Involvement of neutrophils and macrophages in exhaustive exercise-induced liver, kidney, heart, and lung injuries

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ABSTRACT

Moderate exercise is effective for maintaining or improving overall health. However, excessive exercise that exhausts the adaptive reserve of the body or its ability to positively respond to training stimuli can induce tissue damage and dysfunction of multiple organs and systems. Tissue injury, inflammation, and oxidative stress are reportedly induced in the skeletal muscles, liver, and kidneys after exercise. However, the precise mechanisms underlying acute tissue injury after intense exercise have not yet been fully elucidated.

Studies using various experimental models of acute tissue injury, other than intense exercise, have demonstrated infiltration of inflammatory cells, including neutrophils and macrophages. These cells infiltrate injured tissues and induce inflammatory and oxidative stress responses by producing inflammatory cytokines and reactive oxygen species, thereby exacerbating tissue injury. In addition to the activation of blood neutrophils and increase in their levels during and/or after prolonged or intense exercise, chemokines that contribute to leukocyte migration are released, facilitating the migration of neutrophils and monocytes into tissues. Therefore, neutrophils and macrophages, activated by exhaustive exercise, may infiltrate tissues and contribute to exhaustive exercise-induced tissue injury. Recently, the contributions of neutrophils and macrophages to various tissue injuries caused by exhaustive exercise have been reported. In this review, we summarize the involvement of neutrophils and monocytes/macrophages in exhaustive exercise-induced non-skeletal muscle tissue injury. In addition, we present novel data demonstrating the contribution of neutrophils and macrophages to exhaustive exercise-induced cardiac and pulmonary injuries. Our study findings and the evidence presented in this review suggest that neutrophils and macrophages may play pivotal roles in exhaustive exercise-induced tissue injuries.

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INTRODUCTION

Moderate exercise is effective in maintaining and improving overall health, particularly in preventing metabolic syndrome, type 2 diabetes, and cardiovascular diseases [6, 80]. These effects arise from the stimulation of the internal environment of the body, triggering adaptive responses within the musculoskeletal, metabolic, digestive, cardiovascular, and respiratory systems. Specifically, endurance exercises cause an increase in mitochondrial content and function, resulting in enhanced aerobic power [10]. Consequently, various metabolic changes occur, including slower utilization of muscle glycogen and blood glucose, increased reliance on fat as an energy source, glycogen preservation, and lower production of lactate [23]. Furthermore, endurance exercises trigger cardiovascular adaptations, such as increased skeletal muscle capillary density, increased maximal cardiac output, and respiratory changes such as increased ventilation [3, 12, 20]. However, excessive exercises that exhaust the adaptive reserve of the body or its ability to positively respond to training stimuli can counteract the benefits of exercise [42, 62, 74]. Several conditions, including age, fatigue, disease state, and acute or chronic excessive exercise, can impair the adaptive reserve of the body. Recent studies suggest that the benefits of exercise may be decreased in individuals who frequently perform intense exercises, and a J- or U-shaped association has been reported between all-cause or cardiovascular mortality and running amount and frequency [42, 62, 74]. This phenomenon may be influenced by prolonged strenuous exercise-induced organ dysfunction and injury, as evidence suggests that injury occurs in tissues, such as the skeletal muscle, liver, and kidney, after prolonged endurance exercise in humans [78, 79]. Similarly, injury, inflammation, and oxidative stress have been reported in the skeletal muscle, liver, and kidney in animal models following exhaustive exercise using treadmill running and forced swimming methods [47, 52]. Therefore, exhaustion models have been widely utilized to reveal the mechanisms underlying tissue injuries caused by prolonged intense exercise and to establish preventive methods.

Recently, the contribution of neutrophils and macrophages, mediated by inflammation and oxidative stress, to exercise-induced tissue injuries has received increased attention. Prolonged strenuous

exercise increases the number of neutrophils in the blood circulation. This increase is bimodal, with an initial increase in the number of segmented neutrophils recruited from the marginated pool [82]. In contrast, neutrophils whose counts increase 2–3 h after prolonged strenuous exercise show a leftward shift of the nucleus [84] and high expression of CD62L, an adhesion molecule [39], indicating that these cells are derived from the bone marrow reserve. In addition, the plasma levels of neutrophil-derived granular enzymes increase with prolonged exercise [84], suggesting that prolonged and strenuous exercise activates neutrophils and causes degranulation. We have previously reported an increase in blood neutrophil counts after exhaustive exercise in a mouse model [61]. This increase may be attributed to the mobilization of neutrophils from the bone marrow reserve pool, as evidenced by the increased number of CD62L-expressing neutrophils and the decreased percentage of neutrophils in the bone marrow [59], supporting the results of previous studies [39, 84]. We have also noted increased CD11b and CD62L expression [61], suggesting that neutrophil migration is enhanced by exhaustive exercise. Furthermore, the number of monocytes in the blood increases, and these cells are activated after acute strenuous exercise [7]. Leukocyte infiltration into tissues is regulated by chemokines and adhesion molecules. Interleukin (IL)-8, which promotes neutrophil infiltration and activation, is released into the circulatory system during intense exercise [84, 85]. In addition, the level of monocyte chemotactic protein (MCP)-1, a chemokine that promotes monocyte and macrophage infiltration and activation, in the blood increases after prolonged strenuous exercise [84, 85]. Thus, after prolonged or intense exercise, neutrophils and monocytes are likely to migrate into the tissues. These cells have been reported to infiltrate tissues in various tissue injury models and cause inflammation and oxidative stress through the production of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-6, and IL-1β, and reactive oxygen species (ROS), which are involved in tissue injury [15, 40, 68, 87, 92, 93]. In addition, these cells also contribute to exercise-induced muscle, liver, and kidney injuries [30, 31, 60, 61,102].

Exhaustive exercise can induce a systemic inflammatory response, resulting in tissue injury in the heart, lungs as well as in skeletal muscle, kidney and liver [44]. Notably, neutrophils and macrophages contribute to the onset and exacerbation of tissue injury in cardiac and pulmonary damage models, in which injuries have been induced using methods other than exercise [14, 21, 57, 58], suggesting that these cells may also contribute to exhaustive exercise-induced heart and lung injuries. Therefore, the present study aimed to test the hypothesis that increased infiltration of neutrophils and macrophages after exhausting exercise induces secondary cardiac and lung injuries. Although the involvement of neutrophils and macrophages in exercise-induced skeletal muscle injury has been systematically documented [83, 86], to the best of our knowledge, there are no reviews on the mechanisms of tissue injury other than skeletal muscle injuries. Therefore, we provide a comprehensive review that summarizes the involvement of neutrophils and macrophages in exhaustive exercise-induced liver, kidney, heart, and lung injuries.

Involvement of neutrophils and macrophages in exhaustive exercise-induced liver injuries

Prolonged exercise causes injuries to both the skeletal muscle and the liver. The susceptibility of the liver to injury from intensive exercise is attributed to sustained energy depletion and metabolic disturbances [25]. This susceptibility arises because the liver is a tissue that contributes to the neutralization of toxic substances, requiring substantial amounts of ATP [73], and becomes exposed to toxic metabolites when its production of ATP is suppressed by energy depletion and metabolic disturbances. In addition, the liver receives approximately 30% of the total cardiac output, 70% of which is via the portal venous system and 30% via the hepatic artery; this complex vascular supply renders hepatocytes susceptible to circulatory disturbances [4, 53]. After endurance exercise, the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), biomarkers of liver injury in the blood, increase in both animals and humans [72, 79]. In addition, we have previously observed histological changes, including hemorrhage, cytoplasmic vacuolation, inflammatory cell infiltration, impaired radial arrangement of hepatocytes, and cytoplasmic fragmentation, in the liver after exhaustive exercise [61].

Various experimental models of acute liver injury other than exhaustive exercise have demonstrated the infiltration of inflammatory cells, including neutrophils and macrophages [68, 91]. The role of neutrophils as major amplifiers of liver injury via the production of inflammatory cytokines and ROS has been previously discussed [5, 16]. neutrophil depletion using anti-GR1 antibodies ameliorates liver injury [55]. Blocking neutrophil infiltration in MMP-9-knockout mice or mice treated with MMP-9-inhibitory antibodies alleviates ischemia/reperfusion-induced liver injury and decreases the production of inflammatory cytokines [17]. Furthermore, alleviating oxidative stress via the inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase alleviates neutrophil-induced liver injury after ischemia/reperfusion [18]. Recently, we have demonstrated that suppressing neutrophil infiltration into the liver using anti-neutrophil antibodies decreases the production of inflammatory cytokines and ROS in the liver after exhaustive exercise and alleviates exhaustive exercise-induced liver injury, assessed by blood ALT and AST activity and the liver stress score based on hematoxylin and eosin (HE) staining [61].

Macrophages are mobilized to the liver by chemokines, such as MCP-1, and play a role in acute liver injury [28]. They trigger an inflammatory response in the liver by activating the production of inflammatory cytokines and ROS, which exacerbate liver injury [26, 63]. Notably, Zhou et al. have recently reported increased macrophage infiltration in the injured liver after exhaustive exercise [102]. They reported that the administration of anti-inflammatory and antioxidant substances suppressed macrophage infiltration, inflammatory cytokine release, and liver injury, which was assessed by blood AST and ALT levels or histologically (HE staining), suggesting that macrophages are mediators of the inflammatory response in exhaustive exercise-induced liver injury. Furthermore, we have reported that the administration of anti-neutrophil antibodies suppresses macrophage infiltration and MCP-1 production [61]. As neutrophils release MCP-1, which modulates macrophage infiltration into local tissues and triggers an inflammatory response [77], neutrophils may serve as a source of chemokines in the injured liver, thereby contributing to liver injury via macrophage infiltration after exhaustive exercise.

Involvement of neutrophils and macrophages in exhaustive exercise-induced renal injuries

Strenuous exercise-induced tissue injury extends to the kidneys, with the incidence of acute kidney injury after endurance exercise events, such as marathon and triathlon races, reported to be 4%–86% [22, 29, 46, 54, 70]. A recent systematic review reported 27 cases of acute kidney injury requiring hospital treatment following an endurance event, as described in 11 case reports [13]. In addition, assessment of renal function before and after endurance exercise in 800 endurance event participants in 21 studies showed increased levels of serum creatinine, a marker of kidney injury, after endurance exercise. These findings were corroborated in an animal model in which exhaustive exercise increased plasma blood urea nitrogen and creatinine levels [45]. Moreover, histological changes, such as enlarged glomeruli, collapsed tubular epithelial cells, loss of brush border membranes in proximal epithelial cells, dilatation of tubules, and intratubular cast formation, have also been observed in animal models [45].

Exercise-induced acute kidney injury is attributed to several causes, including muscle injury, sympathetic tone, hypohydration, and ischemia/reperfusion. In particular, ischemia/reperfusion induces injury in various tissues, including the kidney. During exercise, blood flow to the kidneys is reduced by up to 25%; therefore, strenuous exercise can cause acute kidney injury [69], similar to that induced by ischemia/reperfusion. In ischemia/reperfusion-induced acute kidney injury, inflammatory cells, such as Neutrophils and macrophages, infiltrate and exacerbate tissue injury through the production of inflammatory cytokines and ROS. Neutrophils are among the early responders, entering the kidney within hours after ischemia/reperfusion-induced injury [43]. Notably, neutrophil depletion by anti-neutrophil serum administration ameliorates acute kidney injury induced by ischemia/reperfusion [19, 36]. Similar results have been reported with neutrophil depletion following the administration of antibodies against ICAM-1 or knockout of ICAM-1 [33, 34]. In addition. macrophage infiltration increases within 24 hours [99]. Studies on clodronate liposome-induced macrophage depletion have suggested that macrophages promote early injury after ischemia/reperfusion [11, 66]. Furthermore, Jo et al. reported that macrophage depletion with clodronate suppresses the production of inflammatory cytokines and chemokines after ischemia/reperfusion-induced acute kidney injury, indicating that macrophages are important mediators of this injury [27]. We tested our hypothesis that neutrophils and macrophages are also likely involved in exhaustive exercise-induced acute kidney injury. We found that exhaustive exercise induced marked histological changes, including congested and swollen glomeruli, tubular dilatation, and nuclear infiltration, as well as increased levels of kidney injury markers, apoptosis, and inflammatory responses in the kidneys. Notably, these exhaustive exercise-induced responses were suppressed by macrophage depletion [60]. Furthermore, we found that blocking neutrophil infiltration in the kidneys using anti-neutrophil antibody alleviates exhaustive exercise-induced renal injury (unpublished data).

Exhaustive exercise-induced injury to the heart, lungs, and pancreas

The cardiovascular response to exercise involves an increase in the heart rate and contractility due to increased sympathetic activity, resulting in increased cardiac output [41]. Exhaustive exercise induces adverse responses in the heart, such as impaired cardiomyocyte Ca2+ handling, mitochondrial dysfunction, and enhanced apoptotic signaling [48, 67]. Liao et al. reported increased levels of creatine kinase MB isozyme (CK-MB) and cardiac troponin I (cTnI), which are markers of myocardial injury [35], after exhaustive exercise [44]. Furthermore, focal necrosis, intravascular coagulation, scattered interstitial hemorrhage, and inflammatory cell infiltration have been observed in cardiac tissues [44]. Although exhaustive exercise-induced cardiac injury is generally attributed to the increased load on the heart resulting from increased cardiac output during exercise [48], in the present review, we focused on injuries secondary to neutrophils and macrophages. In ischemia/reperfusion-induced cardiac injury, neutrophils infiltrate into the heart where they are activated and produce ROS via NADPH oxidase and myeloperoxidase (MPO) [8, 21, 94]. Hiroi et al. reported that neutrophil accumulation via the activation of transient receptor potential melastatin-2, which is highly expressed in immune cells, promotes inflammatory responses and exacerbates cardiac injury [21]. Macrophages, which are mobilized during the initial inflammatory phase, also promote inflammation during cardiac injury through the production of TNF-α and IL-1β [64]. These reports suggest the possible involvement of neutrophils and macrophages in exhaustive exercise-induced cardiac injury.

In the lung tissue, histological changes, such as thickening of the alveolar septum, infiltration of inflammatory cells, and massive necrosis, occur after exhaustive exercise [44]. These changes may be caused by direct injury due to increased ventilation in response to intense exercise or ischemia/reperfusion-induced injury during exercise. Notably, both neutrophils and macrophages are involved in ischemia/reperfusion-induced lung injury [13, 15]. In particular, alveolar macrophages are important for initiating ischemia/reperfusion-induced lung injury [21]. TNF-α and IL-1β, produced by alveolar macrophages, are important mediators of intercellular signaling in the pathogenesis of lung injury [58]. Furthermore, neutrophil activation and infiltration are reportedly involved in ischemia/reperfusion-induced lung injury [58, 96]. However, it remains unclear whether neutrophils and macrophages contribute to exhaustive exercise-induced lung injury.

Despite limited studies, pancreatic tissue injury has also been reported [71]. In dogs, reduced blood flow to the pancreas during exercise has been reported [37], suggesting that ischemia/reperfusion may also be involved in exercise-induced pancreatic injury. Therefore, it is necessary to comprehensively examine the organs affected by exercise-induced tissue injury, warranting future investigations to reveal the mechanisms underlying tissue injuries.

METHODS

All animal experimental procedures for this study complied with the Guiding Principles for the Care and Use of Animals at Waseda University and were approved by the Institutional Animal Care and Use Committee of Waseda University (2013-A110). We investigated the effects of neutrophil and macrophage

depletion in male C57BL/6J mice. To investigate the effects of neutrophil depletion, male C57BL/6J mice were divided into four groups: sedentary with control antibody (n = 10), sedentary with anti-neutrophil antibody (n = 10), exhaustive exercise with control antibody (n = 10), and exhaustive exercise with anti-neutrophil antibody (n = 10). Anti-neutrophil (1A8) or control antibodies were administered intraperitoneally to mice 48 h before treadmill running experiments. To determine the effects of macrophage depletion, C57BL/6J mice were divided into four groups: sedentary with control liposomes (n = 8), sedentary with clodronate liposomes (n = 8), exhaustive exercise with control liposomes (n = 8), and exhaustive exercise with clodronate liposomes (n = 8). The mice were intraperitoneally administered clodronate or control liposomes 48 h before exhaustive exercise. The blood, heart, and lungs were collected 24 h after running until exhaustion on a treadmill with a 7% gradient and a speed of 24 m/min. Although a previous study has reported cardiac and pulmonary injuries during 7 days of exhaustive exercise (37), it was not clear whether these injuries occur during acute exhaustive exercise. As the present study was conducted as an exploratory study using cardiac and pulmonary injury markers and inflammatory cytokines to determine whether these injuries also occur during acute exhaustive exercise, no histological samples were prepared. Moreover, the levels of MPO in the heart and lung and CK-MB and cTnI in the plasma were measured using enzyme-linked immunosorbent assay (ELISA) (details are reported in the Supplementary Methods). The mRNA expression levels of Ly-6G, a neutrophil marker; F4/80, a macrophage marker; receptors for advanced glycation end-products (RAGE), a marker of lung injury; and inflammatory cytokines in the lungs and heart were measured using quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR). The NADPH oxidase activity and hydrogen peroxide levels in the heart were also measured. Details are shown in Supplementary Methods.

RESULTS

Effect of neutrophil depletion on exhaustive exercise-induced heart and lung injuries

To determine the effect of 1A8 antibody treatment on exhaustive exercise-induced neutrophil infiltration in the heart and lungs, we analyzed the mRNA expression of Ly-6G, a specific marker of neutrophils. Exhaustive exercise significantly increased Ly-6G levels in the heart and lungs, whereas treatment with the 1A8 antibody decreased these levels (Figures 1 and 2). Consistently, while exhaustive exercise increased MPO levels in the heart and lung, injection of the 1A8 antibody reduced them (Figures 1 and 2). IL-8 mRNA expression in the heart and lung was not significantly altered by exercise or 1A8 treatment. Plasma CK-MB and cTnI levels, which are specific indicators of myocardial injury, increased with exhaustive exercise; however, these increases were significantly suppressed by treatment with 1A8 (Figure 1). In addition, the wet/dry weight ratio of the lung, a marker of pulmonary edema, and level of RAGE, a marker of lung injury, were significantly increased by exhaustive exercise and suppressed by anti-neutrophil antibody administration. The changes in the mRNA expression of several cytokines are shown in Figures 1 and 2. The inflammatory response induced by exhaustive exercise was suppressed by treatment with the 1A8 antibody (Figures 1 and 2). Moreover, NADPH oxidase and hydrogen peroxide levels in the heart were measured. Exhaustive exercise significantly increased cardiac NADPH oxidase and hydrogen peroxide levels, whereas treatment with the 1A8 antibody significantly suppressed these effects (Figure 1). The effect of exhaustive exercise and neutrophil depletion on macrophage infiltration in the heart and lung was evaluated using F4/80 mRNA. Although exhaustive exercise increased the F4/80 mRNA level in the heart and lung, injection of the 1A8 antibody reduced them (Figures 1 and 2). The mRNA expression of MCP-1 was also increased by exercise but ameliorated by the 1A8 antibody.

Effect of macrophage depletion on exhaustive exercise-induced heart and lung injuries

The effects of exhaustive exercise and clodronate liposome treatment were assessed based on F4/80 expression, which was significantly higher in the heart and lungs of the exhaustive exercise group than in those of the sedentary group. However, F4/80 expression was markedly lower in the exhaustive exercise with the clodronate liposome administration group (Figures 3 and 4). Exhaustive exercise significantly increased MCP-1 mRNA levels in both cardiac and pulmonary tissue, and these increases were suppressed by clodronate liposome treatment (Figures 3 and 4). Exhaustive exercise increased the CK-MB, cTnI, and RAGE levels and wet/dry weight ratio of the lung, confirming the results described above, and this increase was suppressed by clodronate liposome treatment (Figure 3). To elucidate the tissue inflammatory response after exercise, we measured the expression of TNF-α IL-6, and IL-1β in these tissues. In both tissues, the TNF-α and IL-1β levels were significantly increased after exhaustive exercise, and these increases were suppressed by macrophage depletion (Figures 3 and 4).

Discussion of original data

Exhaustive exercise induces cardiac and lung injuries [44]; however, it is not clear whether secondary injury by neutrophils and macrophages contributes to these injuries. In this study, we aimed to further elucidate the mechanisms underlying exhaustive exercise-induced tissue injury using neutrophil and macrophage depletion models. We observed increased Ly-6G mRNA expression in the heart and lung tissues of model mice after exhaustive exercise. In addition, treatment with an anti-neutrophil antibody suppressed these effects. MPO, one of the most abundant proteins in neutrophils reported to be associated with the number of infiltrating neutrophils in the heart and lungs [2, 101], was assessed, and the results were consistent with the Ly-6G levels. F4/80 mRNA level has been reported to be associated with the number of macrophages infiltrating the heart and lungs [24, 51]. In this study, its level was increased by exhaustive exercise and suppressed by clodronate liposome administration. Thus, these results suggest the efficacy of our protocol in blocking neutrophil and macrophage infiltration into tissues. In this study, no increase in IL-8 was observed after exhaustive exercise, neither in the heart nor in the lungs. We have previously reported increased CD11b and CD62L expression on blood neutrophils after exhaustive exercise [59], suggesting that adhesion factors might play a vital role in neutrophil infiltration into these tissues after exercise. In the present study, immunostaining and flow cytometric analysis were not performed to confirm neutrophil and macrophage

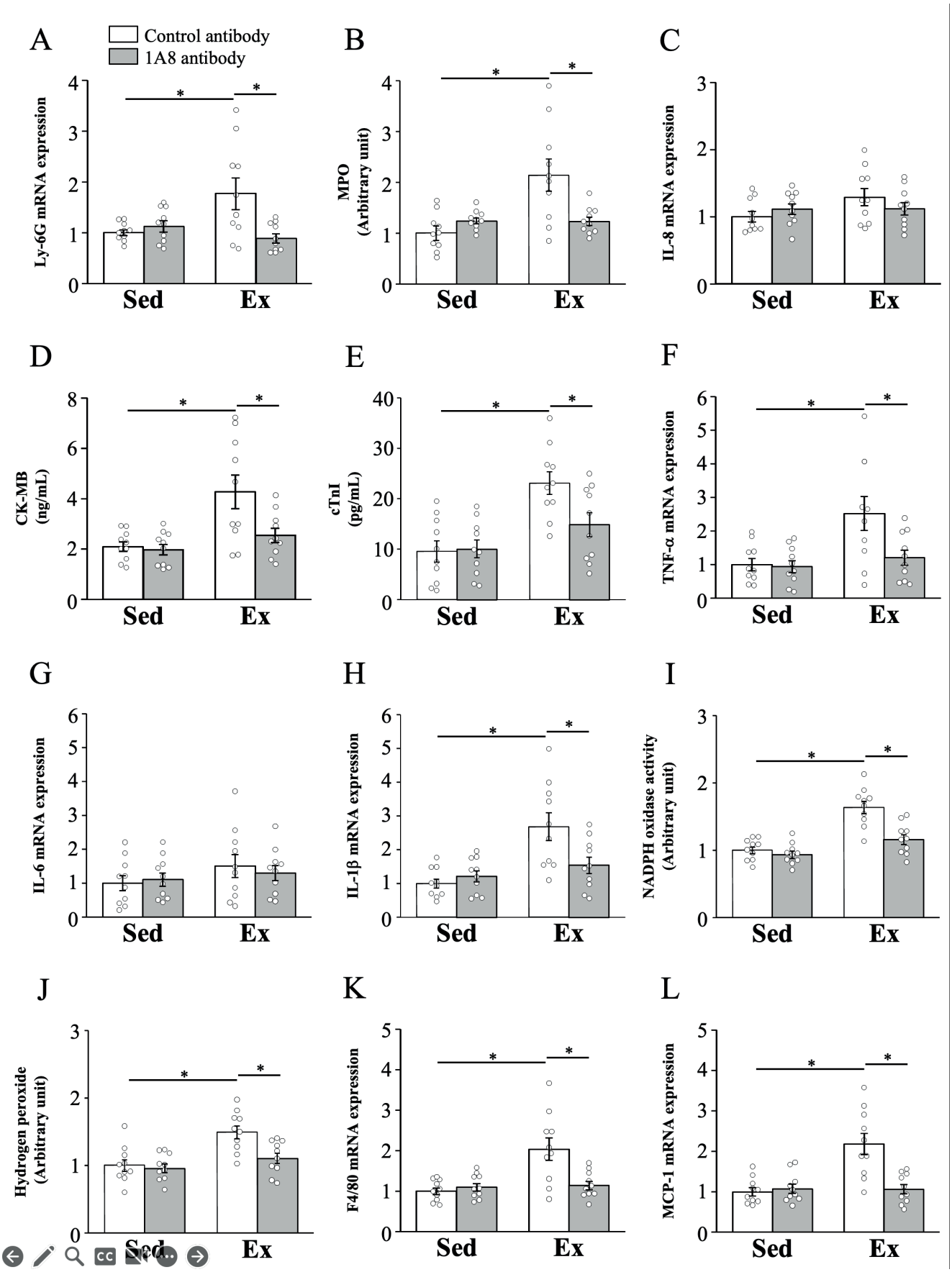

Figure 1. Effects of exhaustive exercise and treatment with the 1A8 antibody on heart injury. (A) Ly-6G expression in the heart. (B) MPO level. (C) IL-8 mRNA expression in the heart. (D and E) Plasma CK-MB and cTnI concentrations. (F) TNF-α, (G) IL-6, and (H) IL-1β expression in the heart. (I) NADPH oxidase activity, and (J) hydrogen peroxide concentration in the heart. (K) F4/80 and (L) MCP-1 mRNA expression in the heart. Values represent mean ± standard error of the mean. Analyses were performed using a two-way analysis of variance for multiple comparisons. *$P < 0.05$. Sed, sedentary; Ex, exercise; MPO, myeloperoxidase; CK-MB, creatine kinase MB isozyme; cTnI, cardiac troponin I; TNF, tumor necrosis factor; IL, interleukin; NADPH, nicotinamide adenine dinucleotide phosphate; MCP, monocyte chemoattractant protein.

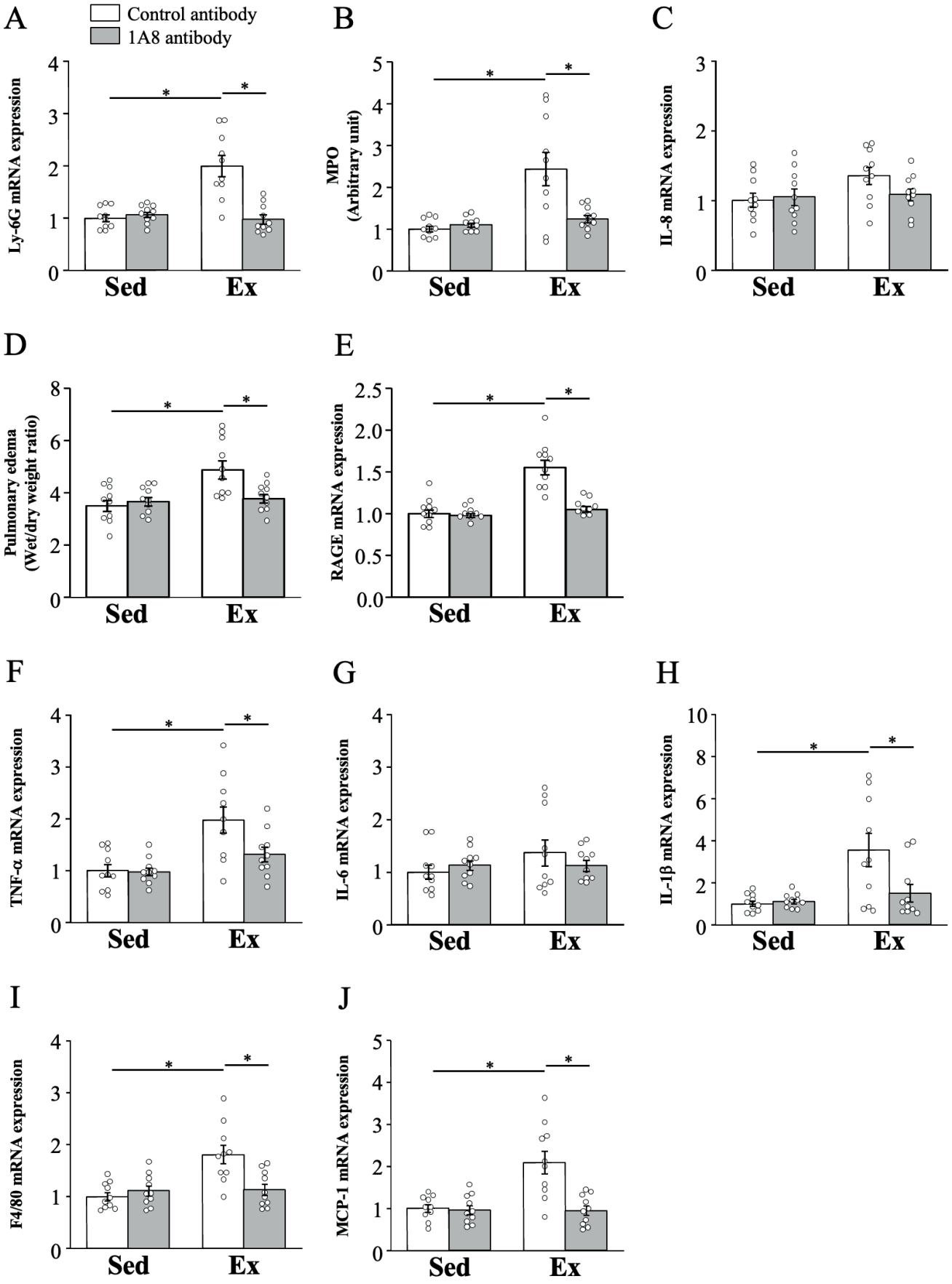

Figure 2. Effects of exhaustive exercise and treatment with the 1A8 antibody on lung injury. (A) Ly-6G expression in the lungs. (B) MPO level. (C) IL-8 mRNA expression in the lung. (D) Pulmonary edema. (E) RAGE, (F) TNF-α, (G) IL-6, (H) IL-1β, (I) F4/80, and (L) MCP-1 expression in the lung. Values represent mean ± standard error of the mean. Analyses were performed using a two-way analysis of variance for multiple comparisons. *P < 0.05. Sed, sedentary; Ex, exercise; MPO, myeloperoxidase; RAGE, receptors for advanced glycation end-products; TNF, tumor necrosis factor; IL, interleukin; MCP, monocyte chemoattractant protein.

tissue infiltration because this study was conducted as an exploratory study examining the involvement of neutrophils and macrophages in acute exhaustive exercise-induced cardiac and pulmonary injuries, rather than continuous exhaustive exercise. As mentioned above, the number of neutrophils and monocytes in the blood increases after exercise [7, 61]; therefore, the Ly-6G, MPO and F4/80 results obtained in this study may reflect the dynamics of these cells in the blood. Thus, further studies on the infiltration of immune cells in various tissues after exhaustive exercise are required.

A previous study has reported cardiac injury with increased blood levels of CK-MB and cTnI in rats subjected to exhaustive exercise for 7 days [44]. In the present study, increased CK-MB and cTnI levels were observed after acute exhaustive exercise, suggesting that cardiac injury may have occurred. During ischemia/reperfusion-induced cardiac injury, neutrophils are recruited to the injured myocardium and activated, further exacerbating the cardiac injury by producing inflammatory cytokines and ROS [8, 21, 94]. Hiroi et al. have shown that H2O2-mediated activation of the neutrophil transient receptor potential melastatin-2 exacerbates ischemia/reperfusion-induced cardiac injury and promotes neutrophil infiltration and inflammation [21]. In addition, neutrophils secrete harmful degradative enzymes, such as MPO [8], and produce ROS via NADPH oxidase [94]. The superoxide generated by NADPH oxidase directly inactivates NO, thereby causing secondary endothelial dysfunction. Furthermore, the lack of NADPH oxidase suppresses ischemia/reperfusion-induced endothelial injury in humans [49]. Consistent with these reports, we demonstrated that anti-neutrophil antibody administration reduced exhaustive exercise-induced ROS production in terms of NADPH oxidase and H2O2 levels, as well as inflammatory cytokine expression, resulting in reduced cardiac injury in mice. Moreover, macrophage recruitment during the initial inflammatory phase of ischemia/reperfusion-induced cardiac injury may further damage the cardiomyocytes by releasing proteolytic enzymes, ROS, and inflammatory cytokines [64]. In the present study, macrophage depletion suppressed the mRNA expression of inflammatory cytokines and levels of cardiac injury markers in the blood, similar to the results observed in the anti-neutrophil antibody administration model. Notably, the administration of anti-neutrophil antibodies in this study prevented increases in F4/80 and MCP-1 mRNA after exhaustive exercise. Neutrophils have been reported to play a role in monocyte recruitment in ischemia/reperfusion-induced cardiac injury [1] and macrophage infiltration in exercise-induced cardiac injury via the production of MCP-1. However, further comprehensive studies are required to elucidate the interactions between neutrophils and macrophages. Few reports have examined changes over time in the mobilization and tissue invasion of these cells after exhaustive exercise; therefore, data collection over extended periods is necessary to fill this knowledge gap. In addition, future studies should aim to accumulate data over time in scenarios where either neutrophils or macrophages are depleted, as was done in this study.

Seven days of exhaustive exercise results in not only cardiac injury but also lung injury, with pulmonary alveolar swelling, inflammatory cell infiltration, and extensive necrosis of lung tissue [44]. In the present study, we assessed the levels of the wet/dry weight ratio of the lung, a marker of pulmonary edema, and RAGE, a membrane receptor expressed on alveolar type-1 epithelial cells in the lungs and a marker of epithelial damage [90], and found them to be increased by exhaustive exercise. These results suggest that acute exhaustive exercise induces lung injury. Neutrophil involvement has also been reported in lung injury [8], and Eppinger et al. have found that neutrophil depletion had no protective effect 30 min after reperfusion, but it attenuated the injury after 4 h [13]. Moreover, neutrophil depletion in the blood using a leukocyte filter alleviates post-reperfusion-induced lung injury [13]. Similarly, macrophages reportedly contribute to lung injury, and Eppinger et al. have reported the contribution of TNF-α and MCP-1 in lung injury [13], indicating an important role of alveolar macrophages immediately post-reperfusion. In the present study, treatment with anti-neutrophil antibody, as well as macrophage depletion, suppressed TNF-α and IL-1βmRNA expression, suggesting that neutrophils and macrophages may contribute to the inflammatory response in the lungs after exhaustive exercise. Furthermore, as in the heart, F4/80 and MCP-1 mRNA expression were suppressed in the anti-neutrophil antibody-treated group. Therefore, even in exercise-induced lung injury, neutrophils may contribute to macrophage infiltration through the production of MCP-1.

In this study, exhaustive exercise-induced cardiac and pulmonary injuries were reduced by neutrophil and macrophage depletion. In particular, injury marker levels in the macrophage-depleted group were decreased to the same level as in the control group. These results suggest that a secondary injury, involving inflammatory cytokine and ROS production by immune cells, might significantly contribute to cardiac and pulmonary injuries induced by exhaustive exercise. However, as the running exercise used in this study elicits a systemic stimulus, it is difficult to determine whether a primary or secondary injury is the major contributor. In a previous study, the administration of anti-inflammatory and antioxidant substances before exhaustive exercise prevented exercise-induced pulmonary and cardiac injuries [32, 101], supporting the hypothesis that inflammatory mediators released by neutrophils and macrophages may play an important role in exercise-induced tissue injury. Liao et al. reported cardiac and pulmonary injuries following seven consecutive days of exhaustive exercise, making comparisons between uphill and downhill exercise types [44]. The degree of lung lesion due to exhaustive exercise was higher in downhill exercise types than in uphill exercise types, and the CK-MB level was significantly higher during downhill exercise than during uphill exercise in cardiac injury [44]. Moreover, previous studies have reported an increase in blood neutrophil and monocyte counts after downhill exercise compared to those after uphill running [44, 76], suggesting that these variations in the infiltrating cells may influence differences in the extent of injuries associated with the type of exercise.

Histological changes in the heart and lungs have been reported seven days after exhaustive exercise [44]. In the present study, acute exhaustive exercise may have caused heart and lung tissue injuries, which may have been suppressed by the depletion of neutrophils and macrophages. However, the lack of histological evaluation showing tissue damage is a limitation of the present study, and a more detailed study is required.

Another important issue that should be addressed in future studies is the extent to which the exhaustive exercise

model used in this study applies to humans. In this study, exhaustive exercise increased plasma CK-MB and pulmonary edema by approximately 1.7–to 2.0-fold and 1.5-fold, respectively. Exhaustive exercise-induced tissue injuries are expected to be comparable or less severe than physical injury, such as ischemia/reperfusion, as CK-MB was increased by approximately 2.5-fold in ischemia/reperfusion-induced cardiac injury and edema was increased by approximately 1.5-fold in pulmonary injury [95, 97]. In human studies, approximately 3.75-fold and 14-fold increases in CK-MB levels have been reported immediately after a marathon race and a triathlon, respectively [65, 75]. In addition, CK-MB increased by 1.8-fold immediately after a half-ironman triathlon, but this increase was maintained up to 48 h after exercise [98]. Therefore, the CK-MB values suggest that the exhaustive exercise used in this study may not be of an intensity beyond that of exhaustive exercises in humans. While pulmonary function has been reported to be reduced after prolonged endurance exercise in humans [88], it is not clear whether lung injury occurs, partly due to the lack of useful biomarkers. In this study, pulmonary RAGE mRNA expression, a marker of lung injury, increased after exhaustive exercise; to the best of our knowledge, this is the first report of such an increase following exhaustive exercise. Plasma RAGE concentrations are increased in patients with acute liver injury and higher concentrations have been detected in patients with more severe lung dysfunction [56], highlighting the potential of RAGE as a blood biomarker for acute lung injury [8]. Unfortunately, in the present study, plasma RAGE levels could not be measured owing to insufficient sample volume. In future studies, we will aim to investigate whether plasma RAGE level can act as a marker of post-exercise lung injury and whether it increases after prolonged intense exercise.

This review focused on the involvement of neutrophils and macrophages in the initial response in exhaustive exercise-induced acute tissue injury. Although excessive inflammation causes tissue injury, the inflammatory response to moderate exercise contributes to tissue adaptation and recovery [9]. Notably, non-steroidal anti-inflammatory drugs have been shown to adversely affect skeletal muscle regeneration and adaptation [50, 89, 91]. Therefore, completely blocking inflammation may prevent certain tissues from adapting to exercise. The threshold of the inflammatory response is not clear and requires further investigation. Moreover, macrophages are believed to be associated with tissue repair and protection [59, 66], and the effects of these cell functions may not be entirely detrimental, as they may aid in the resolution of inflammatory reactions. The contribution of these cells to the recovery phase of exhaustive exercise-induced tissue injury is not clear and should be investigated in future studies.

CONCLUSION

This review summarizes the involvement of neutrophils and macrophages in exhaustive exercise-induced tissue injuries and provides original findings supporting the involvement of neutrophils and macrophages in exhaustive exercise-induced cardiac and pulmonary injuries.

SUPPLEMENTARY METHODS

Animals

Male C57BL/6J mice, aged 10 weeks, were purchased from Kiwa Laboratory Animals (Wakayama, Japan) and housed, 4 mice per cage, in a controlled environment under a 12-h light/dark cycle (lights on at 9:00 h and off at 21:00 h). All mice had free access to standard chow and water. The experimental procedures complied with the Guiding Principles for the Care and Use of Animals at Waseda University and were approved by the Institutional Animal Care and Use Committee of Waseda University (2013-A110).

Neutrophil depletion

The neutrophil-specific antibody anti-Ly-6G (clone 1A8) and isotype control antibody (clone 2A3) were purchased from Bio X Cell (Sunnyvale, CA, USA). Furthermore, 1A8 (0.5 μg) and 2A3 (0.5 μg) antibodies were individually diluted in phosphate-buffered saline, and the mice were intraperitoneally administered 150 μL of either antibody solution, according to their respective experimental groups. In our previous study under the same conditions, the efficiency of neutrophil depletion in blood was 49% immediately after exercise. After 24 h of exercise, it was 25% in the control group [61].

Macrophage depletion

To deplete macrophages, 150 μL of Clophosome-A (TM)-Clodronate Liposomes (Anionic) (Funakoshi, Tokyo, Japan) was administered intraperitoneally to the mice under anesthesia with 2% isoflurane inhalation at 0.8 L/min (Abbott Japan, Tokyo, Japan) using a gas anesthesia system for small laboratory animals (DS Pharma Biomedical, Osaka, Japan). Control mice were administered 150 μL of plain control Clophosome-A liposomes (Funakoshi) in a similar manner. In a preliminary study, we investigated the effects of the administration of clodronate liposomes or control liposomes on blood monocyte counts. The results are shown in Supplemental Table 1.

Exercise protocol

Mice in the sedentary groups remained under resting conditions in the cage, whereas mice in the exercise groups were subjected to exhaustive exercise 48 h after injection. One week before the exhaustive exercise regimen, the mice in all groups were familiarized with running on a motorized treadmill (Natsume, Tokyo, Japan). On the day of the experiment, the mice were forced, using a shock grid,

Table 1: Effects of clodronate liposomes or control liposomes on blood monocytes

	Blood monocytes (10^2/μL)			
	After administration of clodronate liposomes or control liposomes			
	Pre	24h	48h	72h
Control liposome administration group	3.4 ± 0.3	3.2 ± 0.4	3.5 ± 0.3	3.4 ± 0.3
Clodronate liposome administration group	3.3 ± 0.2	1.7 ± 0.2*, §	1.9 ± 0.3*, §	2.0 ± 0.4*, §

Values represent means ± SEM (n=5). Analyses were performed using mixed effect models for repeated measures.
*P<0.05 vs Pre, §P<0.05 vs Control liposome administration group at the same time point

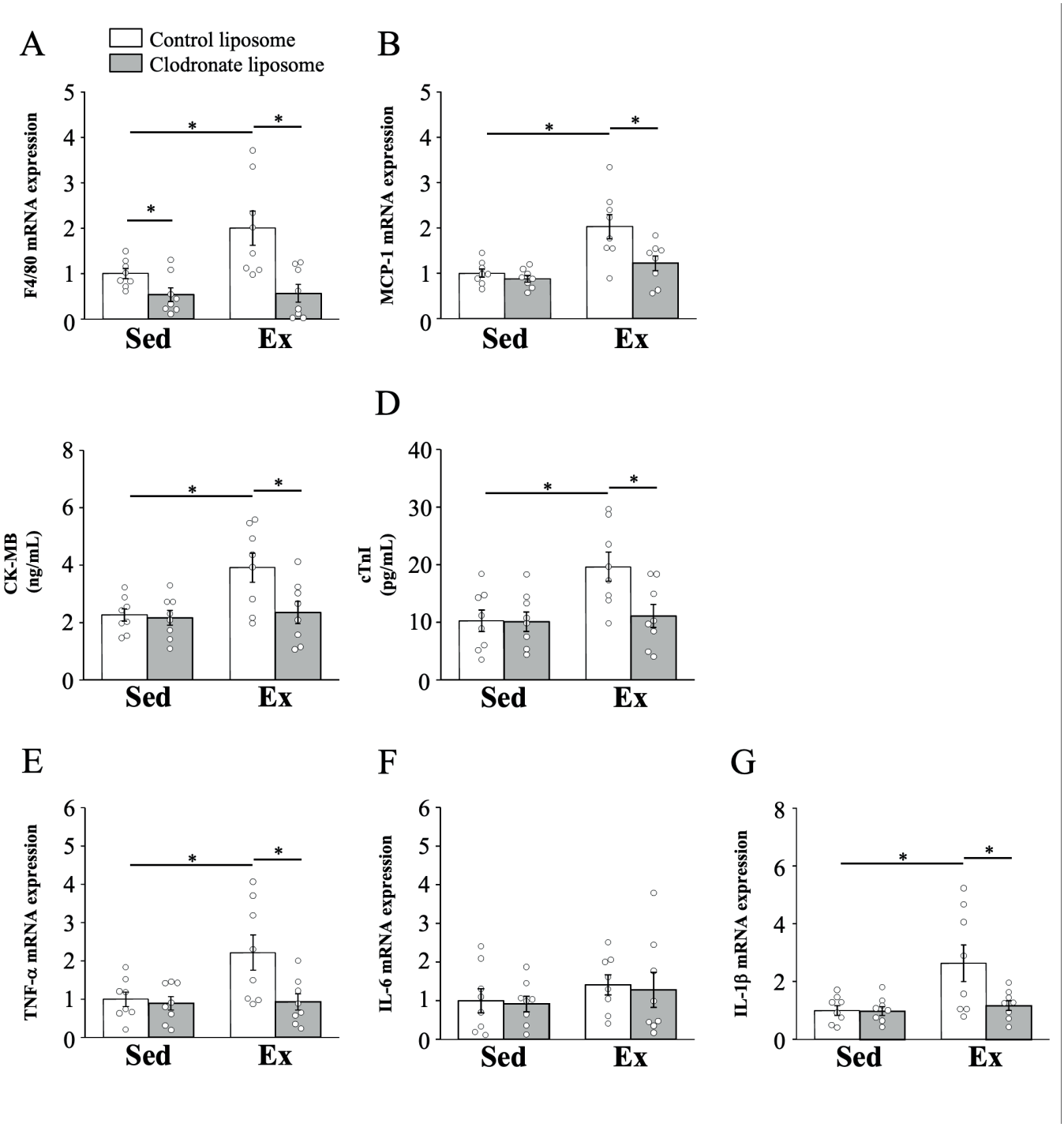

Figure 3. Effects of exhaustive exercise and clodronate liposome administration on heart injury. (A) F4/80 and (B) MCP-1 mRNA expression in the heart. (C and D) Plasma CK-MB and cTnI concentrations. (E) TNF-α, (F) IL-6, and (G) IL-1β expression in the heart. Values represent mean ± standard error of the mean. Analyses were performed using a two-way analysis of variance for multiple comparisons. *P < 0.05. Sed, sedentary; Ex, exercise; MCP, monocyte chemoattractant protein; CK-MB, creatine kinase MB isozyme; cTnI, cardiac troponin I; TNF, tumor necrosis factor; IL, interleukin.

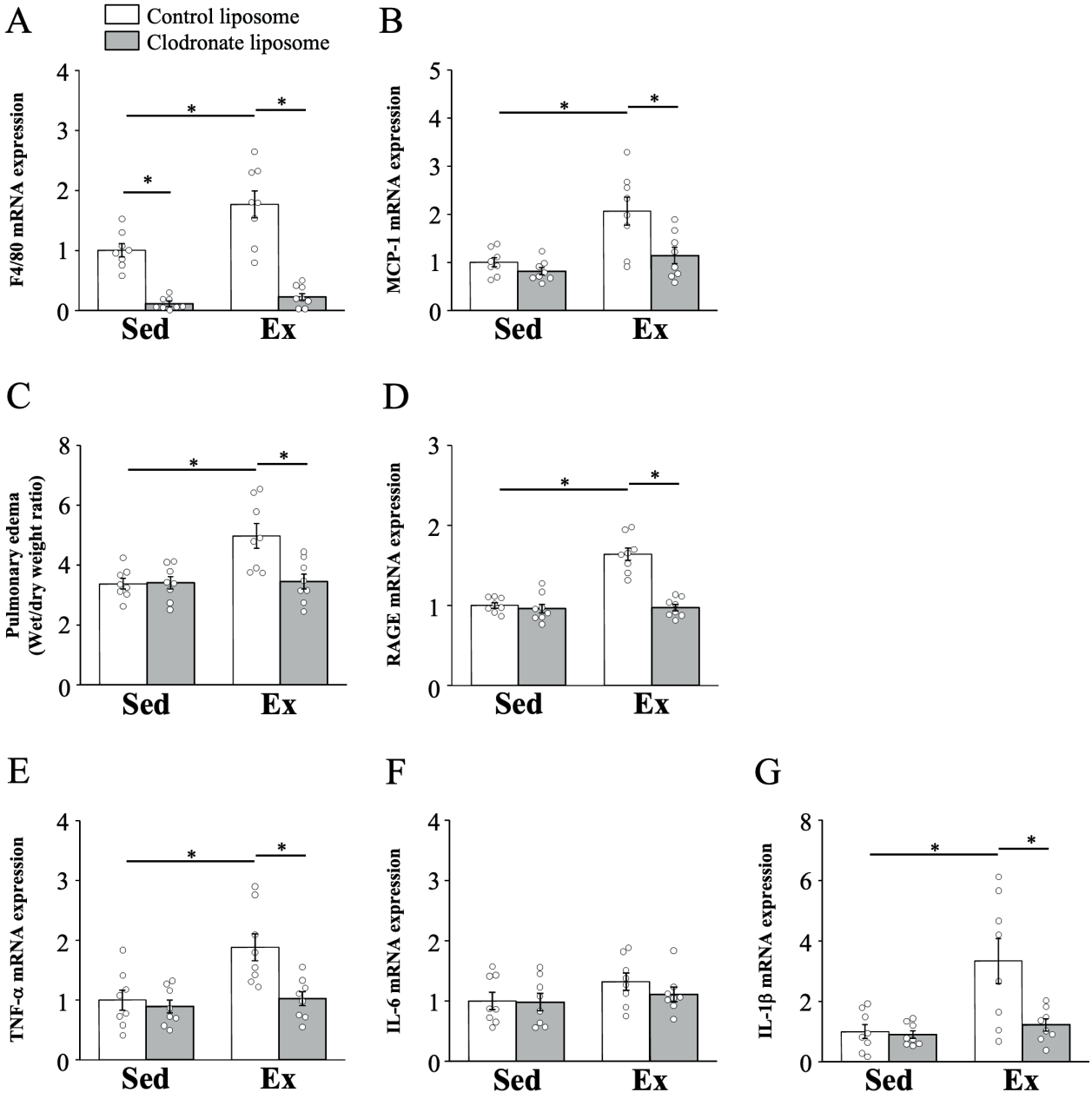

Figure 4. Effects of exhaustive exercise and clodronate liposome administration on lung injury. (A) F4/80 and (B) MCP-1 mRNA expression in the heart. (C) Pulmonary edema. (D) RAGE, (E) TNF-α, (F) IL-6, and (G) IL-1β expression in the heart. Values represent mean ± standard error of the mean. Analyses were performed using a two-way analysis of variance for multiple comparisons. *P < 0.05. Sed, sedentary; Ex, exercise; MCP, monocyte chemoattractant protein; RAGE, receptors for advanced glycation end-products; TNF, tumor necrosis factor; IL, interleukin.

to run on a treadmill with a 7% gradient at a speed of 10 m/min for 15 min, followed by 15 m/min for 15 min, 20 m/min for 15 min, and finally, 24 m/min until exhaustion. Exhaustion was defined as the point at which the mouse refused to run despite touching the shock grid five times. In an experiment examining the effects of neutrophil depletion, the mean running time until mice were exhausted was 338.5 ± 50.2 min in the control antibody group and 342.43± 11.2 min in the 1A8 antibody group. However, in an experiment examining the effects of macrophage depletion, the mean running time until the mice became exhausted was 161.0 ± 14.2 min in the control liposomes groups and 187.7 ± 13.8 min in the clodronate groups, which were not significantly different.

Blood, heart, and lung tissue sampling

The mice in all groups were euthanized 24 h after exhaustive exercise. Anesthesia was maintained by inhalation of 2% isoflurane at 0.8 L/min until exsanguination. The depth of anesthesia in this study was determined to be adequate based on the absence of any flexion response to a noxious stimulus, such as pinching the digit for approximately 2 s. Blood samples were collected from the abdominal aorta using a 1-mL syringe mounted with a 20-gauge needle and coated with heparin (5000 UI/mL; Nipro, Osaka, Japan). Blood samples were transferred to a heparin-coated tube and centrifuged at 2,600 × g for 10 min, and the plasma was stored at -80°C until analysis. The heart and lung tissues were snap-frozen by immersing in liquid nitrogen and stored at -80°C until analysis.

Assessment of blood biomarkers
Plasma CK-MB level was assayed using the Mouse Creatine Kinase MB ELISA Kit (Abcam, Cambridge, UK). Plasma cTnI level was measured using an ELISA kit (CSB-E08421m; CUSABIO, China). All procedures were performed according to the manufacturer's instructions. Optical density was analyzed using a SpectraMax 190 microplate reader (Molecular Devices LLC., San Jose, CA, USA). No standardization by protein content was performed. The inter- and intra-assay coefficient of variation for the same sample was less than 5%.

Measurement of pulmonary edema
After removal, the lungs were blotted briefly on a paper towel; then, they were weighted (wet lung weight). After 72 h of drying at 80°C in an incubator (dry lung weight), the lungs were weighed again, and the wet and dry lung weight ratio was determined.

NADPH oxidase activity
NADPH oxidase activity in the heart tissue was determined based on NADPH oxidation, measured at 340 nm, in a reaction mixture containing 50 mM Tris, 50 mM 2-(N-morpholino) ethanesulfonic acid (pH 7.0), 1 mM KCN (to inhibit low levels of mitochondrial oxidase activity), and 150 mM NADPH (Sigma) for 1 min at 37°C.

Hydrogen peroxide assay
To examine hydrogen peroxide levels in the heart, heart tissues were homogenized using a tissue protein extraction reagent with a protease inhibitor (Thermo, Rockford, IL, USA). Protein concentrations were measured using the BCA Protein Assay Kit (Thermo). Hydrogen peroxide levels were measured using the SensoLyte ADHP Hydrogen Peroxide Assay Kit (Fremont, CA, USA) according to the manufacturer's instructions.

Myeloperoxidase level
Myeloperoxidase (MPO) level in the heart and lung was measured in the homogenate using the Myeloperoxidase Mouse ELISA Kit (Thermo Fisher Scientific, Waltham, MA, USA). MPO levels were normalized to protein concentrations using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR)
Total RNA was extracted from the heart and lung tissues of mice using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of total RNA was assessed using the NanoDrop system (NanoDrop Technologies, Wilmington, DE, USA), and samples with A260/A280 ratios between 1.9 and 2.1 were used for analysis. Total RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). RT-qPCR was performed with the Fast 7500 real-time PCR system (Applied Biosystems) using 10 ng of cDNA and Fast SYBR Green PCR Master Mix (Applied Biosystems). The thermal profile consisted of denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 3 s and annealing at 60°C for 15 s. The expression of target genes was normalized to that of 18S ribosomal RNA, a housekeeping control, using the 2-ΔΔCT method. The data are presented as fold change relative to the expression level in the sedentary group treated with the control antibody. The specific PCR primer pairs used for each gene are listed in Supplemental Table 2.

Statistical analyses
All data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using SAS software version 9.4. To evaluate the statistical significance of the relationship between exhaustive exercise and neutrophil or macrophage depletion, data were analyzed using a two-way analysis of variance. If significant interactions were observed, further comparisons were performed using Tukey's HSD post-hoc test.

Conflicts of interest statement
The authors declare no conflict of interest.

Gene	Forward	Reverse
18s ribosomal RNA	CGGCTACCA-CATCCAAGGA	AGCTGGAATTAC-CGCGGC
Ly-6G	TGGACTCTCA-CAGAAGCAAAG	GCAGAG-GTCTTCCTTC-CAACA
F4/80	CTTTGGCTATGG-GCTTCCAGTC	GCAAGGAGGA-CAGAG-TTTATC-GTG
RAGE	ACTACCGAGTC-CGAGTCTACC	GTAGCTTCCCT-CAGACACACA
TNF-α	TCTTCTCAT-TCCTGCTTGTGG	GAGGCCATTTG-GGAACTTCT
IL-6	AACGATGATG-CACTTGCAGA	TGGTACTC-CAGAAGACCA-GAGG
IL-1β	GGGCCTCAAAG-GAAAGAATC	TTGCTTGGGATC-CACACTCT
IL-8	AGAAGTTTT-GAAGAGGGCT-GAGA	AGTTTCACTG-GCATCTTCACT-GATT
MCP-1	CTTCTGGGCCT-GCTGTTCA	CCAGCCTACT-CATTGGGATCA

Tumor necrosis factor (TNF); interleukin (IL); receptors for advanced glycation end-products (RAGE); monocyte chemoattractant protein (MCP)

Table 2: Primer sequences for RT-PCR analysis.

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