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Nanoparticle incorporating Toll-like receptor 4 inhibitor attenuates myocardial ischaemia–reperfusion injury by inhibiting monocyte-mediated inflammation in mice

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Aims Myocardial ischaemia–reperfusion (IR) injury hampers the therapeutic effect of revascularization in patients with acute myocardial infarction (AMI). Innate immunity for damage-associated protein patterns promotes the process of IR injury; however, the blockade of Toll-like receptor 4 (TLR4) in myocardial IR injury has not been translated into clinical practice. Therefore, we aimed to examine whether the nanoparticle-mediated administration of TAK-242, a chemical inhibitor of TLR4, attenuates myocardial IR injury in a clinically feasible protocol in a mouse model.

Methods and results We have prepared poly-(lactic-co-glycolic acid) nanoparticles containing TAK-242 (TAK-242-NP). TAK-242-NP significantly enhanced the drug delivery to monocytes/macrophages in the spleen, blood, and the heart in mice. Intravenous administration of TAK-242-NP (containing 1.0 or 3.0 mg/kg TAK-242) at the time of reperfusion decreased the infarct size, but the TAK-242 solution did not even when administered at a dosage of 10.0 mg/kg. TAK-242-NP inhibited the recruitment of Ly-6C^{high} monocytes to the heart, which was accompanied by decreased circulating HMGB1, and NF-κB activation and cytokine expressions in the heart. TAK-242-NP did not decrease the infarct size further in TLR4-deficient mice, confirming the TLR4-specific mechanism in the effects of TAK-242-NP. Furthermore, TAK-242-NP did not decrease the infarct size further in CCR2-deficient mice, suggesting that monocyte/macrophage-mediated inflammation is the primary therapeutic target of TAK-242-NP.

Conclusion The nanoparticle-mediated delivery of TAK-242-NP represent a novel and clinical feasible strategy in patients undergone coronary revascularization for AMI by regulating TLR4-dependent monocytes/macrophages-mediated inflammation.

Keywords Innate immunity • TLR4 • Ischaemia–reperfusion • Monocyte • Nanoparticle

This article is part of the Spotlight Issue on Cardioprotection Beyond the Cardiomyocyte.

1. Introduction

Coronary heart disease (CHD) is among the leading causes of death worldwide.[1] Specifically, acute myocardial infarction (AMI) is the most important life-threatening type of CHD requiring emergency care for life saving. The aim of emergency care for AMI is early coronary reperfusion to restore the blood supply and reduce necrotic cell death in the ischaemic myocardium.[2] However, the therapeutic efficacy of reperfusion therapy is hampered by ischaemia–reperfusion (IR) induced by mitochondrial injury and inflammation. Intracellular environmental changes such as calcium overload, oxidative stress, and intracellular acidosis induce the opening of mitochondrial permeability transition pore (mPTP).[3] As a result of mPTP opening, mitochondrial swelling and the release of cytochrome c to the cytosol occur. Cytochrome c activates the caspases (caspase-9 and caspase-3) and subsequently induces subsequent apoptotic cell death.[4,5] In turn, myocardial IR injury causes the

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releases of proinflammatory cytokines and damage-associated protein patterns (DAMPs) including HMGB1, DNA, RNA, S100, and heat shock protein from damaged cardiomyocytes, including the recruitment of neutrophils and monocytes/macrophages to the heart.[6,7] Accumulating evidence supports the hypotheses that there are heterogeneous monocyte/macrophage populations and that inflammatory monocytes/macrophages (Ly-6C^{high}CCR2^{+} in mice) accelerates myocardial cell death during the acute phase after reperfusion.[8,9] We previously reported that the macrophage-targeting delivery of irbesartan (an angiotensin receptor blocker) and pioglitazone (an agonist of peroxisome proliferator-activated receptor γ) skews monocyte/macrophage polarization to less-inflammatory reparative subsets and attenuated IR injury.[10,11] Other studies have reported that the inhibition of monocyte accumulation by an antibody blocking adhesion molecules, e.g. P-selectin, CD11/18, or ICAM-1, reduces myocardial IR injury.[12–14]

During the acute phase after coronary occlusion, DAMPs released by dead cardiomyocytes not only induce cell death in adjacent cardiomyocytes but also activate innate immune systems through receptors, such as Toll-like receptors (TLRs) and Nod-like receptors, further accelerating cardiomyocyte damage.[7] TLRs belong to the pattern recognition receptors family, which recognizes and responds to pathogen-associated molecular patterns and DAMPs. To date, 10 TLRs have been identified in humans, and TLR2, 3, 4, 5, and 9 have been reported to be associated with myocardial IR injury.[15–19] In mice, the deletion of TLR2, 3, and 4 decreases the infarct size after myocardial IR injury. The TLR4 competitive inhibitor, Eritoran, decreases the myocardial infarct size after IR injury in C57Bl/6 mice when administered 10 min before the induction of ischaemia.[20] However, this animal study has not been translated into clinical practice that may require the administration of drugs at the time of reperfusion, a clinically-feasible time point.

Recent advances in nanotechnology and nanomedicine have provided opportunities for drug repositioning by facilitating drug delivery specifically to target organs.[21] We and other researchers tested the efficacy of polymeric nanoparticles composed of bioabsorbable poly-(lactic-co-glycolic acid) (PLGA-NP) in the treatment of cardiovascular diseases including atherosclerosis, pulmonary hypertension, and myocardial IR injury.[22–24] We have previously reported that intravenously administered PLGA-NP are effectively delivered to circulating monocytes through phagocytosis,[11,22] and the IR cardiomyocytes depending on an enhanced vascular permeability and a myocardial endocytic activity after IR.[24,25] Consequently, PLGA-NP enhanced the efficacy of incorporated drugs to attenuate myocardial IR injury when administered at the time of reperfusion.[10,11,21,22,24–27] In this study, we developed PLGA-NP containing TAK-242 (TAK-242-NP), a chemical inhibitor of TLR4 intracellular domain, in order to enhance the efficacy of TAK-242 to block TLR4-mediated inflammation. We examined whether TAK-242-NP could attenuate myocardial IR injury, focusing on the clinically feasible protocol that administers the nano-medicine at the time of reperfusion in a mouse model of myocardial IR injury.

2. Methods

2.1 Myocardial IR injury and AMI

The study protocol was reviewed and approved by the Ethics of Animal Experiments Committee of Kyushu University Faculty of Medicine and was conducted in accordance with the American Physiological Society guidelines and the NIH Guide for the Care and Use of Laboratory Animals, 8th edition. Adult male C57BL/6J mice (9–13 weeks old) (CLEA Japan, Inc., Tokyo, Japan), CCR2-deficient mice on a C57BL/6J background (9–13 weeks old) (CLEA Japan, Inc., Tokyo, Japan) and TLR4-deficient mice on a C57BL/6 background (9–13 weeks old) (Oriental Bio Service, Inc., Kyoto, Japan) were used in this study. A murine model of myocardial IR injury was established as previously described.[28] In brief, the mice were intubated and anaesthetized with 2% isoflurane in 2 L/min oxygen. The hearts were exposed by a left thoracotomy and pericardiotomy. Then, the left anterior descending artery was temporary ligated with 8-0 nylon suture for 30 min and the blood flow was restored. The mice were euthanized by intraperitoneal administration of 200 mg/kg pentobarbital. The detailed methods are described in the Supplementary material online.

2.2 Flowcytometry

Cells isolated from the peripheral blood, spleen, and the heart were analysed with a Gallios Flow Cytometer (Beckman Coulter, Inc., CA, USA) as previously described.[10,11] In brief, the monocytes/macrophages were identified as CD45^{+}CD11b^{+}Lin^{-}, the neutrophils were identified as CD45^{+}CD11b^{+}Lin^{+}, and the lymphocytes were identified as CD45^{+}CD11b^{-}Lin^{+}. To identify the monocyte/macrophage subsets, the cells were stained with an anti-Ly-6C antibody. Additional details are provided in the Supplementary material online.

2.3 Statistical analysis

The data are expressed as the mean ± standard deviation. The statistical analyses of the differences were performed using the unpaired *t*-tests between two groups. To compare three or more groups, ANOVA, followed by Bonferroni's *post hoc* multiple comparison tests or Dunnett's test, was performed using Prism Software version 7.0 (Graph Pad Software, San Diego, CA, USA). *P*-values less than 0.05 were considered to be statistically significant.

Other methods are provided in the Supplementary material online.

3. Results

3.1 Monocytes/macrophages express more TLR4 in the heart than in the spleen and blood

We first quantified cell surface TLR4 by flowcytometry in CD45^{+} cells isolated from the spleen, blood, and heart at 24 h after IR (Supplementary material online, *Figure S1*). The staining with the PE-conjugated anti-TLR4 antibody revealed that the monocytes/macrophages, i.e. both inflammatory Ly-6C^{high} cells and Ly-6C^{low} non-inflammatory cells, express TLR4 more in the heart than in the spleen and blood (*Figure 1A* and *B*).

We examined the effects of IR on the leucocyte population in the heart. Compared with the data from the mouse hearts without IR injury (Supplementary material online, *Figure S2A*), the TLR4-positive neutrophils and Ly-6C^{high} inflammatory monocytes/macrophages were significantly increased in the heart after IR. In contrast, the Ly-6C^{low} monocytes/macrophages were decreased after IR, and the total number of monocytes/macrophages was comparable between the sham and IR groups (*Figure 1C*, Supplementary material online, *Table S2*).

3.2 PLGA-NP delivered to monocytes/macrophages in mice after IR injury

Given the abundant expression of TLR4 in monocytes/macrophages in the heart, we focused on these cells in the treatment with drug delivery system (DDS). Initially, we formulated PLGA-NP incorporating

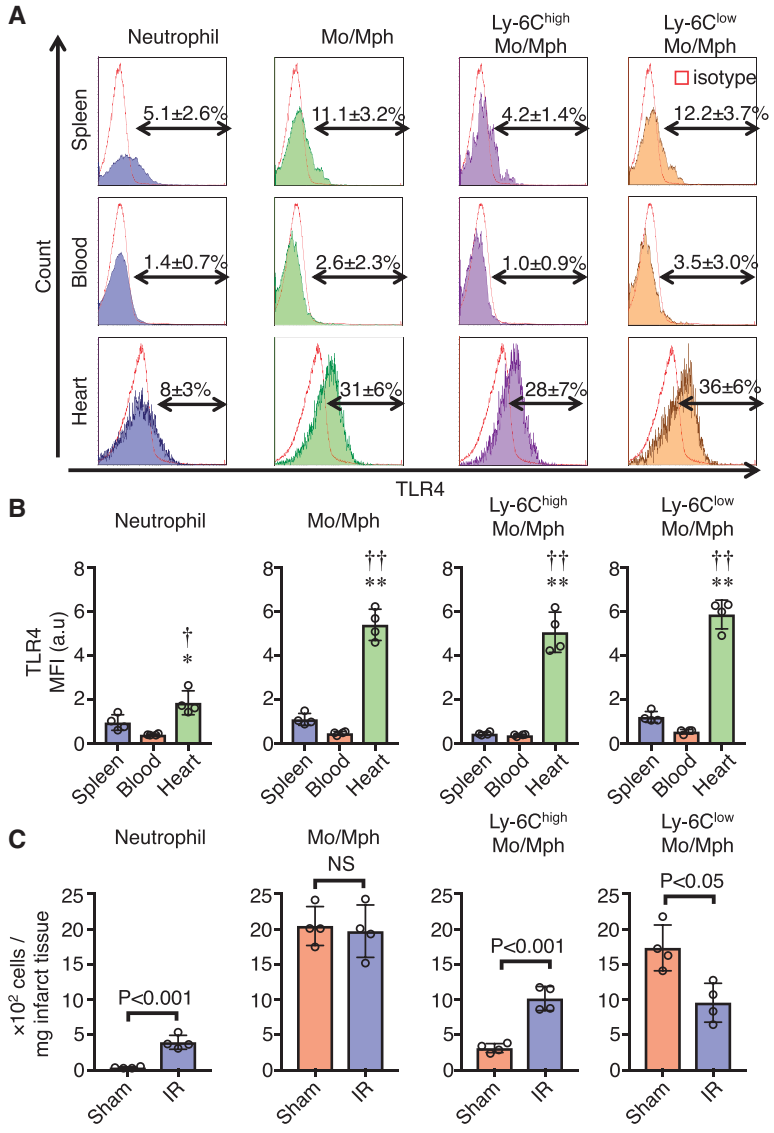

Figure 1 Flowcytometric analyses of TLR4 on inflammatory cells after IR. (*A*) Representative histograms illustrating the expression of TLR4/MD2 at 24 h after IR injury. Isotype IgG was used as a control (red histogram). (*B*) Quantification of TLR4/MD-2 expression as determined by the mean fluorescent intensity (MFI). The data are shown as the mean ± standard deviation ($N = 4$ per group) and were analysed by one-way ANOVA, followed by Bonferroni's multiple comparison test. *$P < 0.05$ vs. spleen, **$P < 0.0001$ vs. spleen, †$P < 0.05$ vs. blood, ††$P < 0.0001$ vs. blood. (*C*) Number of $CD45^+TLR4^+$ cells in mg heart tissues. The data are shown as the mean ± standard deviation ($N = 4$ per group) and were compared by unpaired *t*-test. Mo, monocytes; Mph, macrophages; NS, not significant.

fluorescein isothiocyanate (FITC-NP) and examined the *in vivo* distribution of FITC to elucidate the efficacy of PLGA-NP as a DDS after IR. The saline, FITC solution or FITC-NP were intravenously injected via the femoral vein at the time of reperfusion. Fluorescent stereomicroscopy revealed that the PLGA-NP enhanced the delivery of FITC to the heart and spleen in the mice after the myocardial IR injury (*Figure 2A* and *B*). In the heart, FITC was selectively delivered to the area exposed to IR. The flowcytometric analysis demonstrated that the FITC-NP were effectively delivered to the heart and spleen where the $CD45^+$ leucocytes were analysed and the FITC fluorescent intensity was the highest in the FITC-NP groups, especially in the monocytes/macrophages. In the circulating blood and bone marrow, the FITC fluorescent intensity was the highest in the monocytes/macrophages, too (Supplementary material online, *Figure S3*). In contrast, there were no significant differences in mean fluorescent intensity between the saline and FITC solution groups in all organs. The transmission electron microscopy revealed that nanoparticles were present in the myocardium exposed IR. The nanoparticles were found in macrophages, endothelial cells and border zones of ischaemic and infarcted cardiomyocytes with swollen mitochondria (*Figure 2C*, Supplementary material online, *Figure S5*).

We also measured the plasma and tissue concentration of TAK-242 to ensure the delivery of TAK-242-NP. After the intravenous injection of the TAK-242 solution (3.0 mg/kg) or TAK-242-NP (containing 3.0 mg/kg TAK-242), the plasma concentrations of TAK-242 reached their peaks

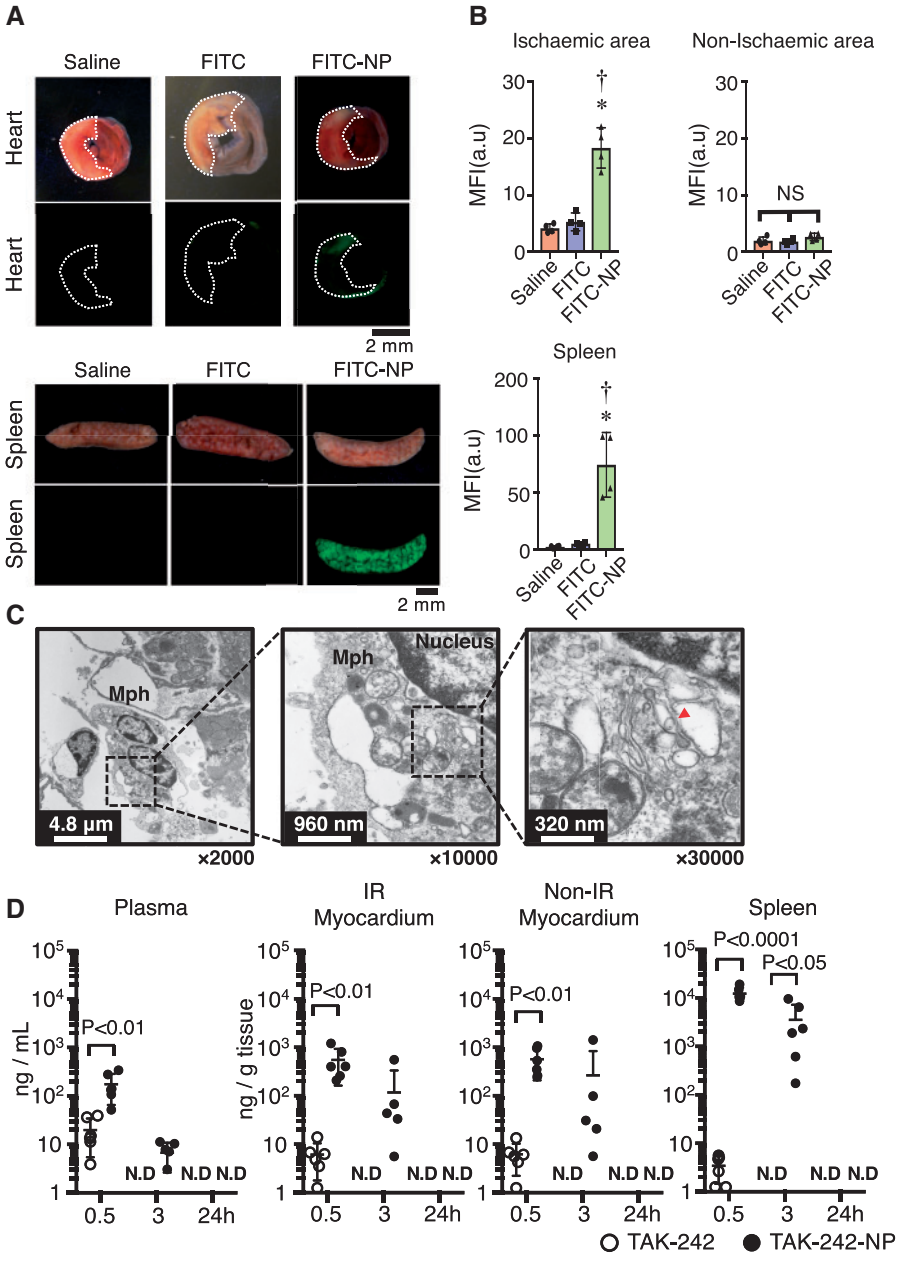

Figure 2 PLGA nanoparticles as a drug delivery system for IR injury. (*A*) Representative light (upper) and fluorescence (lower) stereomicrographs of whole hearts and spleens harvested 3 h after the intravenous administration of saline, FITC solution or FITC-NP. White area indicates the MI area (TTC-negative), red area indicates the non-MI area within the AAR, and blue area indicates the non-ischaemic area. AAR is surrounded by dotted lines. Scale bar: 2 mm. (*B*) Quantitative analyses of fluorescence intensity (MFI). The data are shown as the mean ± standard deviation ($N = 4$ per group) and were analysed by one-way ANOVA, followed by Bonferroni's multiple comparison test. $^{*}P < 0.001$ vs. saline, $^{\dagger}P < 0.001$ vs. FITC. NS, not significant. (*C*) Representative electron micrographs of cardiac tissue 30 min after reperfusion and intravenous administration of TAK-242-NP. Arrowhead indicates nanoparticle. Mph, macrophage (2000× magnification, scale bar: 4.8 μm), (10 000× magnification, scale bar: 960 nm), (30 000× magnification, scale bar: 320 nm). (*D*) Tissue concentration of TAK-242 in the plasma, heart and spleen. The data are shown as the mean ± standard deviation ($N = 6$ per group) and were analysed by two-way ANOVA, followed by Bonferroni's multiple comparison test.

after 0.5 h and decreased to an undetectable level at 24 h (*Figure 2D*). In the plasma, heart and spleen, the concentrations of TAK-242 in the TAK-242-NP group were significantly higher than those in the TAK-242 group at 0.5 and 3 h after injection. These data suggest that PLGA-NPs function as DDS, enabling the effective delivery of incorporated drugs to monocytes/macrophages after myocardial IR.

3.3 TAK-242-NP decreased the infarct size and accumulation of inflammatory cells in the heart

To elucidate the *in vivo* efficacy of TAK-242-NP on the infarct size after myocardial IR injury, TAK-242 (1, 3, or 10 mg/kg) or TAK-242-NP

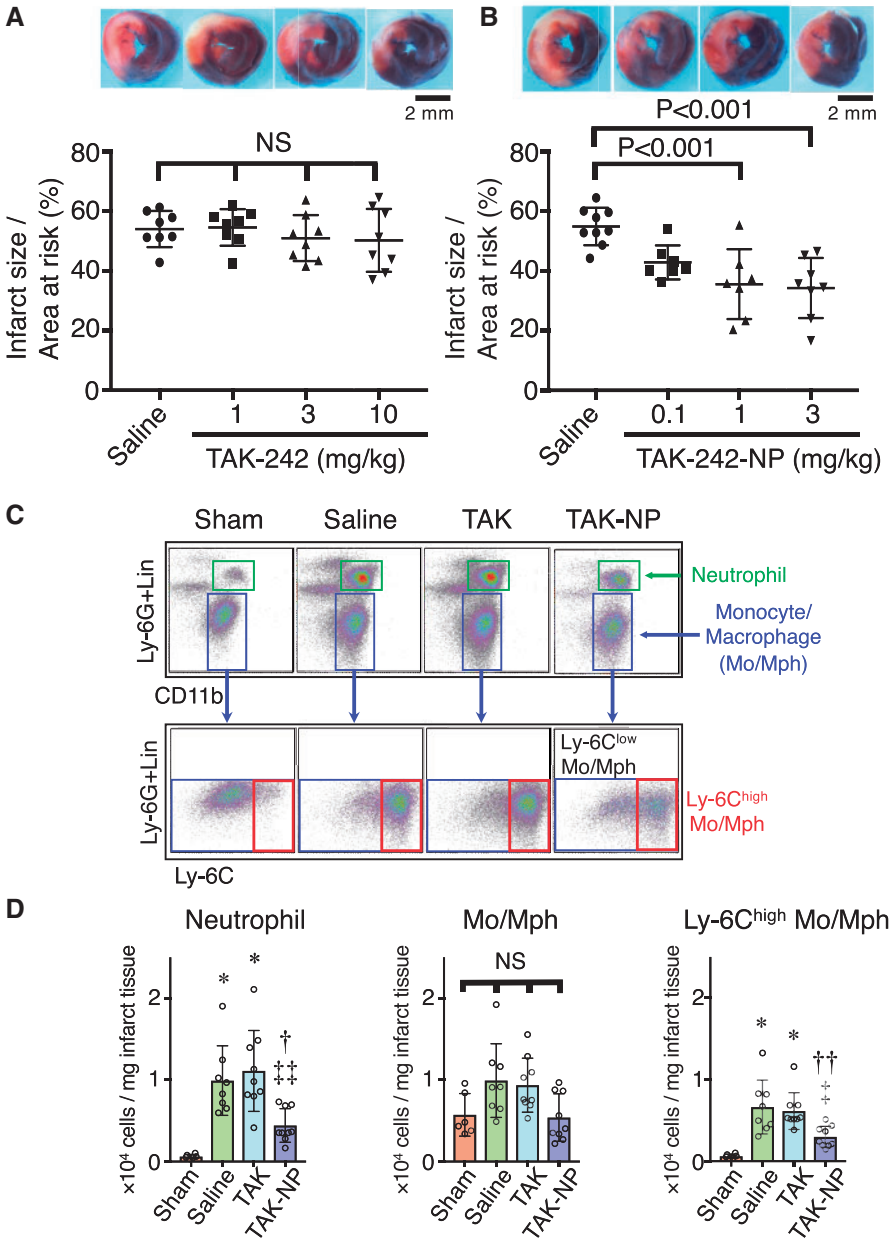

Figure 3 The therapeutic effect of TAK-242-NP on the infarct size and inflammatory cells. (*A*) The effect of saline or TAK-242 solution administered at the time of reperfusion on the infarct size after IR injury quantified 24 h after IR injury. The data are shown as the mean ± standard deviation ($N = 8$ per group) and were compared using one-way ANOVA, followed by Bonferroni's multiple comparison test. (*B*) The effect of saline or TAK-242-NP administered at the time of reperfusion on infarct size after IR injury quantified 24 h after IR injury. The data are shown as the mean ± standard deviation ($N = 9$ saline, $N = 7$ 0.1 mg/kg TAK-242-NP, $N = 7.1$ mg/kg TAK-242-NP, $N = 8.3$ mg/kg TAK-242-NP). The data were compared using one-way ANOVA, followed by Bonferroni's multiple comparison test. (*C*) Flowcytometric gating strategy in the heart after IR injury. (*D*) Flowcytometric analysis of leucocytes in IR hearts treated with saline, TAK-242 solution or TAK-242-NP 24 h after IR injury. The data are shown as the mean ± standard deviation ($N = 6$ sham, $N = 8$ saline, $N = 9$ TAK, $N = 9$ TAK-NP) and were analysed by one-way ANOVA, followed by Bonferroni's multiple comparison test. $^{*}P < 0.001$ vs. sham, $^{\dagger}P < 0.05$ vs. saline, $^{\dagger\dagger}P < 0.01$ vs. saline, $^{\ddagger}P < 0.05$ vs. TAK, $^{\ddagger\ddagger}P < 0.01$ vs. TAK. Mo, monocytes; Mph, macrophages; NS, not significant; TAK, TAK-242 solution; TAK-NP, TAK-242-NP.

(0.1, 1, or 3 mg/kg as incorporated TAK-242) was injected at the time of reperfusion. The dose of TAK-242 was determined based on previous reports in which systemically administered TAK-242 (1–10 mg/kg) showed efficacy in mouse models of sepsis or cerebral IR injury.[29–32] As shown in Supplementary material online, *Figure S8*, there were no significant differences in the AAR/LV among the treatment groups. In the mice treated with TAK-242, even 10 mg/kg of TAK-242 administered at the time of reperfusion did not affect the infarct size, whereas 3 mg/kg significantly decreased the infarct size when administered before the induction of ischaemia (*Figure 3A*, Supplementary material online,

Figure S7). In contrast, 1 mg/kg of TAK-242-NP significantly reduced the infarct size compared with saline (*Figure 3B*). The flowcytometric analysis demonstrated accumulation of neutrophils and Ly-6C^{high} inflammatory monocytes in the heart after IR, both of which were decreased by TAK-242-NP (*Figure 3C* and *D*). TAK-242-NP had no significant effects on the haemodynamic parameters 6 h after reperfusion (Supplementary material online, *Table S1*).

3.4 TAK-242-NP inhibited the activation of monocytes/macrophages after IR

Subsequently, we examined the protease activity, NF-κB binding activity and cytokine expression, all of which are associated with inflammatory monocyte/macrophage subsets, in the heart after IR injury. The *in vivo* and *ex vivo* fluorescent molecular tomography was performed using the near infrared fluorescent probe ProSense in which the quenched fluorescence is activated by cathepsin B, L, S, and plasmin, demonstrating an increase in protease activity in the heart after IR. TAK-242-NP (containing 3 mg/kg TAK-242), but not the equivalent dose of TAK-242, decreased the protease activity (*Figure 4A*). The NF-κB binding activity in the IR myocardium was also increased by IR injury, and this induction was attenuated by TAK-242-NP (*Figure 4B*). The protein levels of IL-1β, IL-6, and MCP-1 were increased in the heart by the IR injury, and this induction of IL-6 and MCP-1 was attenuated by TAK-242-NP (*Figure 4C*). Then, we quantified the levels of circulating HMGB1, one of the DAMPs associated with prognosis after AMI.[33] As shown in *Figure 4D*, plasma HMGB1 exhibited two peaks at 0.5 and 24 h after reperfusion. TAK-242-NP suppressed the later, but not earlier, peak of HMGB1 (*Figure 4E* and *F*).

3.5 Monocyte/macrophage recruitment is a primary therapeutic target of TAK-242-NP

The data presented above suggest that monocyte/macrophage-mediated inflammation plays a critical role as a therapeutic target of TAK-242-NP. Therefore, we employed CCR2-deficient mice to elucidate the role of monocyte-mediated inflammation in the therapeutic effects by TAK-242-NP. The CCR2 deficiency decreased the infarct size after IR compared with that in the wild-type mice (data not shown), and TAK-242-NP had no additional effects on the infract size in CCR2-deficient mice (*Figure 5A*). TAK-242-NP had no significant effects on the protein levels of IL-1β, IL-6, and MCP-1 measured in the homogenized IR myocardial tissue from the CCR2-deficient mice (*Figure 5B*). The flowcytometric analyses indicated that the accumulation of monocytes/macrophages was abrogated in the CCR2-deficient mice (*Figure 5C*). These data suggest that the recruitment of monocytes/macrophages is the primary mechanism of the therapeutic effects of TAK-242-NP.

3.6 Deletion of TLR4 abrogated the induction of CCR2 in splenic monocytes

Next, we employed TLR4-deficient mice to examine the interaction between TLR4 and CCR2-dependent inflammation. The TLR4 deficiency itself decreased the infarct size compared with that in the wild type control mice (data not shown). In these TLR4-deficient mice, the treatment with TAK-242-NP did not further decrease the infarct size, confirming that TAK-242-NP decreased the infarct size via solely TLR4-dependent mechanisms (*Figure 6A*). The expression of inflammatory cytokines, including IL-1β, IL-6, and MCP-1, was unaffected by TAK-242-NP (*Figure 6B*). The flowcytometric analyses of leucocytes isolated from the heart after IR demonstrated that the recruitment of neutrophils and monocytes/macrophages was inhibited by the genetic deletion of TLR4 (Supplemental material online, *Figure S6*). In the spleen, in a reservoir of myeloid cells,[32] the expression of CCR2 increased after IR injury in the neutrophils and monocytes/macrophages (in both the Ly-6C^{high} and Ly-6C^{low} populations) in the TLR4$^{+/+}$ control mice, which was abrogated by the deletion of TLR4 (*Figure 6C*), suggesting that TLR4-dependent signals play a role in preconditioning splenic myeloid cells to express CCR2 and respond to MCP-1 expressed in the heart after IR.

3.7 TAK-242-NP attenuated cardiac remodelling after IR injury

We finally examined cardiac remodelling by echocardiography and histopathology (*Figure 7*). TAK-242-NP (3 mg/kg as TAK-242) was intravenously administered at the time of reperfusion, and left ventricular remodelling was evaluated after 1, 2, and 4 weeks later. In the control mice, an equivalent volume of saline was injected. The haemodynamic parameters were measured by the tale cuff method and were unaffected by the administration of saline or TAK-242-NP (Supplementary material online, *Table S1*). The left ventricular dimensions, including left ventricular end-diastolic diameter and left ventricular end-systolic diameter, were increased 7 days after IR injury in the saline group but was attenuated by TAK-242-NP on Day 28. Similarly, the Left ventricular systolic function determined as left ventricular ejection fraction and fractional shortening was impaired at 7 days after IR injury, but TAK-242-NP attenuated left ventricular systolic dysfunction on Day 28 (*Figure 7A* and *B*). Histopathological analysis showed that TAK-242-NP reduced Masson-trichrome positive fibrotic area in the left ventricle and prevented from expansion of left ventricle at 28 days after reperfusion (*Figure 7C* and *D*).

4. Discussion

The novel findings of this study are as follows: (i) PLGA-NP rapidly and effectively delivered TAK-242 to monocytes/macrophages after myocardial IR injury, (ii) the monocytes/macrophages in the heart express more TLR4 than the monocytes in the spleen and circulating blood, (iii) the nanoparticle-mediated delivery of TAK-242 to monocytes/macrophages at the time of reperfusion reduced the infarct size by inhibiting monocyte-mediated inflammation, and (iv) the plasma HMGB1 levels were increased biphasically after IR injury, and the blockade of TLR4-mediated signals suppressed the later peak of plasma HMGB1.

We previously reported that PLGA-NP was delivered to the ischaemic myocardium and inflammatory cells in the blood, heart, and spleen.[10,11] In this study, we measured the tissue concentrations of TAK-242 by HPLC to confirm the delivery of the incorporated TAK-242 to the target organs. Compared with the injection of the TAK-242 solution, the concentrations of TAK-242 were approximately nine-fold higher in the plasma, 90-fold higher in the heart and 3600-fold higher in the spleen 30 min after reperfusion (*Figure 2D*). In addition, TAK-242 was detected in these organs 3 h after reperfusion, whereas TAK-242 was not detected in the TAK-242 solution group 3 h after reperfusion, indicating that PLGA-NP effectively delivered TAK-242 to the target tissues and were retained longer than TAK-242. The *in vivo* kinetic data indicated that the spleen was a major target of PLGA-NP at least immediately after the intravenous injection in mice after myocardial IR injury. Accumulating data suggest that the spleen functions as a monocyte reservoir in the mammalian body and that splenic monocytes are released into the blood as a result of sympathetic nerve activation, followed by the proliferation of myeloid progenitor cells after cardiac ischaemia.[34,35]

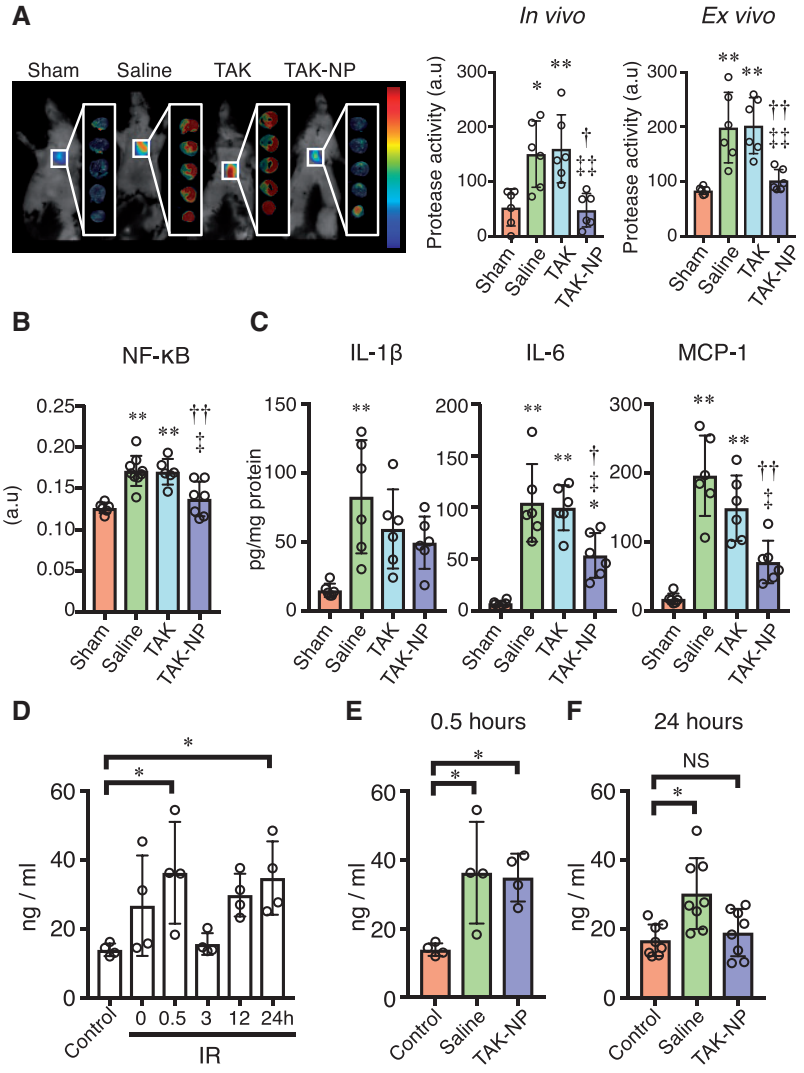

Figure 4 Effect of TAK-242-NP on inflammation and circulating HMGB1. (*A*) FMT images visualizing protease activity by ProSense-680 in mice 48 h after IR injury. Right graphs show the quantitative data. The data are shown as the mean ± standard deviation ($N = 6$ per group). FMT, fluorescence molecular tomography. $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. sham, $^{\dagger}P < 0.05$ vs. saline, $^{\dagger\dagger}P < 0.01$ vs. saline, $^{\ddagger}P < 0.05$ vs. TAK, $^{\ddagger\ddagger}P < 0.01$ vs. TAK. The data were analysed by one-way ANOVA, followed by Bonferroni's multiple comparison test. (*B*) NF-κB binding activity was measured as proteins extracted from the infarcted myocardium 12 h after IR. The data are shown as the mean ± standard deviation and were analysed by one-way ANOVA, followed by Bonferroni's multiple comparison test ($N = 6$ sham, $N = 9$ saline group, $N = 6$ TAK, $N = 7$ TAK-NP). $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. sham, $^{\dagger}P < 0.05$ vs. saline, $^{\dagger\dagger}P < 0.01$ vs. saline, $^{\ddagger}P < 0.05$ vs. TAK, $^{\ddagger\ddagger}P < 0.01$ vs. TAK. (*C*) Quantitative data of IL-1β, IL-6, and MCP-1 proteins in the infarcted myocardium at 12 h after reperfusion. The data are shown as the mean ± standard deviation ($N = 6$ per group) and were analysed by one-way ANOVA, followed by Bonferroni's multiple comparison test. $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. sham, $^{\dagger}P < 0.05$ vs. saline, $^{\dagger\dagger}P < 0.01$ vs. saline, $^{\ddagger}P < 0.05$ vs. TAK, $^{\ddagger\ddagger}P < 0.01$ vs. TAK. (*D*) Time course of circulating HMGB1 level after IR. The data are shown as the mean ± standard deviation ($N = 4$ per group). $^{*}P < 0.05$ vs. control. The data were analysed by one-way ANOVA, followed by Dunnett's multiple comparison test. (*E* and *F*) HMGB1 level at indicated time points after reperfusion. The data are shown as the mean ± standard deviation ($N = 4$ and 8) and were analysed by one-way ANOVA, followed by Dunnett's multiple comparison test. $^{*}P < 0.05$ vs. control.

This hypothesis suggests that splenic monocytes engulfed FITC-NP are released into the bloodstream and then recruited to the myocardial tissue by chemokines, including MCP-1.

Several studies have reported that the expression of TLR4 on monocytes is increased in patients with AMI.[36,37] Ishikawa *et al.*[37] reported that the expression of TLR4 on monocytes in ruptured plaques was also increased compared with monocytes in circulating blood. We quantified TLR4 expression on neutrophils and monocytes/macrophages accumulating in the heart after IR and found that TLR4 expression was increased compared with that in the cells from the spleen and the blood at 3 and 24 h after reperfusion (*Figure 1B* and *C*, Supplementary material online, *Figure S9*). A similar phenomenon was observed in mice without IR injury (Supplementary material online, *Figure S2A* and *B*). One possible mechanism of TLR4 induction is hypoxia-induced expression of TLR4 via HIF-1α (hypoxia inducible factor 1α)-mediated mechanisms. In the liver, lung, and pancreas, hypoxia caused by ischaemia has been reported to

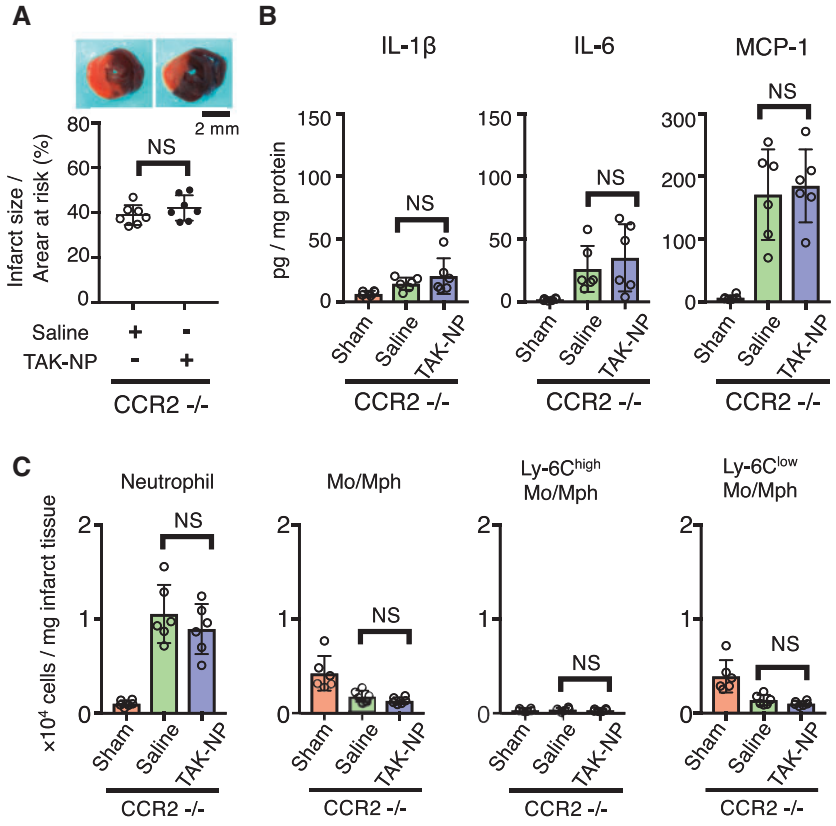

Figure 5 Effect of TAK-242-NP in CCR2-deficient mice. (*A*) Infarct area quantified 24 h after reperfusion. The data are shown as the mean ± standard deviation ($N = 7$ per group) and were analysed by unpaired *t*-test. Scale bar: 2 mm. (*B*) Quantitative data of IL-1β, IL-6 and MCP-1 extracted from the heart 12 h after reperfusion. The data are shown as the mean ± standard deviation ($N = 6$ per group) and analysed by one-way ANOVA followed by Bonferroni's multiple comparison test. (*C*) Flowcytometric analysis of the ischaemic myocardium 24 h after reperfusion in CCR2-deficient mice. The data are shown as the mean ± standard deviation ($N = 6$ per group). The data were compared using one-way ANOVA, followed by Bonferroni's multiple comparison test. Mo, monocytes; Mph, macrophages; TAK-NP, TAK-242-NP; NS, not significant.

increase TLR4 by HIF-1α-mediated mechanisms.[38–40] These results suggest that TLR4 in monocytes/macrophages is a promising therapeutic target for IR injury.

TAK-242 is a newly developed, small-molecule TLR4 inhibitor that specifically binds TLR4 among 10 TLRs in humans.[41,42] TAK-242 has been tested in clinical trials in patients with sepsis, however, its development was terminated due to cost-effectiveness.[29] However, TAK-242 is expected to be a novel drug for the treatment of IR injury when combined with a nanotechnology-based DDS. The effects of TAK-242 on IR injury have been reported in the brain, liver and kidney in preclinical animal models.[32,33,43,44] Regarding myocardial IR injury, TAK-242 shows its efficacy only when administered before the induction of ischaemia (Supplementary material online, *Figure S7*). This study is the first time that demonstrated the efficacy of TAK-242 by using PLGA-NP. The data regarding the tissue concentration of TAK-242 indicated that the concentrations of the TAK-242 solution were not enough to reduce myocardial IR injury. The up-titration of the doses of TAK-242 might improve its efficacy, however, it was not feasible due to the limited solubility of TAK-242.

Oyama *et al.*[17] reported that the deletion of TLR4 reduced the infarct size after myocardial IR injury, however, the role of TLR4 in the monocytes/macrophages has not been fully understood in terms of the mechanisms of IR injury and subsequent left ventricular remodelling. In this study, we have demonstrated that the nanoparticle-mediated delivery of TAK-242 to inflammatory cells decreased the recruitment of neutrophils and Ly-6C^{high} monocytes to the heart after IR injury, similar to the results of the TLR4 deletion (*Figure 3C* and *D*, Supplementary material online, *Figure S6*). The mechanisms by which the blockade of TLR4 decreased the accumulation of inflammatory cells to the heart are as follows; (i) the decrease in chemokine release from the heart after IR, (ii) the decrease in CCR2 expression in splenic monocytes, and (iii) the inhibition of monocyte/macrophage polarity shift towards inflammatory subsets. The inhibition of the monocyte/macrophage polarity shift is underpinned by the decreased protease activity, decreased NK-κB binding activity and decreased expression of IL-6 and MCP-1 in the heart after IR. The reduction in Ly-6C^{high} inflammatory monocytes/macrophages in the IR heart also supports this interpretation. To clarify the impact of TAK-242-NP on monocyte differentiation after IR injury, we quantified the ratio of Ly-6C^{high}/Ly-6C^{low} monocytes in the spleen after IR. In flowcytometry, the deletion of TLR4 decrease the ratio of Ly-6C^{high}/Ly-6C^{low} monocyte subset and the expression of CCR2 on monocytes, suggesting that TAK-242-NP have significant impacts on the differentiation of monocytes at least 24 h after reperfusion in the spleen (Supplementary material online, *Figure S10*, *Figure 6C*). There are reports

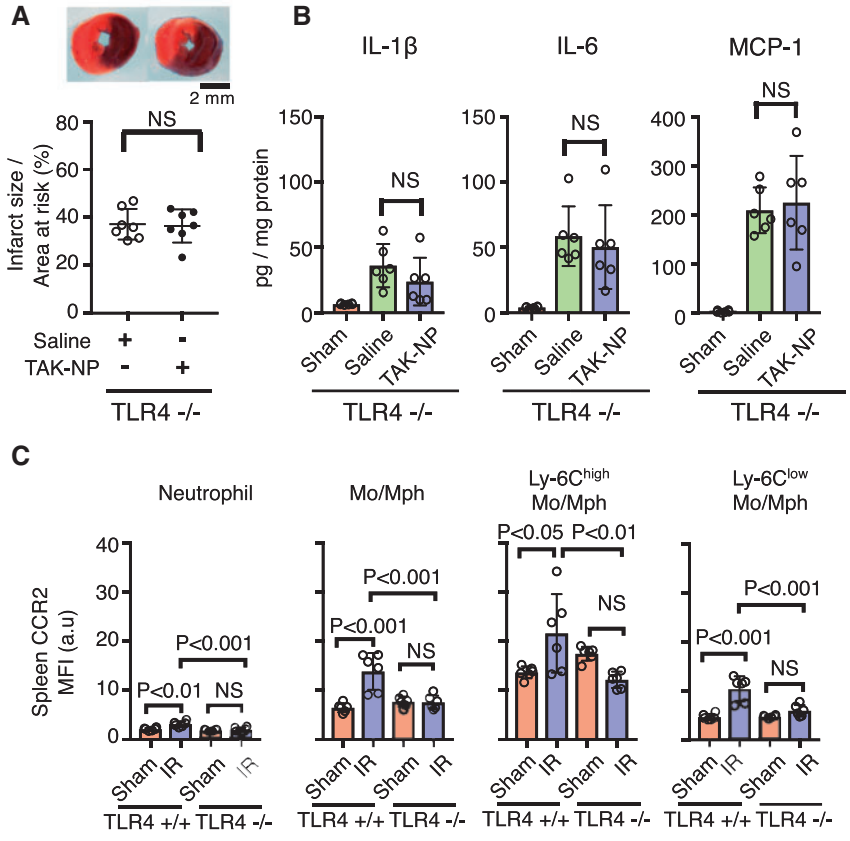

Figure 6 Infarct size and expressions of inflammatory cytokines under therapeutic efficacy of TAK-242-NP, and the expression of CCR2 after IR. (*A*) Infarct area quantified 24 h after reperfusion. The data are shown as the mean ± standard deviation ($N = 7$ per group) and analysed by unpaired *t*-test. Scale bar: 2 mm. (*B*) Quantitative data of IL-1β, IL-6, and MCP-1 extracted from the heart 12 h after reperfusion. The data are shown as the mean ± standard deviation ($N = 6$ per group). (*C*) CCR2 expression on $CD45^+$ cells isolated from the spleen 24 h after reperfusion. The data are shown as the mean ± standard deviation ($N = 6$ per group). The data were compared using one-way ANOVA, followed by Bonferroni's multiple comparison test. Mo, monocytes; Mph, macrophages; NS, not significant.

suggesting that TLR4 signalling protects monocytes from apoptosis.[45,46] Therefore, induction of monocyte apoptosis by blockade of TLR4-mediated signalling could be one of the mechanisms by which monocyte accumulation in the heart was suppressed. TAK-242 binds the intracellular domain of TLR4 and inhibits the binding of the adaptor proteins MyD88 and TRIF. The blockade of MyD88-mediated signalling by TAK-242 is considered to inhibit established downstream pathways, i.e. mitogen-activated protein kinase (MAPK: JNK, p38MAPK) phosphorylation and NF-κB activation via IRAK4 (interleukin-1 receptor-associated kinase 4)-mediated mechanisms.[42,47,48]

The time course of the plasma HMGB1 level showed two peaks at 0.5 h and 24 h after IR. In humans, the circulating HMGB1 level was measured in patients with AMI, and the reported peak of HMGB1 occurred approximately 12 h after the onset of AMI.[33] However, data regarding the super acute phase of AMI have not been reported. In this study, for the first time, we demonstrated that there are two peaks of HMGB1. The results showing that TAK-242-NP abrogated the later peak of HMGB1 suggest that HMGB1 is released from cardiomyocytes immediately after IR injury due to ischaemia- or reperfusion-induced cell death, and then, inflammatory cells, especially monocytes/macrophages recruited to the heart after IR, cause further cardiomyocyte death and release of HMGB1. The data showing that TAK-242-NP did not inhibit the early peak of HMGB1 at the time when few monocytes/macrophages are recruited to the heart again supports the hypothesis that TAK-242-NP decreased the infarct size primarily by inhibiting inflammation rather than directly inhibiting cardiomyocyte cell death.

To confirm this hypothesis, we have administered TAK-242-NP to CCR2-deficient mice. In these mice, TAK-242-NP did not lead to an additional reduction in the infarct size compared with that in the controls, suggesting that CCR2-mediated inflammation is the primary therapeutic target of TAK-242-NP. We previously reported that PLGA-NP are also delivered to Troponin T^+ cardiomyocytes in the ischaemic area of the IR heart after intravenous administration.[24] However, the results of the CCR2-deficient mice clearly indicate that monocytes/macrophages are the primary target of TAK-242-NP and that the direct effects of TAK-242-NP on cardiomyocytes are minor. The results of the CCR2-deficient mice also suggest that the role of neutrophils is minor compared with that of monocytes/macrophages. Previous data showing that neutrophil depletion did not significantly decreased the infarct size after IR injury also support this hypothesis.[10,49]

In addition, we have prepared TLR4-deficient mice to exclude the possibility that TAK-242-NP decreased the infarct size via

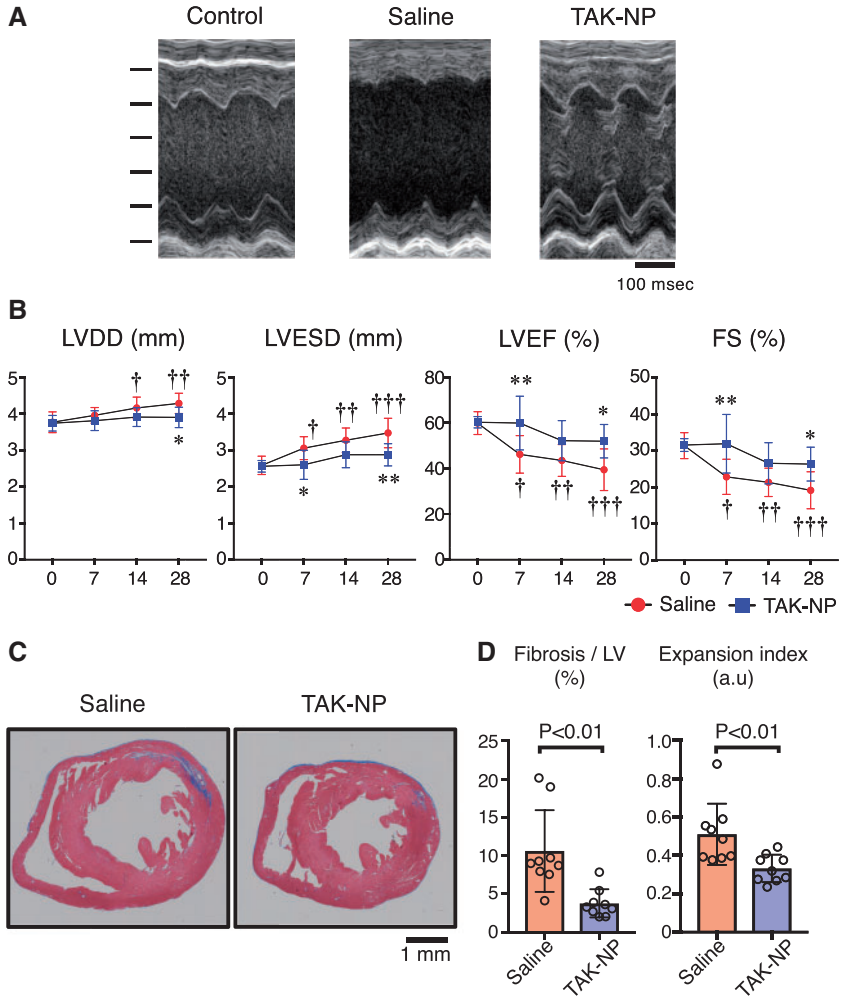

Figure 7 Cardiac remodelling under the therapeutic effect of TAK-242-NP. (*A*) Representative M-mode echocardiographic images recorded 28 days after reperfusion. The interval of each scale bar is 1 mm. The time scale bar is 100 ms. (*B*) Time course of echocardiographic parameters ($N = 10$ saline, $N = 12$ TAK-NP). The data were calculated by two-way ANOVA, followed by Bonferroni's multiple comparison test. $^{*}P < 0.05$ vs. saline, $^{**}P < 0.01$ vs. saline, $^{\dagger}P < 0.05$ vs. saline at Day 0, $^{\dagger\dagger}P < 0.001$ vs. saline at Day 0, $^{\dagger\dagger\dagger}P < 0.0001$ vs. saline at Day 0. (*C*) Representative histopathological images obtained 28 days after reperfusion. The scale bar is 1 mm. (*D*) TAK-242-NP effects on Masson-trichrome-positive fibrotic area in the left ventricle and expansion index at 28 days after reperfusion ($N = 9$ per group). The data were calculated by unpaired *t*-test. TAK-NP, TAK-242-NP.

TLR4-independent mechanisms. However, TAK-242-NP showed no additional effects on the infarct size. These data indicate that TAK-242-NP exerted its efficacy through TLR4-dependent mechanisms in $CCR2^{+}$ monocytes, although animal experiments using knockout mice generally have several limitations such as deletion of CCR2 or TLR4 could decrease infarct size and mask the effect of TAK-242-NP. Several studies have reported the crosstalk between TLR4 and CCR2 on monocytes.[50,51] Liu *et al.*[51] reported that TLR4 signalling augmented monocyte chemotaxis by inhibition of CCR2 internalization and desensitization in bone marrow-derived monocytes. Therefore, we quantified CCR2 expression in TLR4-deficient mice to explore the detailed mechanisms by which TAK-242-NP inhibits monocyte accumulation. In wild-type mice, myocardial IR injury increased CCR2 expression in splenic monocytes. This induction was abrogated in the TLR4-deficient mice. This observation suggests that the suppression of CCR2 induction in splenic monocytes inhibited monocyte release from the spleen and recruitment to the ischaemic area of the heart during the acute phase after IR injury in mice treated with TAK-242-NP. During the chronic phase, TAK-242-NP attenuated left ventricular remodelling and preserved left ventricular systolic function, which was accompanied with reduction of fibrotic area and left ventricular expansion (*Figure 7*). Therefore, intravenous injection of TAK-242-NP during the acute phase after IR injury could be a clinically feasible therapy that not only decreases the infarct size but also attenuates left ventricular remodelling during the chronic phase.

PLGA is a biodegradable polymer, which is approved as a DDS by the US food and Drug Administration and the European Medicine Agency. We developed nanoparticles incorporating a HMG-CoA reductase inhibitor, and have completed a phase I/IIa clinical trial for patients with critical limb ischaemia (UMIN Clinical trial registry ID: UMIN000008011, UMIN00014940, and UMIN000019189). Thus, this nanoparticle-based technology may be applied for myocardial IR injury.

In conclusion, TAK-242-NP reduced myocardial IR injury by inhibiting TLR4-driven, $CCR2^{+}$ monocyte-mediated inflammation. The blockade of TLR4 signalling by TAK-242-NP is a novel and clinically feasible strategy for the treatment of AMI patients undergoing reperfusion therapy.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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