Leukotriene B₄ receptor 1 exacerbates inflammation following myocardial infarction

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Abstract
Leukotriene B₄ receptor 1 (BLT1), a high-affinity G-protein-coupled receptor for leukotriene B₄ (LTB₄), is expressed on various inflammatory cells and plays critical roles in several inflammatory diseases. In myocardial infarction (MI), various inflammatory cells are known to be recruited to the infarcted area, but the function of BLT1 in MI is poorly understood. Here, we investigated the role of BLT1 in MI and the therapeutic effect of a BLT1 antagonist, ONO-4057, on MI. Mice with infarcted hearts showed increased BLT1 expression and LTB₄ levels. BLT1-knockout mice with infarcted hearts exhibited attenuated leukocyte infiltration, proinflammatory cytokine production, and cell death, which led to reduced mortality and improved cardiac function after MI. Bone-marrow transplantation studies showed that BLT1 expressed on bone marrow-derived cells was responsible for the exacerbation of inflammation in infarcted hearts. Furthermore, ONO-4057 administration attenuated the inflammatory responses in hearts surgically treated for MI, which resulted in reduced mortality and improved cardiac function after MI. Our study demonstrated that BLT1 contributes to excessive inflammation after MI and could represent a new therapeutic target for MI.
1 | INTRODUCTION

Myocardial infarction (MI) is the major cause of death and disability worldwide. MI is induced by occlusion of the coronary artery, which supplies oxygen and nourishment to cardiomyocytes. When MI develops, cardiomyocytes governed by the coronary artery undergo cell death. Dead cells release intracellular contents, which initiate inflammatory responses, resulting in the infiltration of various inflammatory cells in the infarcted area. Infiltrated inflammatory cells such as neutrophils, monocytes, and macrophages promote further inflammation by producing proinflammatory cytokines. Such inflammatory responses are essential for cardiac repair in MI. However, excessive inflammation flare-up accelerates cell death and exacerbates tissue injury, resulting in the deterioration of cardiac functions after MI. Accordingly, proper control of the inflammatory responses is important in the treatment of MI. Currently, surgical treatments such as cardiac catheter surgery and bypass surgery, and drug treatments such as sympathetic beta-receptor blockers, angiotensin II antagonists, and calcium antagonists are used to treat MI. However, only few therapies targeting the inflammatory responses that occur after MI have been established. In this study, we aimed to investigate the role of BLT1 in MI. We found that BLT1 and LTB4 were upregulated in the infarcted area after MI. In BLT1-knockout (KO) mice, the inflammatory responses at the infarcted area were attenuated, and the survival of the BLT1-KO mice after MI was significantly increased as compared with that of wild-type (WT) mice. Consistently, administration of a BLT1 antagonist immediately after MI attenuated inflammation and improved cardiac functions after MI. Our study revealed that BLT1 promotes excessive inflammation, and thereby worsens cardiac conditions after MI.

2 | MATERIALS AND METHODS

2.1 | Surgical procedure for inducing MI

We purchased C57BL/6J mice from Charles River Japan (Kanagawa, Japan) and Japan SLC (Shizuoka, Japan). BLT1-KO mice were generated as described previously. Mice (male, 8-10 weeks old) were intubated and ventilated under anesthesia. Through a left thoracotomy, the left anterior descending coronary artery was ligated permanently with a surgical suture (M6-80B2, Natsume Seisakusho, Tokyo, Japan). In sham-operated mice, the same procedure was performed, but the coronary artery was not ligated. In MI-operated hearts, infarct area looked whitish. We defined whitish ventricle as “infarct” area and the remaining ventricle as “remote” area (Supplementary Figure 1A). All experimental procedures were performed in accordance with the guidelines of Kyushu University.

2.2 | Real-time RT-PCR

Total RNA samples from sham- or MI-operated mice heart tissue were extracted using ISOGEN (Nippon Gene, Tokyo, Japan) and purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). To synthesize cDNA, a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) was used in accordance with

KEYWORDS

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with the manufacturer’s instructions. To quantify mRNA expression, we used a StepOne Plus real-time PCR system (Thermo Fisher Scientific) and TaqMan probes. The primer and probe sets were purchased from Thermo Fisher Scientific or Sigma-Aldrich (St. Louis, MO, USA). The assay ID or sequences are listed and described in Supplementary Table 2.

Relative expression was quantified using the comparative C\text{\textsubscript{T}} method. Gapdh mRNA was amplified as an internal control. In the absolute quantification of BLT1 and leukotriene B4 receptor 2 (BLT2), the plasmids pcDNA3-Flag-BLT1 and pcDNA3-HA-BLT2 were used to create standard curves, after which the copy numbers of BLT1 and BLT2 were determined from the standard curves.

### 2.3 LTB\textsubscript{4} production

The hearts of mice after MI were harvested, and the ventricles were cut into “infarct” and “remote” areas, and immediately frozen with liquid nitrogen after removing the blood and atria. The frozen tissues (~100 mg) were powdered using an SK-100 mill (Tokken Inc, Chiba, Japan), and lipids were extracted with 1 mL of methanol for 1 day at −20°C. After addition of \[^{[2H4]}\text{PGE}_2\], \[^{[2H4]}\text{PGD}_2\], \[^{[2H4]}\text{PGF}_{2\alpha}\], \[^{[2H4]}\text{6-keto-PGF}_{1\alpha}\], \[^{[2H4]}\text{TXB}_2\], \[^{[2H4]}\text{LTB}_4\], \[^{[2H8]}\text{12-HETE}\], and \[^{[2H8]}\text{15-HETE}\] (Cayman Chemical, Ann Arbor, MI, USA) as an internal standard, the samples were diluted with water containing 0.1% formic acid to yield a final methanol concentration of 10%, and then, loaded on Oasis HLB cartridges (Waters, Milford, MA, USA). The columns were sequentially washed with water, 15% methanol, and petroleum ether, all containing 0.1% formic acid. The samples were eluted with 200 µL of methanol containing 0.1% formic acid and analyzed with liquid chromatography technique coupled with tandem mass spectrometry (LC-MS/MS) as described previously. All compounds were analyzed in a negative ion polarity mode. Eicosanoids were quantified by multiple reaction monitoring (MRM). The MRM transitions monitored were m/z 351 → 271 for PGE\textsubscript{2} and PGD\textsubscript{2}; m/z 355 → 275 for \[^{[2H4]}\text{PGE}_2\] and \[^{[2H4]}\text{PGD}_2\]; m/z 353 → 193 for PGF\textsubscript{2\alpha}; m/z 357 → 197 for \[^{[2H4]}\text{PGF}_{2\alpha}\]; m/z 369 → 245 for 6-keto-PGF\textsubscript{1\alpha}; m/z 373 → 249 for \[^{[2H4]}\text{6-keto-PGF}_{1\alpha}\]; m/z 369 → 195 for TXB\textsubscript{2}; m/z 373 → 199 for \[^{[2H4]}\text{TXB}_2\]; m/z 319 → 115 for 5-HETE; m/z 319 → 167 for 11-HETE; m/z 335 → 195 for LTB\textsubscript{4}; m/z 339 → 197 for \[^{[2H4]}\text{LTB}_4\]; m/z 319 → 179 for 12-HETE; m/z 327 → 184 for \[^{[2H4]}\text{12-HETE}\]; m/z 319 → 219 for 15-HETE; m/z 327 → 226 for \[^{[2H8]}\text{15-HETE}\]. For accurate quantification, calibration curves were generated for each target eicosanoid using known reference standards and isotope-labeled internal standards. Automated peak detection, calibration, and calculation were carried out using the Xcalibur 2.2 software package.

### 2.4 Echocardiography

An echocardiographic analysis was performed using two-dimensional targeted M-mode tracings obtained using a Nemio GX image-analyzing system (SSA-580A, Canon Medical Systems Corporation, Tochigi, Japan). We performed echocardiography under anesthesia on days 14, 28, or 56 days after MI. To minimize data deviation, heart rate was maintained at approximately 500 bpm in all the mice. Fractional shortening (FS) was calculated in accordance with the following formula: %FS = [(LVIDd – LVIDs)/LVIDd] × 100. Ejection fraction (EF) was calculated using the following formula: %EF = [(end-diastolic volumes (EDV) – end-systolic volume (ESV))/EDV] × 100; EDV and ESV were calculated according to Pombo’s method.

### 2.5 Masson’s trichrome staining

The paraffin-embedded heart sections were stained with Masson’s trichrome by using a trichrome stain kit (ScyTek Laboratories, Inc, Logan, UT) in accordance with the manufacturer’s instructions. The stained sections were observed under fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan), and the infarct and left ventricular areas were assessed using a BZ-II analyzer (Keyence).

### 2.6 Evans blue/TTC staining

At 3 hours after MI, the mice were perfused with 1% Evans blue dye under anesthesia, and their hearts were excised. The hearts were cut into five segments parallel to the short axis, and the obtained heart sections were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 minutes at 37°C, and then, in 10% neutral-buffered formalin overnight. The heart sections were photographed using a digital camera, and the area at risk (negative for Evans blue), infarct area (negative for TTC), and left ventricular area were assessed using Photoshop (Adobe Systems, San Jose, CA, USA).

### 2.7 Immunohistochemistry

The paraffin-embedded mice heart sections (5-µm thick) were deparaffinized and boiled in 10 mM citrate buffer (pH 6.0) for 10 minutes for antigen retrieval. The sections were treated with 0.1% Triton X-100 for 5 minutes and subsequently blocked with phosphate-buffered saline (PBS) containing 5% BSA and 0.01% Triton X-100. The sections were stained with an anti-BLTI antibody (1:100, #120114, Cayman Chemical) at 4°C overnight, washed with PBS, and then, incubated with Alexa Fluor 488 Donkey anti-rabbit IgG antibody (1:200; Invitrogen,
Carlsbad, CA, USA) at room temperature for 1 hour. Fresh-frozen sections (6-μm thick) of mouse hearts embedded in an optimum cutting temperature compound (Sakura Finetek, Tokyo, Japan) were fixed in cold acetone at 4°C for 10 minutes and blocked with PBS containing 3% BSA and 0.05% Triton X-100. In Gr-1 staining, the sections were reacted with an FITC anti-mouse Gr-1 antibody (1:100, RB6-8C5; BioLegend, San Diego, CA, USA) at 4°C overnight. In CD68 staining, the sections were reacted with a rat anti-mouse CD68 antibody (1:200, FA-11; AbD Serotec, Raleigh, NC, USA) at 4°C overnight, washed with PBS, and then, incubated with Alexa Fluor 488 goat anti-rat IgG antibody (1:200; Invitrogen, Carlsbad, CA, USA) at room temperature for 1 hour. After staining with the fluorophore-conjugated antibodies, the sections were incubated with DAPI solution (1:1000; Dojindo, Kumamoto, Japan) for 5 minutes, mounted using the FluorSave reagent (EMD Millipore, Burlington, MA, USA), and covered with a coverslip. TdT-mediated dUTP nick end labeling (TUNEL) staining was performed using an ApoTag fluorescein direct in situ apoptosis detection kit (EMD Millipore), in accordance with the manufacturer’s instructions. The stained sections were examined under confocal microscopy (A1Rsi, Nikon, Tokyo, Japan or LSM700, Carl Zeiss, Oberkochen, Germany), and positive areas and cell numbers were quantified using the ZEN blue edition (Carl Zeiss). Four randomly selected fields per section were analyzed.

The paraffin-embedded heart sections from the patients who had undergone MI were deparaffinized and autoclaved in 10 mM citrate buffer (pH 6.0) at 121°C for 10 minutes for antigen retrieval. The sections were treated with 0.3% H2O2/MeOH solution for 10 minutes and subsequently blocked with PBS containing 10% normal goat serum. The sections were stained with primary antibodies. The stained cells were washed and incubated with primary antibodies for 60 minutes at 4°C. Subsequently, the cells were incubated with fluorophore-conjugated antibodies for 30 minutes on ice. For intracellular staining, the cells were first fixed and permeabilized using an intracellular fixation and buffer set (Thermo Fisher Scientific) in accordance with the manufacturer’s instructions, and then, stained with primary antibodies. The stained cells were washed and resuspended with PBS containing 2% FBS and 0.05% NaN₃, and then, analyzed using the FACScalibur system (BD Biosciences).

2.9 | Antibodies and reagents used for flow cytometry

The following antibodies/reagents were used: biotin-conjugated anti-BLT1 antibody (1:100; a gift from Dr Yokomizo, Juntendo University), biotin-conjugated mlgG1 isotype control (1:50, P3.6.2.8.1; Thermo Fisher Scientific), FITC-anti-Gr-1 (1:100, RB6-8C5; BioLegend), FITC-anti-Ly6C (1:100, HK1.4; BioLegend), APC-anti-F4/80 (1:100, BM8; BioLegend), guinea pig anti-vimentin (1:200, GP53; PROGEN, Heidelberg, Germany), APC-anti-CD31 (1:100, MEC13.3; BioLegend), FITC-anti-α-SMA (1:200, 1A4; Sigma-Aldrich), rabbit anti-α-actin (1:200, EP2528Y; Abcam, Cambridge, UK), streptavidin-PE, and streptavidin-APC (1:200, BioLegend), donkey anti-rabbit-Alexa Fluor 488 (1:200; Invitrogen), and donkey anti-guinea pig Alexa Fluor 488 (1:200; Jackson ImmunoResearch Laboratories, Cambridge, UK).

2.10 | BM transplantation

The recipient 8-week-old WT mice received 10 Gy of γ-irradiation. BM cells were collected from the femurs or tibias of the donor 8-week-old WT or BLT1-KO mice. The donor BM cells were resuspended in PBS and intravenously injected in the recipient mice. At 4 weeks after transplantation, the BM chimeras were subjected to MI surgery.

2.11 | BLT1 antagonist injection

The BLT1 antagonist ONO-4057 was supplied by Ono Pharmaceutical Co (Osaka, Japan). ONO-4057 (40 mg/kg;
dissolved in 7% sodium bicarbonate) or vehicle (7% sodium bicarbonate) was administered via the lateral tail vein of the mice at approximately 13 minutes after coronary artery ligation.

2.12 Statistical analyses

Results are presented as mean ± SEM from at least three independent experiments. For statistical comparisons between two groups, we used a two-tailed Student’s $t$ test, and for comparisons among multiple groups, we used one-way analysis of variance with the Tukey’s range test.

3 RESULTS

3.1 Expressions of BLT1 mRNA and LTB$_4$ are increased in the heart after MI

To examine the possible involvement of BLT1 encoded by Ltb4r1 in MI, we first measured the BLT1 mRNA expression in the MI-operated mouse hearts by real-time reverse-transcription polymerase chain reaction (RT-PCR). In the infarcted area of the hearts on day 3 after MI, the BLT1 mRNA expression level was significantly higher than that in sham-operated mouse hearts and remote areas of the mouse hearts (Figure 1A). Consistent with this result, immunostaining of the hearts on day 3 after MI using an antibody against BLT1 demonstrated that the level of the BLT1 protein was significantly increased in the infarcted area, but not in sham-operated mouse hearts and remote areas of the mouse hearts (Figure 1B). Because BLT2 encoded by Ltb4r2, has been also identified (like BLT1) as a G protein-coupled receptor for LTB$_4$,28,29 we assessed whether BLT2 mRNA expression was also upregulated after MI. Comparison of BLT1 and BLT2 mRNA expression levels in hearts surgically treated for MI by using absolute quantification methods revealed that the BLT2 mRNA expression level was markedly lower than the BLT1 mRNA expression level and did not change after MI (Figure 1C), indicating that BLT1 is the dominant receptor for LTB$_4$ in the inflammatory phase of MI.

We then measured the LTB$_4$ level in infarcted mouse hearts. The mouse hearts after MI were immediately collected,
frozen rapidly, and then, measured with mass spectrometry. The analysis revealed that the LTB₄ levels were remarkably increased in the infarcted area of surgically treated MI hearts (Figure 1D). We also measured the production of other lipid mediators derived from arachidonic acid, such as 6-keto-PGF₁α, PGD₂, PGE₂, PGF₂α, TXB₂, 5-HETE, 11-HETE, 12-HETE, and 15-HETE in surgically treated MI hearts (Supplementary Figure 1B), and found that the production

**FIGURE 2**  BLT1 deficiency reduces the mortality rate and infarct size after MI. A. Kaplan-Meier survival curves of the WT (blue line; n = 27) and BLT1-KO mice (red line; n = 36) after MI. The dotted line represents the data obtained for the sham-operated mice (WT, n = 11; KO, n = 15). Differences in survival rate between the WT-MI and BLT1-KO-MI mice were tested using a log-rank test. ### P < .001. B. Evans blue and triphenyltetrazolium chloride staining of WT (n = 3) and BLT1 KO mouse hearts (n = 3) at 3 hours after MI. Scale bar, 1 mm. The ratios of the infarct area to the area at risk (AAR; infarct area/AAR) and the AAR to the left ventricular (LV) size (AAR/LV) are shown in the right panels. C, Echocardiographic measurements of the WT mice (sham, n = 11; MI, n = 9) or BLT1-KO mice (sham, n = 14; MI, n = 21) at 28 days after MI. The LVIDs, LVIDd, LVPWd, IVSTd, EF, and FS are shown. D, Ratio of heart weight (HW) to body weight (BW) of the WT mice (sham, n = 11; MI, n = 9) or BLT1-KO mice (sham, n = 14; MI, n = 19) at 28 days after MI. E, Masson’s trichrome staining of paraffin-embedded ventricular sections from the WT (n = 10) and BLT1-KO mice (n = 11) on day 28 after MI. The ratio of the infarct area to the LV area is shown in the right panel. Scale bar, 1 mm. Error bars represent the mean ± SEM. B, E, * P < .05; n.s., not significant, unpaired two-tailed Student’s t test. C, D, ** P < .01; *** P < .001, one-way ANOVA followed by Tukey’s range test.
of 6-keto-PGF1α, PGD2, PGE2, PGF2α, TXB2, 5-HETE, 11-HETE, 12-HETE, and 15-HETE were increased in the infarct area of the surgically treated MI hearts (Supplementary Figure 1C). Furthermore, the mRNA expression level of LTA4H, an enzyme required for LTB4 biosynthesis, was also increased in the infarcted area of the surgically treated MI hearts (Figure 1E). These results suggest that LTB4-BLT1 is involved in the pathological condition of hearts after MI.

3.2 | BLT1 deficiency improves survival and cardiac functions after MI

To investigate the pathophysiological role of BLT1 in MI, we compared the pathological conditions of WT and BLT1 KO mice after MI for 4 weeks. The survival rate of the surgically treated BLT1-KO mice with MI was significantly higher than that of the surgically treated WT mice with MI (Figure 2A). Staining of heart sections with Evans blue dye and TTC revealed that the sizes of the infarcted area and area at risk did not differ significantly between the WT and BLT1-KO mice in the initial phase after MI (Figure 2B). Autopsies revealed that the major cause of death was cardiac rupture. Next, we assessed the cardiac morphology and function of the WT and BLT1-KO mice on day 28 after MI by using echocardiography. The echo-cardiographic analysis revealed that the cardiac function parameters, left ventricular end systolic internal diameter (LVIDs), left ventricular end-diastolic internal diameter (LVIDd), and left ventricular posterior wall thickness in diastole (LVPWd) increased after MI operation, and the increases were significantly attenuated in BLT1 KO mice, indicating that cardiac functions were improved in MI-operated BLT1-KO mice as compared with MI-operated WT mice (Figure 2C). Similarly, interventricular septal thickness at end-diastole (IVSTd), ejection fraction (EF), and fractional shortening (FS) decreased after MI operation, and the decreases were significantly attenuated in BLT1 KO mice (Figure 2C). On the contrary, cardiac functions did not differ between the WT and BLT1 KO mice at baseline (Supplementary Table 1). Consistent with these results, on day 28 after MI, the increases in the ratios of heart weight/body and lung weight/body of the BLT1-KO mice were lower than those of the surgically treated MI WT mice (Figure 2D and Supplementary Figure 2). Furthermore, Masson’s trichrome staining of heart sections and quantitative measurement of the infarcted areas revealed that the infarcted area in the BLT1-KO mice was smaller than that in the WT mice on day 28 after MI (Figure 2E), although the BLT1-KO mice did not show reduction in infarcted area in the initial phase of MI as compared with the WT mice (Figure 2B). Collectively, our data indicate that BLT1 exacerbated the cardiac conditions after MI.

3.3 | BLT1 promotes leukocyte infiltration and inflammatory responses in the infarcted mouse hearts

In the aforementioned analysis, the difference in survival rate between the WT and BLT1-KO mice was evident on days 3-7 after MI (Figure 2A). Thus, we compared the heart conditions of the WT and BLT1-KO mice on day 3 after MI. As MI robustly induces leukocyte infiltration and inflammatory responses at the infarcted area, we first examined the degree of infiltration of neutrophils and monocytes/macrophages after MI. Immunofluorescence staining using antibodies against Gr-1 (a neutrophil marker) and CD68 (a monocyte/macrophage marker) revealed that in the peri-infarcted area, infiltration of neutrophils (Gr-1+ cells), and monocytes/macrophages (CD68+ cells) were reduced in the BLT1 KO mice as compared with that infiltration in the WT mice (Figure 3A). These results suggest that inflammatory cytokine production was decreased in the BLT1-KO mice after the reduction of the infiltrated leukocytes. To verify this, we performed real-time RT-PCR to assess the proinflammatory gene expression in the hearts on day 3 after MI. In the WT mice, the mRNA expression levels of the pro-inflammatory genes Tnf, Il1b, and Cxcl2 were significantly increased in the infarcted area, but the increased expressions were significantly attenuated in the BLT1-KO mice (Figure 3B). We also examined the mRNA expressions of the fibrosis-related genes Acta2 and Ctgf, and found that these genes showed increased expression levels in the infarcted area after surgery for MI, and the increase in Acta2 expression level was significantly attenuated in the BLT1-KO mice (Figure 3C). The upregulation of Ctgf in the infarcted area also tended to be decreased in the BLT1-KO mice, although the decrease was not statistically significant.

Excessive inflammation has been reported to lead to cardiac tissue damage and cell death. Thus, we assessed the degree of apoptosis in the infarcted hearts by TUNEL method on day 3 after MI. This experiment revealed that the number of TUNEL-positive cells in the peri-infarcted area of the BLT1-KO mice was decreased as compared to WT mice (Figure 3D), demonstrating that apoptosis of cardiac cells at the peri-infarcted area was attenuated in the BLT1-KO mice. Recently, a new form of programed cell death distinct from apoptosis, called necroptosis, has received attention and is reported to be involved in the pathology of MI. Therefore, we also measured the gene expression of receptor-interacting serine/threonine-protein kinase 3 (RIPK3), which is essential for necroptosis
in surgically treated MI hearts. As previously reported, the Ripk3 expression was induced in the infarcted area, but this induction was significantly decreased in the BLT1-KO mice (Figure 3E), suggesting that necroptosis is also attenuated in the BLT1-KO mice. These results together indicate that BLT1 induces cell death of cardiac cells by promoting leukocyte infiltration and proinflammatory gene expression after MI.

**FIGURE 3** BLT1 promotes leukocyte infiltration and inflammatory responses in the mouse hearts after MI. A, Immunofluorescence micrographs of peri-infarct areas of the WT and BLT1 KO mouse hearts on day 3 after MI. The heart sections were co-stained with antibodies against Gr-1 (green) or CD68 (green), and DAPI (blue). Scale bar, 50 μm. Quantitative data are shown in the right panel (n = 3 each). B, mRNA expression levels of the proinflammatory genes in the sham (sh)-operated ventricles, and in the remote (rem) and infarct (inf) areas of the WT and BLT1 KO mouse hearts on day 3 after MI (WT: sh/rem/inf, n = 3/5/5; KO: sh/rem/inf, n = 3/5/5). C, mRNA expression levels of the fibrosis-related genes in the sham (sh)-operated ventricles, and in the remote (rem) and infarct (inf) areas of the WT and BLT1 KO mouse hearts on day 3 after MI (WT: sh/rem/inf, n = 3/5/5; KO: sh/rem/inf, n = 3/5/5). D, Representative images of TUNEL staining of the peri-infarct areas of the surgically treated mouse hearts with MI. Heart sections were obtained from the WT and BLT1 KO mice on day 3 after MI. Quantitative data are shown in the right panel (WT, n = 4; KO, n = 5). Scale bar, 50 μm. E, RIPK3 mRNA expression level in the sham (sh)-operated mouse ventricles, and in the remote (rem) and infarct (inf) areas of the mouse hearts on day 3 after MI (WT: sh/rem/inf, n = 3/5/5; KO: sh/rem/inf, n = 3/5/5). Error bars represent the mean ± SEM. (A, D) *P < .05; **P < .01, unpaired two-tailed Student’s t test. B, C, E, *P < .05; ***P < .001; n.s., not significant, one-way ANOVA followed by Tukey’s range test.
3.4 | BLT1 is expressed on neutrophils and monocytes/macrophages in the infarcted mouse hearts

Next, we examined the cells in mouse hearts with MI that express BLT1. The mouse hearts 3 days after MI were digested by enzymes, and the cells were stained by antibodies against marker proteins of specific leukocytes such as Gr-1, Ly6C (a monocyte and neutrophil marker), and F4/80 (a macrophage marker), together with an antibody against BLT1. The stained cells were subsequently subjected to flow cytometry analysis. The analysis revealed that BLT1 was expressed on the cells positive for Gr-1, Ly6C, and F4/80, indicating that neutrophils and monocyte/macrophages expressed BLT1 (Figure 4A). In particular, Gr-1+ neutrophils expressed BLT1 at high levels, while F4/80+ monocytes/macrophages expressed BLT1 at lower levels than did neutrophils. We further examined the BLT1 expression on nonleukocytes. Flow cytometry analysis using antibodies against vimentin (a fibroblast marker), CD31 (an endothelial cell marker), α-smooth muscle actin (αSMA; a myofibroblast marker), and α-actin (a cardiomyocyte marker) demonstrated that fibroblasts, endothelial cells, myofibroblasts, and cardiomyocytes did not express BLT1 (Figure 4B). These results indicate that BLT1 was mainly expressed in leukocytes, especially neutrophils in the hearts after MI. The expression of BLT1 in CD11b-positive cells (neutrophils and monocytes/macrophages) were also observed in the patients with MI (Figure 4C), suggesting that BLT1 is linked to the pathogenesis of human MI.

3.5 | BLT1 expression on BM-derived cells promotes leukocyte infiltration and inflammatory responses after MI

To confirm that BLT1 on leukocytes recruited to the infarcted hearts contributes to the deterioration of cardiac conditions after MI, we performed BM transplantation. First, we prepared the following chimeric mice: WT mice transplanted with WT BM cells (WT → WT) and WT mice transplanted with BLT1-KO BM cells (KO → WT). The BM-transplanted mice were subjected to surgery for MI. Immunofluorescence staining revealed that in the peri-infarcted area, the numbers of Gr-1+ neutrophils and CD68+ monocytes/macrophages present in the KO → WT mice were lower than those in the WT → WT mice (Figure 5A). Real-time RT-PCR experiments confirmed that the Ltb4r1 mRNA expression level was markedly decreased in the KO → WT mice on day 3 after MI (Figure 5B) and revealed that the upregulation of proinflammatory genes after MI in the infarcted area was attenuated in the KO → WT mice as compared with the WT → WT mice (Figure 5C). Consistently, the upregulation of the fibrotic gene Ctgf was decreased in the infarcted area of the KO → WT mice (Figure 5D), although the upregulation of another fibrotic gene, Acta2, in the infarcted area was not statistically significant (Figure 5D). Moreover, in agreement with these results, TUNEL-positive cells were fewer and the RIPK3 mRNA expression levels were lower in the KO → WT mice as compared with those in the WT → WT mice (Figure 5E,F).

To further clarify the involvement of BLT1 expression on BM-derived cells in the pathological condition of MI, we conducted the aforementioned experiments in two other chimeric mice generated as follows: BLT1-KO mice transplanted with BLT1-KO BM cells (KO → KO) and BLT1 KO mice transplanted with WT BM cells (WT → KO). The immunostaining experiment revealed that the number of Gr-1+ neutrophils in the KO → KO mouse hearts was lower than that in the WT → KO mouse hearts on day 3 after MI induction (Supplementary Figure 3A). Moreover, the BLT1 mRNA expression level was increased in the infarcted area in the WT → KO mice and, as expected, not detected in the KO → KO mice (Supplementary Figure 3B). The MI-induced upregulations of proinflammatory genes and Ripk3 were more enhanced in the WT → KO mice than in the KO → KO mice (Supplementary Figure 3C, D). These results indicate that BLT1 expressed on BM-derived cells plays a pivotal role in exacerbating the adverse condition in surgically treated MI hearts.

3.6 | Administration of a BLT1 antagonist improves post-MI cardiac condition

The above-mentioned results show that BLT1 worsened the cardiac conditions after MI by promoting leukocyte infiltration and exacerbating the inflammatory responses. Therefore, we conducted experiments by using a BLT1 antagonist (ONO-4057) to examine whether BLT1 could serve as a therapeutic target for MI. ONO-4057 (40 mg/kg) was intravenously injected in the mice immediately after MI surgery, and the mortality of the mice was evaluated over 28 days. We found that the survival rate was improved by ONO-4057 administration (Figure 6A). Consistently, the deterioration of both EF and FS after MI was significantly suppressed in the ONO-4057-treated mice as compared to the vehicle-treated mice (Figure 6B and Supplementary Figure 4A). As BLT1 promotes leukocyte infiltration into the peri-infarcted area after MI, we further examined whether administration of ONO-4057 attenuated this infiltration. Immunofluorescence staining of mouse hearts on day 1 after MI and ONO-4057 administration demonstrated that the infiltration of Gr-1+ neutrophils and CD68+ monocytes/macrophages was
FIGURE 4  BLT1 expression on leukocytes in infarcted mouse and human hearts. Flow cytometry analysis of BLT1 expression on cells isolated from WT mouse hearts on day 3 after MI. A, BLT1 expression on Gr-1+ neutrophils, Ly6C+ neutrophils/monocytes, and F4/80+ monocytes/macrophages. B, BLT1 expression on vimentin+ fibroblasts/myofibroblasts, α-SMA+ myofibroblasts, CD31+ endothelial cells, and α-actinin+ cardiomyocytes. Dotted lines: control. C, Immunofluorescence micrographs of the infarct areas of the MI patients (n = 5). The heart sections were co-stained with antibodies against BLT1 (red), CD11b (green), and DAPI (blue). Scale bar, 50 μm
FIGURE 5  BLT1 expression on BM-derived cells promotes leukocyte infiltration and inflammatory responses. A, Immunofluorescence micrographs of the peri-infarct areas of the BM-transplanted mouse hearts on day 3 after MI. The BM-transplanted mice were generated by transplanting the BM cells from the WT or BLT1 KO mice into the lethally irradiated WT mice (WT → WT, n = 4; KO → WT, n = 4). Heart sections were co-stained with antibodies against Gr-1 (green) or CD68 (green) and DAPI (blue). Quantitative data are shown in the right panels. Scale bar, 50 μm. B-D, mRNA expression levels of (B) Ltb4r1, (C) proinflammatory genes, and (D) fibrosis-related genes in the hearts of the sham (sh)-operated BM-transplanted mice, and in the remote (rem) and infarct (inf) areas of the BM-transplanted mouse hearts on day 3 after MI (WT → WT: sham/rem/inf, n = 4/8/8; KO → WT: sham/rem/inf, n = 4/8/8). E, Representative micrographs of TUNEL staining in the peri-infarct areas of the BM-transplanted mice on day 3 after MI (WT → WT, n = 4; KO → WT, n = 4). Quantitative data are shown in the right panel. Scale bar, 50 μm. F, RIPK3 mRNA expression level in the hearts of the sham (sh)-operated BM-transplanted mice and in the remote (rem) and infarct (inf) areas of the BM-transplanted mouse hearts on day 3 after MI (WT → WT: sham/rem/inf, n = 4/8/8; KO → WT: sham/rem/inf, n = 4/8/8). Error bars represent the mean ± SEM. A, E, *P < .05; **P < .01, unpaired two-tailed Student’s t test. B-D, F, ***P < .01; ****P < .001; n.s., not significant, one-way ANOVA followed by Tukey’s range test.
decreased by the administration (Figure 6C). In addition, the administration attenuated the upregulation of proinflammatory cytokine production after MI. In this study, we revealed that BLT1 expressed on the leukocytes recruited to the infarct hearts worsened the inflammation in the hearts, which led to deterioration of cardiac functions after MI. In the BLT1-KO mice, infiltration of neutrophils/macrophages to the infarct area and the expression levels of inflammatory cytokines and chemokines in the heart were significantly reduced as compared with those in the WT mice. Bone marrow transplantation experiments revealed the role of BLT1 on leukocytes recruited to the infarct area.

**FIGURE 6**  BLT1-antagonist administration improves cardiac condition by attenuating leukocyte infiltration and proinflammatory cytokine production after MI. A, Kaplan-Meier survival curves of the WT mice after MI and intravenous administration of vehicle (7% sodium bicarbonate; n = 23) or ONO-4057 (BLT1 antagonist; 40 mg/kg; n = 25). Differences in survival between the vehicle- and ONO-4057-administered mice were tested using the log-rank test. $^{*}P < .05$. B, Echocardiographic measurements of the vehicle- (n = 10) or ONO-4057-administered mice (n = 19) at 8 weeks after MI. C, Quantification of leukocyte infiltration into the peri-infarct areas of the mouse hearts on day 3 after MI and administration of vehicle (n = 7) or ONO-4057 (n = 8). D, mRNA expression levels of proinflammatory genes in the remote (rem) and infarct (inf) areas of the WT mouse hearts on day 1 after MI and intravenous injection of vehicle (n = 10) or ONO-4057 (n = 9). E, Representative micrographs of TUNEL staining in the peri-infarct areas of the WT mouse hearts on day 1 after MI and intravenous injection of vehicle (n = 8) or ONO-4057 (n = 9). Quantitative data are shown in the right panel. Scale bar, 50 μm. Error bars represent the mean ± SEM. B, C, E, $^{*}P < .05$; $^{**}P < .01$, unpaired two-tailed Student’s t test. D, $^{*}P < .05$; n.s., not significant, one-way ANOVA followed by Tukey’s range test.

**4 DISCUSSION**

Excessive inflammatory responses during MI increase tissue injury and exacerbates cardiac function. In this study, we revealed that BLT1 expressed on the leukocytes recruited to the infarct hearts worsened the inflammation in the hearts, which led to deterioration of cardiac functions after MI. In the BLT1-KO mice, infiltration of neutrophils/macrophages to the infarct area and the expression levels of inflammatory cytokines and chemokines in the heart were significantly reduced as compared with those in the WT mice. Bone marrow transplantation experiments revealed the role of BLT1 on leukocytes recruited to the infarct area.
We also revealed for the first time that the LTB4 expression level increases in infarcted mouse hearts. The LTB4-BLT1 pathway is known to be involved in the chemotaxis of leukocytes and inflammatory responses mainly through Gi protein activation. However, until now, among the molecules in the pathway, only 5-LOX, the LTB4-producing enzyme, clearly revealed its role in MI by using KO mice. For BLT1, only one report demonstrated the favorable effect of a BLT1 antagonist, LSN2792613, on ischemia-reperfusion. Regarding BLT2, only one study reported that BLT2 antagonist administration has no effect on the pathological condition after MI. In this study, we discovered that BLT1 is abundantly expressed and BLT2 is hardly expressed in the heart with MI. This result provides the molecular basis of the previous report that the BLT2 antagonist does not affect the MI condition. Furthermore, by using BLT1-KO mouse, we demonstrated that BLT1 contributes to an excessive inflammatory response after MI. Consistent with this result, we found that ONO-4057, an antagonist of BLT1, improves the pathology of MI (permanent occlusion model).

The emerging evidence shows that necroptosis, a new type of cell death, is tightly linked to the pathogenesis of MI. Deficiency of RIPK3, a main regulator that promotes necroptosis, in mice improved the deterioration of cardiac functions after MI. We found that the mRNA expression level of necroptosis-related gene, RIPK3, was decreased in the infarcted region of the BLT1-KO mice as compared with the infarcted region of the WT mice. Therefore, suppression of necroptosis after MI could contribute to the improvement of cardiac conditions after MI in BLT1 KO mice. On the other hand, RIPK3 has also been reported to be involved in the activation of inflammasome, which is a protein complex that converts IL-1β and IL-18 precursors into IL-1 and IL-18. Considering that inflammasome formation exacerbates the pathological condition after MI, BLT1 may also be involved in the inflammasome formation via RIPK3.

To date, many research studies have been conducted on therapeutic agents targeting inflammation during MI. For example, cardiac function after MI is improved by suppressing inflammatory responses such as inhibition of inflammatory cytokine production at the time of MI. MI treatment options for MI.

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CONFLICT OF INTEREST
The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS
M. Nakaya and H. Kurose designed research; Y. Horii, H. Ohara, H. Nishihara, K. Watari, T. Nakaya, A. Nagasaka, Y. Sugiuara, and T. Okuno performed experiments, and analyzed data; A. Tanaka, T. Yokomizo analyzed data and contributed to the scientific discussion. M. Nakaya and H. Kurose wrote the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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