Alternatively activated macrophages determine repair of the infarcted adult murine heart

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Introduction

The formation of connective tissue is an essential process in the healing and repair of almost every tissue and organ, and this process is of particular importance in the case of myocardial repair (1, 2). Because of its insufficient regenerative ability (3), the adult mammalian heart must permanently compensate for the post-MI loss of cardiomyocytes by producing fibrotic tissues. These connective tissues must be firm and extensive enough to maintain the rigidity and performance of the failing heart in conditions of high mechanical stress. Without this, the fragile ventricular wall will undergo sudden rupture in the worst-case scenario. Even if cardiac rupture does not occur, the insufficient tissue repair will make the damaged heart more vulnerable to adverse ventricular remodeling, and this condition will progressively advance to end-stage heart failure (1, 2).

Alternatively activated (or M2) macrophages play a role in the repair and/or regeneration of various types of organs, and the features of these cells are distinct in different organs and environmental conditions (4–6). Recent studies have implicated the involvement of M2 macrophages in myocardial repair by depleting macrophages with chemicals (e.g., clodronate liposome) or knocking out genes that are relevant to myeloid cell differentiation (7–13). However, these depletion methods have limited specificity for M2 macrophages, lymphocytes, neutrophils, and dendritic cells (DCs) are unaffected (16). We hypothesized that this mouse would be able to offer a more specific depletion of M2 macrophages in the heart and thus enable us to elucidate the precise roles of M2 macrophages in myocardial repair after MI.

Consequently, “rescue” (compensation of depleted M2 macrophages) experiments have not been applied in these studies. Similarly, it was not possible for these models to identify the cellular and molecular mechanisms by which a particular subset of macrophages (i.e., M2 macrophages) induce myocardial repair. Therefore, it is important to accumulate more convincing evidence to determine the precise role of M2 macrophages after MI using a more appropriate model, which will also help to dissect the underlying mechanism (15). To this end, we used mice with a deletion of the Tribl gene (a member of the Ca2+/calmodulin-dependent protein kinase [CAMK] Ser/Thr protein kinase family), which have been shown to possess an impaired ability to form M2 macrophages in the spleen, liver, lung, and adipose tissue, whereas Ly-6Chi inflammatory monocytes or macrophages, lymphocytes, neutrophils, and dendritic cells (DCs) are unaffected (16). We hypothesized that this mouse would be able to offer a more specific depletion of M2 macrophages in the heart and thus enable us to elucidate the precise contributions of M2 macrophages to myocardial repair after MI.

Although recent progress in diagnosis and treatment, including percutaneous coronary interventions, has significantly improved the early survival of patients who suffer an MI, this disease remains one of the major causes of human death and disability (17). Therefore, the development of new, more effective treatments is of urgent importance. One promising approach to achieve this goal may be to increase the number of cardiac M2 and other cells (14). Therefore, these methods are useful for investigating the overall contribution of whole macrophage subsets; however, these methods have a limited capability in dissecting the precise role of a certain subset of macrophages (i.e., M2 macrophages). In addition, “rescue” (compensation of depleted M2 macrophages) experiments have not been applied in these studies. Similarly, it was not possible for these models to identify the cellular and molecular mechanisms by which a particular subset of macrophages (i.e., M2 macrophages) induce myocardial repair. Therefore, it is important to accumulate more convincing evidence to determine the precise role of M2 macrophages after MI using a more appropriate model, which will also help to dissect the underlying mechanism (15). To this end, we used mice with a deletion of the Tribl gene (a member of the Ca2+/calmodulin-dependent protein kinase [CAMK] Ser/Thr protein kinase family), which have been shown to possess an impaired ability to form M2 macrophages in the spleen, liver, lung, and adipose tissue, whereas Ly-6Chi inflammatory monocytes or macrophages, lymphocytes, neutrophils, and dendritic cells (DCs) are unaffected (16). We hypothesized that this mouse would be able to offer a more specific depletion of M2 macrophages in the heart and thus enable us to elucidate the precise contributions of M2 macrophages to myocardial repair after MI.

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macrophages (18, 19); however, the methods used to enhance cardiac M2 macrophages in previous studies are clinically unfeasible, unsuccessful, or under development (20–22). IL-4 is a major Th2 cytokine that drives the differentiation of monocytes and macrophages into M2 phenotypes and/or increases their proliferation in situ, as observed in tissues other than the heart (23–26). Thus, we investigated the potential for IL-4 administration to increase M2 macrophages in the heart after MI, improve the repair of the damaged myocardium, and enhance the maintenance of the function and structure of the infarcted heart.

**Results**

M2-like macrophages were present in the adult murine heart and were predominantly increased in the infarct area after MI, exhibiting strengthened reparative abilities. We confirmed that the left ventricular (LV) myocardium of adult mice contained CD11b^+ F4/80^+ macrophages, more than 90% of which were positive for CD206 (Figure 1A). On day 7 after MI that was induced by coronary artery ligation, the ratio of CD206^+ CD11b^+ F4/80^+ macrophages was reduced, with an increase in the CD206^- subset. Conversely, the majority of CD206^+ cardiac cells were positive for both F4/80 and CD11b in both normal (no MI) and post-MI hearts (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI85782DS1). CD11b^+CD206^- cardiac cells were primarily negative for CCR2 and Ly6C, whereas CD11b^+CD206^- cells were positive for these markers (Figure 1B).

In addition, these CD206^+F4/80^+CD11b^+ cells expressed high levels of other M2 macrophage markers including *Chil3* (*Ym1*) and *Retnla* (*Fizz1*) (4–6, 27), both before and after MI, compared with what was detected in primary cardiac fibroblasts and CD11b^+CD206^- cardiac cells (Figure 1C).

Consistent with these findings, a sizable number of CD206^+ cells were detected by IHC in the normal heart (Figure 2A). Approximately 96.9% ± 2.0% of these cells were positive for CD68, whereas 92.8% ± 3.0% of the CD68^+ macrophages were CD206^+. After MI, CD206^+ cells exhibited dynamic spatiotemporal changes in their distribution. These cells were predominantly increased in the infarct area (and in the border area to a lesser degree), with a peak on day 7 (Figure 2, B and C). In contrast, CD11c^+ cells were rarely observed in the intact (no-MI) heart but increased by day 3 after MI in the infarct and border areas (Figure 2C and Supplemental Figure 2B). Although it is not entirely specific (i.e., inclusion of DCs), CD11c is a frequently used cell-surface marker for M1-like inflammatory macrophages and monocytes (28–30). Our data supported the utility of CD11c by demonstrating that most of the CD11c^+ cells from the day-7 post-MI heart were
hearts (Figure 3, B and C). The expression levels of these repair-associated genes in the CD206+ cardiac M2-like macrophages were increased compared with levels in the CD206–CD11b+ inflammatory (M1-like) macrophages and monocytes from post-MI hearts. These results suggest that M2-like macrophages increase in number and also exhibit increases in post-MI antiinflammation and tissue repair activity at the appropriate location and time at which inflammation resolution and tissue healing are necessary. These findings imply a potential role for these cells in the repair of infarcted myocardium.

Trib1–/– mice showed a depletion of the post-MI augmentation of M2-like macrophages, without altering M1-like macrophages. We next aimed to clarify the reparative role of post-MI CD206+ M2-like macrophages using Trib1–/– mice, which are known to have an impaired ability to form M2-like macrophages in the spleen, liver, lung, and adipose tissue, while their ability to form other inflammatory cells remains intact (16). We first confirmed that the heart function and structure, extracellular collagen deposition, occurrence of cardiac fibroblasts, and capillary density of the intact, CCR2+/Ly6C–CD206+ (Supplemental Figure 3) and that CD11c was expressed in CD11b+CD206– cells, but not in CD11b+CD206+ cells (Supplemental Figure 4). Additionally, it is known that the post-MI frequency of cardiac DCs is much lower (fewer than 1 in 20 cells) that that of cardiac macrophages throughout (31), suggesting that contamination of CD11c+cardiac cells by DCs is insignificant, if it occurs at all. Furthermore, the number of CD68+ cells (pan-macrophages) was generally equal to the sum of CD206+ cells and CD11c+ cells in each area at each time point after MI (Figure 2C and Supplemental Figure 2).

Microarray analysis revealed that CD206+F4/80+CD11b+ M2-like macrophages collected from day-7 post-MI hearts had a different molecular signature than did those from intact hearts, although both were clustered in a group that was distinct from peritoneal M2 macrophages (Figure 3A; full data are available in the Gene Expression Omnibus [GEO] database; GSE69879). A range of antiinflammatory and repair-associated genes, including Il10, Il1r1, Vegfa, Il1α, and Spp1 (osteopontin) (32–38), were upregulated in M2-like macrophages from MI hearts compared with intact hearts (Figure 3, B and C). The expression levels of these repair-associated genes in the CD206+ cardiac M2-like macrophages were increased compared with levels in the CD206+CD11b+ inflammatory (M1-like) macrophages and monocytes from post-MI hearts.

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Figure 3. Cardiac M2-like macrophages strengthened their post-MI reparative ability. (A) CD206 F4/80 CD11b+ M2-like macrophages were collected by FACS from intact hearts [M2 (no MI)], day-7 post-MI hearts [M2 (MI)], and from the peritoneal cavity of intact mice [M2 (Peritoneal)] and subjected to microarray analysis. Macrophages from different origins showed distinct molecular signatures. M2 (MI) had a different expression profile from that of M2 (no MI), although both were clustered distinctly from the M2 (Peritoneal) profile. (B) Heatmap revealed that among the genes encoding secreted proteins, 13 genes were upregulated and 3 were downregulated in M2 (MI) compared with M2 (no MI) macrophages. The upregulated genes included antiinflammatory and repair-associated genes. (C) Quantitative RT-PCR (qRT-PCR) analysis confirmed the post-MI upregulation of these repair-associated genes in CD206 F4/80 CD11b+ cardiac M2-like macrophages [M2 (MI)]. CD11b CD206 M1-like macrophages from day-7 post-MI hearts [M1 (MI)] expressed these genes at lower levels. Expression levels relative to those of M2 (Peritoneal) are presented. n = 6 in each group. *P < 0.05 versus both M2 (Peritoneal) and M1 (MI); †P < 0.05 versus M2 (no MI); #P < 0.05 versus M2 (Peritoneal); 1-way ANOVA.
These data indicate that M2-like macrophages are essential for post-MI fibroblast-mediated cardiac repair. IL-4 administration amplified the post-MI augmentation of cardiac M2-like macrophages. The establishment of an effective method to amplify cardiac M2-like macrophages is valuable for the purposes of strengthening the above data to support the biological roles of these cells in myocardial repair as well as developing an innovative treatment for heart failure (18, 19). Here, we demonstrated that a pharmacological approach using IL-4 helped to achieve this goal. WT mice received i.p. injections of either a long-acting IL-4 complex (23) or PBS (control) and then underwent the MI-induction surgery. As expected, IL-4 treatment amplified (>2-fold) the post-MI increase in CD206+ M2-like macrophage numbers in the damaged myocardium (Figure 7). The percentage of Ki67+-expressing CD206+ cardiac M2-like macrophages originated from the donor cells (host, CD206+ CM-DiI+ cells; donor, CD206+ CM-DiI- cells). Scale bar: 50 μm. n = 6 hearts.
proliferation of CD206+ cells in the control group was increased after MI, this post-MI increase in the proliferation of CD206+ cardiac cells was more extensive following IL-4 treatment.

IL-4 treatment improved post-MI cardiac performance by strengthening the fibroblast-mediated formation of connective tissues in the infarct area. IL-4–mediated amplification of the post-MI increase in M2-like macrophages was associated with improved survival and prevented cardiac rupture (Figure 8, A and B). Furthermore, IL-4 treatment enhanced cardiac function and attenuated post-MI ventricular dilatation (Figure 8, C and D, and Supplemental Tables 2 and 3). Underpinning these benefits, the size of the infarct area was reduced, and the thickness of the infarcted ventricular wall was increased in the IL-4–treated hearts (Figure 9), together with production of more solid and supportive scar tissue. An increased amount and improved organization (alignment) of collagen fibrils in the infarct area were observed (Figure 10A). In contrast, the post-MI increase in collagen deposition in the remote and border areas (representing adverse post-MI ventricular remodeling) was unchanged or attenuated by IL-4 treatment. Myocardial expression of the Col1a1 and Col3a1 genes on day 7, but not day 28, after MI was upregulated in the IL-4–treated hearts (Figure 10B). Furthermore, an increased number, amplified activation (ratio of α smooth muscle actin+ [αSMA+] Thy1+ myofibroblasts to Thy1+ fibroblasts), and attenuated fibroblast apoptosis were noted in the infarct area in the IL-4–treated mice, whereas no alteration in the dynamics of fibroblasts in the remote and border areas was observed (Figure 11 and Supplemental Figure 12). The increased accumulation of M2-like macrophages following IL-4 treatment was also associated with improved capillary formation in the ischemic areas (Supplemental Figure 13), which would increase local perfusion and improve the viability and functionality of fibroblasts and myofibroblasts to enhance the formation of connective tissues.

Improved cardiac repair following IL-4 treatment was primarily achieved via cardiac M2-like macrophages. Several pieces of our data support the idea that the effects of IL-4–induced strengthening of fibrotic tissue formation described above were more likely to be achieved via cardiac M2-like macrophages rather than through direct effects of IL-4 on fibroblasts or other cell types. First, in Trib1−/− mice, in which the post-MI augmentation of M2 macrophages was selectively suppressed, IL-4 treatment did not affect post-MI mortality, cardiac rupture, ventricular function, activation of cardiac fibroblasts, collagen deposition, or capillary density (Figure 12 and Supplemental Table 1). Second, expression levels of IL-4 receptor α (Il4ra) in cardiac fibroblasts were remarkably reduced compared with expression in cardiac M2-like macrophages (Supplemental Figure 14A). Accordingly, our in vitro study showed that IL-4 administration did not activate cultured primary cardiac fibroblasts (Supplemental Figure 14B). Additionally, IL-4 treatment in vivo did not significantly affect collagen deposition, cardiac fibroblasts, or capillary density in the intact hearts of WT mice (Supplemental Figure 15). Taken together, these results suggest that IL-4 treatment increases the number of cardiac M2-like macrophages, which increases the activation of cardiac fibroblasts, enabling them to develop more stable fibrotic tissues to reinforce the fragile, infarcted ventricular wall. This effect improves the post-MI vital and functional prognosis by preventing rupture, dilatation, and dysfunction of the heart.

Cardiac M2-like macrophages activated fibroblasts by secreting IL-1α and osteopontin. We next used the Boyden chamber system to investigate the mechanisms underlying the ability of M2-like macrophages to regulate fibroblasts in an in vitro setting, in which primary cardiac fibroblasts were cocultured with CD206−/F4/80−/CD11b+ M2-like macrophages isolated from WT mouse hearts (with or without MI) (Figure 13A). Although coculture with M2-like macrophages from intact hearts did not activate fibroblasts, coculture with M2-like macrophages from day-7 post-MI hearts markedly increased fibroblast activation (transformation into αSMA+ myofibroblasts; Figure 13, B and C). Given the differences in gene expression in cardiac M2-like macrophages before and after MI (Figure 3, B and C), we hypothesized...
that IL-1α (35, 36) and osteopontin (37, 38) might be key mediators of the M2-like macrophage-induced activation of cardiac fibroblasts. Supporting this hypothesis, expression of Il1a and Spp1 in the myocardium on day 7 after MI tended to be increased by IL-4 treatment (Supplemental Figure 16A). In contrast, the post-MI upregulation of other profibrotic factors in M2-like macrophages, including Tgfb1 and Pdgfa (1, 2), was not significant (<1.3-fold compared with that observed in intact hearts). Indeed, Ab-mediated neutralization of IL-1α and/or osteopontin significantly suppressed the activation of cardiac fibroblast transformation into αSMA+ myofibroblasts when the cells were cocultured with post-MI M2-like macrophages (Figure 13, B and C, and Supplemental Figure 16B). These data suggest that IL-1α and osteopontin may act as paracrine mediators that are produced by cardiac M2 macrophages to regulate the activation of cardiac fibroblasts.

**Discussion**

Here, we describe the spatiotemporal-specific changes in cardiac M2-like macrophages. The healthy adult murine heart contained CD206+F4/80+CD11b+ M2-like macrophages, which increased in number after MI, peaking on day 7 (the post-MI “healing” stage) predominantly in the infarct area. In parallel, cardiac M2-like macrophages strengthened their tissue-repairing abilities by upregulating various antiinflammatory and repair-associated genes after MI. Consistent with previous reports (7, 8), these findings suggested a role for these cells in the repair of infarcted myocardium.

To precisely elucidate this role of cardiac M2-like macrophages, we conducted a “specific depletion” study using Trib1−/− mice. The functional, structural, and histological properties, including the occurrence of M2-like macrophages, of the intact, no-MI hearts of Trib1−/− mice were all comparable to those of their WT littermates.
However, these mice exhibited a complete loss of the post-MI augmentation of M2-like macrophages in the heart, without affecting other cell types including proinflammatory monocytes and macrophages. This model allowed us to dissect the precise role of M2-like macrophages after MI without bias from other subsets of macrophages, which is different from previous models using pan-macrophage depletion via techniques such as clodronate liposome injection (7–9). The results were dramatic: the selective suppression of M2-like macrophages after MI resulted in a largely reduced survival rate, with 9-fold more frequent cardiac ruptures and exacerbated cardiac dysfunction than occurred in their WT littermates. This result corresponded to a defective formation of fibrotic connective tissues in the infarct area, along with impaired accumulation and activation of cardiac fibroblasts. This finding was strengthened by an additional rescue experiment, in which M2-like macrophages were supplied externally by transplanting BMDMs collected from WT mice. The transplanted macrophages exhibited an M2 phenotype in the damaged myocardium, achieving a post-MI frequency of M2-like macrophages equivalent to that in WT mice, which is consistent with findings in the lung (39). Notably, this recovery of M2-like macrophages successfully rescued the poor post-MI prognosis and reduced tissue repair in Trib1–/– mice. This series of studies using Trib1–/– mice provides evidence for the essential role of cardiac M2-like macrophages in fibroblast-mediated repair of the infarcted, fragile myocardium via the formation of secure fibrotic tissues. This repair allows a certain degree of maintenance (imperfect, but almost sufficient to survive) of the geometry, integrity, and function of the heart after MI.

In addition, we demonstrated that external IL-4 stimulation amplified the post-MI augmentation of cardiac M2-like macrophages in WT mice. IL-4 treatment substantially strengthened the formation of connective tissues in the infarcted myocardium, which was underpinned by increased accumulation, amplified transformation into myofibroblasts, and attenuated apoptosis of fibroblasts. This “physiological” fibrotic tissue formation resulted in an improved post-MI prognosis and cardiac structure and function. In contrast, the extracellular collagen deposition in the remote and border areas, which represents adverse ventricular remodeling, was not increased by IL-4 treatment, alleviating concerns that excessive, pathological myocardial fibrosis would be induced by this treatment. Our results also demonstrated an insignificant response of cardiac fibroblasts to IL-4 stimulation in vitro, a markedly reduced expression of Il4ra in cardiac fibroblasts compared with expression in cardiac M2-like macrophages, and an absence of IL-4–induced repair in Trib1–/– mice, in which M2 macrophages, but not fibroblasts, were selectively depleted. All of these data suggested that the observed IL-4–induced activation of cardiac fibroblasts was mainly mediated by M2-like macrophages, rather than a direct effect of IL-4 stimulation on fibroblasts. Investigations using cell type–specific conditional Il4ra-KO mice will be required to further confirm the direct role of IL-4 in cardiac M2-like macrophages. Collectively, our results from the study of
selective depletion followed by rescue and the specific augmentation experiment indicated that cardiac M2 macrophages govern the fibroblast-mediated repair of infarcted myocardial tissue by forming secure fibrotic tissues, which critically determines the overall post-MI prognosis.

As regards the mechanism underlying M2-like macrophage-mediated cardiac repair, the coculture of primary cardiac fibroblasts and isolated cardiac M2-like macrophages indicated that M2-like macrophages from infarcted heart, but not those from normal heart, were able to increase the activation of cardiac fibroblast transformation into myofibroblasts. Il1a and spp1 (osteopontin) expression was upregulated by more than 3-fold in M2-like macrophages from the MI heart compared with those from the normal heart. In contrast, other known fibrotic factors, including Tgfb1 and Pdgfa, were not significantly upregulated. Indeed, the studies with neutralizing Abs confirmed that IL-1α and osteopontin are essential mediators of M2-like macrophage–induced activation of fibroblasts, at least in vitro. These data provide new biological insights

Figure 8. IL-4 treatment improved post-MI prognosis and cardiac function. (A) Post-MI survival rates of the mice were improved in the IL-4-treated group (n = 31) compared with those of the PBS-treated group (n = 29). *P < 0.05 versus the PBS-treated group, log-rank test. (B) The cardiac rupture rate over a 28-day post-MI period was reduced in the IL-4-treated group (n = 31) compared with that of the PBS-treated group (n = 29). *P < 0.05 versus the PBS-treated group, χ² test. (C) Echocardiographic analysis revealed that the IL-4–treated group showed improved cardiac function and attenuated ventricular dilatation compared with the PBS-treated group. n = 12 for each point in each group. *P < 0.05 versus the PBS-treated group at the corresponding time point, repeated-measures ANOVA. See Supplemental Table 2 for additional pre- and post-MI echocardiographic data. (D) Cardiac catheterization assessments detected improved hemodynamics and enhanced cardiac performance at day 28 after MI in the IL-4–treated group (n = 10) compared with the PBS-treated group (n = 10). *P < 0.05 versus the PBS-treated group, 2-tailed, unpaired Student’s t test. See Supplemental Table 3 for more detailed data. dP/dt, LV contractility; LVEDP, LV end-diastolic pressure; Max, maximum.

Figure 9. IL-4 treatment reduced infarct size and increased infarcted wall thickness. Picrosirius red staining of day-28 post-MI heart samples indicated that the infarct size was reduced (A) and the thickness of the walls of the infarct area was increased (B) in the IL-4–treated group compared with the PBS–treated group. Scale bars: 1 mm. n = 6 in each group. *P < 0.05 versus the PBS–treated group, 2-tailed, unpaired Student’s t test.
The “original” cardiac-resident M2-like macrophages in intact adult hearts primarily originate from the yolk sac, and upon MI injury, most of these cells disappear (via cell death and/or migration out) by day 1 after MI (7). We confirmed this attrition of original cardiac M2-like macrophages early after MI (Supplemental Figure 17). Subsequently, “new” M2-like macrophages develop in the MI heart, predominantly originating from the BM (7–9). Therefore, the major subsets of pre- and post-MI cardiac M2-like macrophages are likely to have different origins and thus may utilize different mechanisms (different roles of and/or different compensatory pathways to TRIB1) for their differentiation.

Although the cellular mechanism by which Trib1 deficiency results in a reduced frequency of M2-like macrophages after MI remains uncertain, the previously reported reduced differentiation of BM cells into M2-like macrophages (16) may be involved. In addition, the reduced post-MI proliferation of cardiac M2-like macrophages in Trib1−/− mice (Supplemental Figure 5) may also contribute to the reduced post-MI augmentation of these macrophages. The influence of Trib1 deficiency on the migration and recruitment of BM-derived M2-like macrophages and/or their precursors remains to be determined. Given that the recruitment of M1-like macrophages and monocytes from the circulation was not reduced in Trib1−/− mice (ref. 16 and Supplemental Figure 7) and that some of cardiac M2-like macrophages share the same origin with M1-like macrophages and monocytes (Ly6C+ monocytes), we hypothesize that the migration of the precursors of BM-derived M2-like macrophages might not be severely affected. However, it is not currently feasible to prove this hypothesis, because the precise identification of the circulating precursors of cardiac M2-like macrophages remains uncertain.

On the other hand, IL-4 treatment increased CD206+ cardiac M2-like macrophages both before and after MI. We believe that the pre-MI increase depends on amplified local proliferation of the original cardiac-resident M2-like macrophages. Previous studies have demonstrated that IL-4 treatment is sufficient to increase the number of M2-like macrophages through local proliferation (23, 24). Consistent with this observation, our study (Ki67 staining) revealed an increase in the local proliferation of CD206+ cardiac macrophages (16) may be involved. In addition, the reduced post-MI proliferation of cardiac M2-like macrophages in Trib1−/− mice (Supplemental Figure 5) may also contribute to the reduced post-MI augmentation of these macrophages. The influence of Trib1 deficiency on the migration and recruitment of BM-derived M2-like macrophages and/or their precursors remains to be determined. Given that the recruitment of M1-like macrophages and monocytes from the circulation was not reduced in Trib1−/− mice (ref. 16 and Supplemental Figure 7) and that some of cardiac M2-like macrophages share the same origin with M1-like macrophages and monocytes (Ly6C+ monocytes), we hypothesize that the migration of the precursors of BM-derived M2-like macrophages might not be severely affected. However, it is not currently feasible to prove this hypothesis, because the precise identification of the circulating precursors of cardiac M2-like macrophages remains uncertain.

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We used the intrapericardial injection method to deliver BMDMs (incorporated in Matrigel) into the heart after MI. This might not be a standard method, but it reproducibly resulted in the successful accumulation of BMDMs in the infarcted myocardium. One concern associated with this method is that the procedure itself (transplantation of Matrigel without BMDMs) might affect the post-MI survival or development of cardiac rupture in Trib1–/– mice, but we confirmed that this was not the case (Figure 5, A and B). Although an i.v. injection of BMDMs may be more technically straightforward, our previous study revealed that this cell-delivery method did not result in a sufficient post-MI engraftment of BMDMs into the heart (data not shown).

It is reasonable to suggest that strategies that enhance M2 macrophage-mediated cardiac repair will be useful for the treatment of MI, which remains a major cause of human death and disability (17). The therapeutic effect of this approach is based on enhancement of the intrinsic self-repair system that is mediated by naturally occurring M2 macrophages, which activate multiple M2-like macrophages in non-MI hearts following IL-4 treatment. In contrast, the augmented post-MI increase in the number of CD206+ cardiac M2-like macrophages following IL-4 treatment is likely to be underpinned by the increased differentiation and local proliferation of BM-derived M2-like macrophages. It has been repeatedly reported that IL-4 stimulates the differentiation of BMDMs into an M2-like phenotype (25, 26). Our study (Ki67 staining) revealed that the increased proliferation of CD206+ cardiac M2-like macrophages in post-MI hearts was further amplified by IL-4 treatment. Given our data showing that the original cardiac M2-like macrophages mostly disappeared early after MI, even following IL-4 treatment (Supplemental Figure 17B), it is unlikely that the original yolk-sac–derived cardiac-resident M2-like macrophages significantly contributed to the post-MI increase in CD206+ cardiac M2-like macrophage numbers following IL-4 treatment. Further investigations are needed to fully elucidate these speculated mechanisms and determine the detailed dynamics of cardiac macrophages following Trib1 KO or IL-4 treatment.

Figure 11. IL-4 treatment increased the post-MI number and activation of cardiac fibroblasts, with attenuated apoptosis. (A) IHC demonstrated that the number and activation (transformation into Thy1+αSMA+ myofibroblasts) of Thy1+ cardiac fibroblasts in the infarct area of the IL-4–treated group were increased at post-MI day 7 compared with the PBS-treated group. Scale bars: 50 μm. αSMA ratio is the percentage of αSMA+Thy1+ myofibroblasts to the percentage of Thy1+ fibroblasts. n = 6 different hearts in each group. *P < 0.05 versus the PBS-treated group, 2-tailed, unpaired Student’s t test. See Supplemental Figure 7 for additional data in other areas and at other time points. (B) Double immunofluorescence staining for Thy1 and cleaved caspase 3 demonstrated that apoptosis of cardiac fibroblasts in the infarct area of the IL-4–treated group was attenuated at post-MI day 7 compared with the PBS-treated group. Scale bars: 50 μm. n = 6 in each group. *P < 0.05 versus the PBS-treated group, 2-tailed, unpaired Student’s t test.
fully proven) (43, 44). In addition, our study did not observe any evident adverse effect of IL-4, including delayed healing or excessive fibrotic scar formation of anastomosed skin. Nevertheless, additional preclinical studies are required to completely address the safety issues surrounding IL-4 treatment. Local targeted delivery of IL-4 to the heart through an intracoronary injection or use of biomaterials for controlled drug delivery may ease the safety concerns.

In conclusion, our data provide evidence that cardiac M2-like macrophages critically govern the activity of fibroblasts and myofibroblasts in the infarcted adult murine heart. This process plays a vital role in the repair of the infarcted, fragile ventricular wall by forming firm fibrotic tissues. This tissue repair mechanism controls the degree of maintenance of the rigidity and function of the infarcted heart, thus determining the post-MI prognosis by preventing cardiac rupture and attenuating the development of heart failure. It was suggested that IL-1\(\alpha\) and osteopontin might play a role in the regulation of fibroblasts by cardiac M2-like macrophages. In addition, the targeted activation of M2-like macrophages following IL-4 administration enhances this endogenous repair mechanism, suggesting that this approach may have therapeutic potential for the treatment of MI.

Methods

Animals. Six- to eight-week-old mice were used in the experiments. C57BL/6 mice were purchased from Charles River Laboratories. Trib1\(^{-/-}\) mice were a gift of Shizuo Akira (Osaka University, Osaka, Japan) and were a hybrid of a C57BL/6 and SV129 mixed background (16). Age-matched WT littermates were used as a control. The mice were maintained in a specific pathogen-free room in our animal facility on a 12-hour light/12-dark cycle with free access to food and water. The mice were randomly assigned to different groups, and, where possible, the in vitro studies and in vivo procedures and assessments were performed in a blinded manner.

Induction of MI. MI was induced in mice by ligating the left coronary artery under 1.0% isoflurane anesthesia and mechanical venti-
The isolated cells were resuspended in FACS buffer (HBSS plus 2 mM EDTA plus 0.5% BSA) and preincubated with an anti-mouse CD16/CD32 Ab (rat, 1:100 dilution; eBioscience; catalog 14-0161) to block the Fc receptor. The dead cells and debris were excluded by forward scatter/side scatter (FSC/SSC) and DAPI staining (1:1,000 dilution; Sigma-Aldrich). To determine their phenotypes, the cells were stained with the following Abs for 3 hours at 37°C for 15 minutes, and filtered using a 40-μm cell strainer (BD Falcon). The remnant heart tissues were again digested with a fresh digestion solution and filtered similarly; this cycle was repeated 5 times. The suspension obtained at each cycle was combined and subjected to flow cytometric analyses or FACS after erythrocytes were depleted using Red Cell Lysis Buffer (BioLegend) according to the manufacturer’s protocol.

Flow cytometry and FACS. The isolated cells were resuspended in FACS buffer (HBSS plus 2 mM EDTA plus 0.5% BSA) and preincubated with an anti-mouse CD16/CD32 Ab (rat, 1:100 dilution; eBioscience; catalog 14-0161) to block the Fc receptor. The dead cells and debris were excluded by forward scatter/side scatter (FSC/SSC) and DAPI staining (1:1,000 dilution; Sigma-Aldrich). To determine their phenotypes, the cells were stained with the following Abs for 3 hours at

![Diagram](image)

**Figure 13. IL-1α and osteopontin played a vital role in cardiac M2-like macrophage–induced fibroblast activation.** (A) Schematic of the coculture experimental protocol. Primary cardiac fibroblasts were cocultured with or without CD206+F4/80+CD11b+ M2-like macrophages from intact, no-MI or MI (day 7 after MI) hearts in the Boyden chamber culture system for 48 hours. Neutralizing Abs against osteopontin and/or IL-1α were added to the relevant groups at the beginning of the coculture with M2-like macrophages. (B) Activation of cardiac fibroblasts (ratio of vimentin+αSMA+ myofibroblasts to vimentin+ fibroblasts) was increased in the cocultures with M2 (MI) macrophages but not with M2 (no MI) macrophages. Representative images are presented in C. This increased activation was eliminated by neutralizing Abs against osteopontin and IL-1α. n= 8–10 in each group. *P < 0.05, 1-way ANOVA. (C) Representative images of immunocytochemical staining for vimentin and αSMA. Nuclei were counterstained with DAPI. Scale bars: 200 μm.
4°C: APC-conjugated anti-CD11b Ab (rat, 1:100 dilution; eBioscience; catalog 17-0112); phycoerythrin– (PE–) or Alexa Fluor 488–conjugated anti-F4/80 Ab (rat, 1:20 dilution; eBioscience; catalog 12-4801 or 53-4801); Alexa Fluor 488–conjugated anti-CD206 Ab (rat, 1:50 dilution; BioLegend; catalog 141709); PerCP-conjugated anti-CD11c Ab (hamster, 1:20 dilution; BioLegend; catalog 17-5932); allophycocyanin– (APC–) or PE-conjugated anti-CCR2 Ab (rat, 1:20 dilution; R&D Systems; catalog FAB5538A or FAB5538P); and APC- or PerCP-conjugated anti-Ly6C Ab (rat, 1:25 dilution; eBioscience; catalog 17-5932 or 45-5932). Flow cytometric analyses were performed using FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star). Cell sorting was performed using a FACSaria II (BD Biosciences).

Isolation of peritoneal macrophages. Mouse peritoneal cells were collected as previously described (47). Immediately after cervical dislocation, 5 ml cold HBSS was injected into the peritoneal cavity and was lased with Red Cell Lysis Buffer (BioLegend) according to the manufacturer’s protocol, and the resulting cell suspension was subjected to FACS, as described above.

Preparation of BMDMs. Mouse BMDMs were prepared from the femurs and tibiae of 8-week-old WT mice as previously described (39). BM mononuclear cells were collected by centrifugation on Ficoll-Paque (GE Healthcare) and were cultured in DMEM (Sigma-Aldrich) containing 10% FBS, 50 μU/ml penicillin, 50 μg/ml streptomycin, and 10 ng/ml granulocyte-macrophage CSF (GM-CSF) (R&D Systems; 415-ML) overnight in a CO2 incubator. The non– or weakly attached cells were collected and transferred to new dishes and cultivated for an additional 5 days. The cells were prepared for immunocytochemistry or transplantation. Immunocytochemical staining was performed to evaluate monocyte-to-macrophage differentiation. The attached cells were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) in PBS for 5 minutes. The cells were blocked in 5% goat serum in PBS for 30 minutes. The samples were incubated with the following primary Abs overnight at 4°C: anti-CD68 Ab (1:100 dilution; AbD Serotec; catalog MCA1957); PE-conjugated anti-CD14 Ab (1:20 dilution; eBioscience; catalog 12-0141); and PE-conjugated anti-F4/80 Ab (1:20 dilution; eBioscience; catalog 12-4801). CD68 was visualized by incubating the cells with an Alexa Fluor 488–conjugated secondary Ab (1:300 dilution; Invitrogen; catalog A-11006) for 1 hour at room temperature. DAPI (1:500 dilution; Sigma-Aldrich) was used to stain the nuclei for 1 hour at room temperature. The images were acquired with an All-in-One microscope (Keyence). Immunocytochemical results are shown in Supplemental Figure 18A. Most of the produced cells were positive for CD68, CD14, and F4/80.

BMDM transplantation. For histological tracing after transplantation, BMDMs were labeled with CM-Dil (Molecular Probes) as previously described (48). CM-Dil-labeled BMDMs (2 × 105 cells) in 25 μl PBS were suspended in 25 μl Matrigel (Corning). Shortly after left coronary artery ligation (as described above, but without opening the pericardium), the BMDM-containing Matrigel was injected into the pericardial cavity. A scheme describing the BMDM transplantation is shown in Supplemental Figure 18B.

Isolation of cardiac fibroblasts. Cardiac fibroblasts were isolated from C57BL/6 mice (Charles River Laboratories) as previously described (49). The isolated mouse heart was cut into 1-mm pieces, and the fragments were plated evenly in 10-cm 0.1% gelatin-coated dishes so that they did not contact each other. Each fragment was covered by a droplet of RPMI1640 medium containing 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin and incubated for 24 hours at 37°C in a CO2 incubator. Then, a sufficient amount of medium was added to cover the entire bottom of the culture dish, and the culture was incubated for an additional 24 hours. Sufficient medium was then added to completely cover the heart fragments, followed by an additional 5 days of culture. The cells were collected by trypsinization when fibroblast outgrowth was observed. The fibroblasts were maintained in RPMI 1640 medium containing 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. After 48 hours, 2 × 104 M2-like macrophages were seeded onto polycarbonate membrane inserts ( pore size, 0.4 μm; Thermo Scientific), which were set into the wells containing fibroblasts. Neutralizing anti-osteopontin Ab (10 μg/ml; R&D Systems; catalog AF808) and/or neutralizing anti-IL-1α Ab (10 μg/ml; R&D Systems; catalog MAB400) was added to the culture medium at the beginning of coculture. After 48 hours of coculture, fibroblasts were prepared for the analysis.

Routine analysis methods. Detailed protocols of routine research methods including cardiac function measurement, RNA extraction and real-time PCR, microarray analysis, IHC, Picrosirius red staining, and immunocytochemistry are described in the Supplemental Methods.

Statistics. All statistical tests were performed using GraphPad Prism software, version 6 (GraphPad Software). Data represent the mean ± SEM. For comparisons between multiple groups, repeated-measures ANOVA or 1- or 2-way ANOVA was performed, followed by Bonferroni’s post-hoc test. Two groups were compared using the 2-tailed, unpaired Student’s t test. Cardiac rupture rates were compared with the χ2 test, whereas the survival curves were compared with the log-rank test.

Study approval. The investigations using animals conformed to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and to NIH guidelines (Guide for the Care and Use of Laboratory Animals. National Academies Press. 1996.), and all in vivo procedures were performed by UK Home Office Personal License holders. All animal studies were performed with the approval of the ethics committee of the Queen Mary University of London and the UK Home Office (project license PPL70/7254).

Author contributions
MS, Yasunori Shintani, and KS conceived and designed the research studies. MS and Yusuke Shintani conducted most of the experiments and acquired data, with professional support from Yasunori Shintani, HI, RS, AY, HA, KY, and KS. All authors participated in the analysis and interpretation of the data. KS primarily wrote and edited the manuscript, with input from all other authors.

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