the wound healing process.

Discussion

In the present study, we confirmed that the deletion of CD28 impaired cardiac function and survival rate, and cause the progression of LV remodeling and cardiac rupture after MI. Deletion of CD28 decreased the number of α-SMA positive myofibroblasts and increased the activation of MMP-9, leading to a decrease in the extent of myocardial fibrosis in the infarct area by degradation of collagen fiber. Deletion of CD28 extended the inflammatory period and delayed the time-course of the wound healing process in the infarct area.

A recent study suggested that there is a relationship between the progression of LV remodeling and the wound healing process of infarct heart tissue in the mice MI model.13 Three phases of the wound healing process (inflammatory phase, reparative phase and maturation phase) are critical following MI.14 In the inflammatory phase, M1 macrophages clear dead myocyte debris through phagocytosis and proteolysis, and secrete inflammatory cytokines including IL-1β, IL-6 and TNF-α, as well as proteases including MMP-13,16 In the reparative phase, M2 macrophages secrete the anti-inflammatory cytokines, IL-10 and TGF-β1, which in turn recruit and activate reparative myofibroblasts. Myofibroblasts secrete large amounts of extracellular matrix (ECM) in order to replace lost ventricular tissue with a stable scar.17,18 The maturation phase is marked by apoptosis of the majority of inflammatory and reparative cells, scar maturation and remodeling. During the replacement process of necrotic tissue to collagen fibers, both MMP-2 and MMP-9 play an important role in the rearrangement of collagen fibers; myofibroblasts also produce collagen fibers.12,17-20 It has been reported that MMP-9 is produced by M1 macrophages.21 The activity of MMP-9 is increased at 3 days after MI and attenuated at 7 days after MI, while MMP-2 was increased during 3 days to 7 days after MI.4,12,22 In our experiments, we confirmed a significant increase in the activity of MMP-9, with the increases in the M1 macrophage in CD28KO mice compared with Control mice at 5 days after MI. Therefore, we think that the increased in number M1 macrophage during the infarcted area in production of collagen fibers by myofibroblasts in the infarcted area.17,18 The mRNA expression of TGF-β was higher in CD28KO mice compared with Control mice at 3 days after MI in our experiments. Some reports revealed that IL-1β inhibits the proliferation and differentiation of cardiac fibroblasts to myofibroblasts in the healing heart after MI, independent of TGF-β.23,24 We confirmed the significant increases of the mRNA expression of IL-1β in CD28KO mice compared with Control mice. These results suggest that both the decrease of collagen production by myofibroblasts and degradation of collagen fiber by MMP-9 are the main reasons for the reduction of collagen fiber in the scar area of CD28KO mice after MI. The expression levels of IL-10 in the myocardium were increased at 3 and 5 days after MI in both Control mice and CD28KO mice in the present study. A previous paper also reported that IL-10 in the myocardium is increased at 7 day after MI.1 Because there were no significant differences in the expression levels of IL-10 between Control mice and CD28KO at 3 and 5 days after MI, we believe that IL-10 is not involved in cardiac repair in CD28KO mice. Another paper reported that the level of IL-10 does not affect the repair of ischemic heart after MI using IL-10 KO mice.25 Both iNOS and Arginase-1 are known as the marker of M1 and M2 macrophages, respectively. We checked the expression levels of iNOS and Arginase-1 to confirm the M1 and M2 macrophage polarization, respectively. In fluorescence activated cell sorting (FACS) analysis, the M1 macrophage was increased and the M2 macrophage was decreased in the infarcted area after MI in CD28KO mice compared to Control mice (Figures 5A,B). Foxp3 is a transcriptional factor that is expressed specifically in Tregs and controls the differentiation of Tregs. We believe that the decrease in expression level of Foxp3 is associated with the decrease in number of Tregs in the infarcted area in CD28KO mice (Figure 5D). Although there were no significant histopathological changes in the non-infarcted area (data not shown), we evaluated the gene expression in the non-infarcted area in the heart. There were no significant differences in gene expression levels of TNF-α, IL-1β, IFN-γ, IL-10, TGF-β, iNOS, Arg-1, Foxp3, and Collagen I/III in the non-infarcted area at 5 days after MI (data not shown). Therefore, we believe that the protective response induced by CD28 does not occur in the non-ischemic area. By the prolongation of inflammation in the infarcted area in CD28KO mice, the increase in IL-1β released by the M1.
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### Conclusions

Deletion of CD28 exacerbates LV remodeling and increases cardiac rupture after MI by the prolongation of the inflammatory period and reduction of collagen fiber in the infarct hearts in a mice MI model.

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### Disclosures


### References


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