lower doses.[40] The Japanese Chronic Heart Failure (J-CHF) study, however, assessed 3 doses of carvedilol (2.5, 5, and 20 mg/day), but dose dependency in mortality and morbidity was not confirmed.[39] Surrogate markers of chronic HF prognosis, however, include LVEF,[41] BNP,[42] and HR,[43] and the J-CHF noted that decreases in BNP and HR were dose dependent.[39] Despite the fact that the present results were not statistically examined because of the small number of patients, large SD, and confounding by treatment duration, the present week 12 results for LVEF, BNP, and HR do not contradict the notion of dose-dependent improvement. Clearly, further investigation of the efficacy of carvedilol CR doses >40 mg/day is warranted in Japanese chronic HF patients.

The present results suggest that dose dependency of decrease in HR, which has been identified as a prognostic factor in HF,[44] will last to 80 mg carvedilol CR in Japanese patients (data not shown). This finding is consistent with a study by Konishi et al, who noted a significant decrease in HR among 110 Japanese patients assigned to a low-dose carvedilol regimen for 18 months.[45] Moreover, in a meta-analysis of HF β-blocker trials, McAlister et al observed a relationship between the extent of the reduction in HR, not the dose of β-blocker, and the magnitude of increased survival.[43] HR reduction is regarded as a marker of sympathetic suppression. Thus, given the dose dependency of the reduction in HR, uptitration to a dose of 60 or 80 mg of carvedilol CR might be more efficacious in Japanese patients with chronic HF.

The limitations of the present study were the small number of enrolled patients and the absence of a comparison group. In addition, because the primary goal of the study was to confirm the safety and tolerability of a once-daily dose of 10–40 mg carvedilol CR, the present findings regarding efficacy and PD should be regarded as preliminary. Additional studies are needed to examine the tolerability, safety, and efficacy of a once-daily dose of 10–80 mg carvedilol CR.

Conclusions

The characteristics of AEs did not substantially differ in patients receiving the CR and IR formulations of carvedilol. In addition, no new safety issues emerged in patients receiving carvedilol CR in contrast to those known in carvedilol IR. With increasing doses up to 80 mg once daily of carvedilol CR, exposure to plasma S(–)-carvedilol increased in an approximately dose-proportional manner. Seven of 19 patients receiving carvedilol CR reached a daily dose of 80 mg. Thus, further investigation of the tolerability, safety, and efficacy of carvedilol CR doses up to 80 mg/day is warranted in Japanese patients with chronic HF.

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Deficiency of type 1 cannabinoid receptors worsens acute heart failure induced by pressure overload in mice

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Aims We investigated the influence of type one cannabinoid receptor (CB1) deficiency on acute heart failure (AHF) and the underlying mechanism. Acute heart failure syndrome is an important clinical problem because of its high morbidity and mortality rates. Activation of CB1 induces vascular dilation and reinforces the properties of morphine, long-standing therapies for AHF syndrome, but the effect of endogenous CB1 activation on AHF is largely unknown.

Methods and results Acute heart failure mouse model characterized by hypertension and pulmonary oedema was created by using transverse aortic constriction (TAC). Mortality, echocardiography, haemodynamic, morphology, and circulatory catecholamine levels in response to TAC were evaluated in CB1 knockout (KO) and wild-type mice. Type one cannabinoid receptor KO mice had a much higher mortality rate at 1 week after TAC attributable to AHF (65 vs. 11%, $P < 0.001$). One hour after TAC, CB1 KO mice had significant larger lung weight to body weight ratio (LW/BW, $14.53 + 1.09$ mg/g in KO vs. $10.42 + 0.36$ mg/g in WT, $P < 0.01$) and higher plasma epinephrine levels ($9720 + 1226$ pg/mL vs. $6378 + 832$ pg/mL, $P < 0.05$). Pharmacological activation of CB1 reduced LW/BW in wild-type mice. Administration of epinephrine to wild-type TAC mice significantly increased left ventricular end-diastolic pressure and LW/BW, while CB1 agonists reduced the LW/BW and the plasma levels of catecholamine and increased myocardial activity of AMP-activated protein kinase.

Conclusion Endogenous activation of CB1 in mice has cardiac protection in AHF, which is attributable to the inhibition of excessive sympathetic activation.

Keywords Cannabinoid receptor • Acute heart failure • Catecholamine • Mortality • Mouse

Introduction

Patients with cardiovascular diseases are increasingly hospitalized due to acute heart failure syndromes (AHFS). However, traditional therapies for AHFS, such as oxygen, loop diuretics, nitrates, and morphine have significant limitations, and the mortality rate remains relatively high,[1] indicating the need to develop more effective treatments. Cannabinoids have not only neurobehavioural, but also cardiovascular effects.[2,3] Both of cannabinoids receptors 1 and 2 (CB1 and CB2, respectively) exist in the cardiovascular system.[4,5] Recent published literature has addressed the influence of CB1 activation on the uncommon forms of heart failure induced by hepatic cirrhosis or doxorubicin,[6] however, the influence of endogenous CB1 activation on the classical AHFS remains poorly understood.

Type one cannabinoid receptor agonists have been shown to exhibit a vasodilatory effect,[7] inhibit the release of neurohormonal factors,[8] improve myocardial energy metabolism,[9] and suppress

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vasopressin-induced vasoconstriction.[10] More importantly, it was reported that a CB1 antagonist increased the acute mortality rate after myocardial infarction in rats.[11] These findings raise the possibility that CB1 signalling may play an important role in AHFS.

In the present study, we hypothesized that activation of CB1 might be beneficial for AHF. To verify this hypothesis, we first established a murine model of AHF mimicking the clinic profiles of pulmonary oedema and high blood pressure, and then investigated the influence of the CB1 activity in AHF by studying the cardiac changes in CB1 knockout (KO) mice and the influence of CB1 agonists or antagonists on heart function in wild-type (WT) mice as well as the underlying mechanism.

Methods

Agents

WIN 55,212-2 (WIN), and AM 251, [*N*-(piperidin-1-yl)-5-(4-iodophonyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], were purchased from Tocris Bioscience (Ellisville, MO). 2-Arachidonyl glycerol (2-AG) was purchased from Sigma RBI. Epinephrine was purchased from Sigma Chemical Company (see Supplementary material online).

Transverse aortic constriction model

All procedures were performed in accordance with our institutional guidelines for animal research that conformed to the 'Position of the

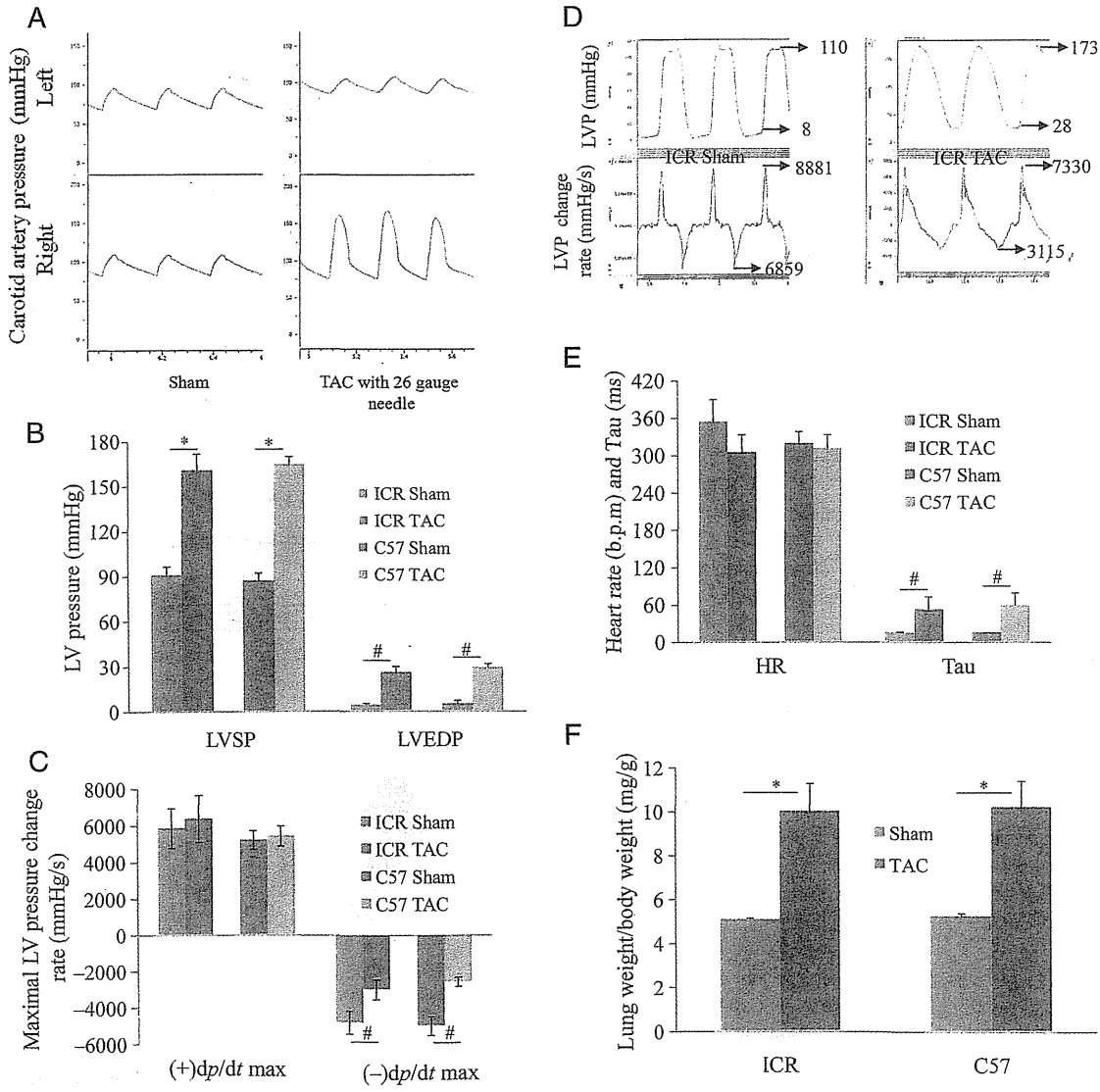

Figure 1 Acute heart failure induced by transverse aortic constriction in both ICR and C57 mice. (*A*) Representative recordings of blood pressure made simultaneously from the right and left carotid arteries. (*B*) Left ventricular systolic and end-diastolic pressure. (*C*) Maximal left ventricular pressure rise and fall rate ($\pm dp/dt$ max). (*D*) Representative recordings of left ventricular pressure and pressure change rate. (*E*) Heart rate and the exponential time constant of relaxation (Tau). (*F*) The lung weight to body weight ratio. $^{\#}P < 0.05$, $^{*}P < 0.01$, $n = 7$ in each group.

American Heart Association on Research Animal Use' adopted by the AHA on 11 November 1984. Type one cannabinoid receptor(−/−), CB1(+/+) or C57 mice were used. Development of mice lacking CB_1 was previously described by Ledent *et al.*[2] Male C57BL/6 mice (7 weeks old and weighing 20–24 g) or ICR or CB1 mice (5–7 weeks) were anaesthetized with pentobarbital sodium (50 mg/kg). To induce acute pulmonary oedema, transverse aortic constriction (TAC) was performed as described elsewhere,[12] degree of aortic stenosis was controlled by the size of the banded needle which was withdraw after aortic ligation and confirmed by the pressure gradient across the banded site (*Figure 1A*), which was usually >50 mmHg at the 3rd day after TAC in our laboratory.

Transthoracic echocardiography was performed with a Sonos 4500 and a 15-6 L MHz transducer (Philips, the Netherlands) and invasive assessment of haemodynamic was carried out using a Millar catheter (see Supplementary material online).

Measurement of catecholamine and endocannabinoids and blood cell counts

Plasma catecholamine (epinephrine, norepinephrine, and dopamine) were measured by SRL, Inc. (Kyoto, Japan) using high-performance liquid chromatography (HPLC). Anandamide (AEA) and *N*-oleoylethanolamine (OEA) in the heart and lung samples HPLC–tandem mass spectrometry (HPLC/MS-MS) (see Supplementary material online).

Circulating blood cell counts (red cells, leucocytes, and platelets) were measured using a Sysmex KX-21 hematology analyzer (Sysmex, Japan) (see Supplementary material online, Methods).

Cell culture and immunoblotting

Ventricular myocytes were isolated from neonatal rats at 2–3 days of life and cultured. Proteins were prepared from cultured cardiomyocytes or whole hearts of mice. Then immunoblotting was performed using mouse antibodies directed against phosphorylated AMP-activated protein kinase (AMPK), which recognizes the AMPK pan-subunit phosphorylated at Thr-172, or AMPK (Cell Signaling). Immunoreactive bands were visualized by the enhanced chemiluminescence method (Amersham) and then were quantified by densitometry with Scion Image software.

Knockdown of rat type one cannabinoid receptor using siRNA and real-time PCR

Neonatal rat cardiomyocytes at 50–70% confluence were transfected with CB1 siRNA (designed and synthesized by B-Bridge International, Inc.) with Optifect (Invitrogen Co.). SiRNA for CB1 was transfected at 66 nM at 4–6 h after plating of cells into a 60-mm culture dish. The siRNA sequences were 5′-ggg aag aug aac aag cuu a-TT (sense) and 5′-uaa gcu ugu uca ucu ucc c-TT (antisense), while the control siRNA was uag cga cua aac aca uca a-dTdT.

Real-time PCR for fatty acid amide hydrolase (FAAH) and the monoacylglycerol lipase (MAGL) in heart and lung was performed using a Quantitect SYBR Green RT-PCR kit (DRR420A,Takara, Japan) (See Supplementary material online, Methods).

Statistical analysis

The SPSS 16.0 (Chicago, USA) software was used for analysis. The unpaired and paired *t*-tests were used for comparisons between two groups and between different conditions within the same group, respectively. One-way ANOVA with post hoc analysis by the Tukey–Kramer exact probability test was employed for multiple comparisons. Survival analysis was performed using Kaplan Meier curves with Log-rank test for comparison between the groups. Results were expressed as the mean ± SEM and $P < 0.05$ was considered to indicate statistical significance. All the tests were two-sided.

Results

Confirmation of acute heart failure model

One hour after TAC, left ventricular systolic pressure (LVSP) was increased to ~170 mmHg in both ICR and C57 mice (*Figure 1B*). Left ventricular end-diastolic pressure (LVEDP) was increased by more than 2 folds, d*p*/d*t* min was dramatically reduced, and the exponential time constant of relaxation (*tau*) was markedly extended (*Figure 1B–E*), indicating a diastolic AHF was successfully induced by TAC. Lung weight/body weight ratio (LW/BW) was significantly increased by ~90% in both ICR and C57 TAC mice (*Figure 1F*), indicating an acute pulmonary oedema occurred.

Higher mortality of type one cannabinoid receptor deficiency mice in response to transverse aortic constriction

The survival curve showed that nearly 65% of CB1 KO mice vs. 11% of WT mice died during the first week after TAC

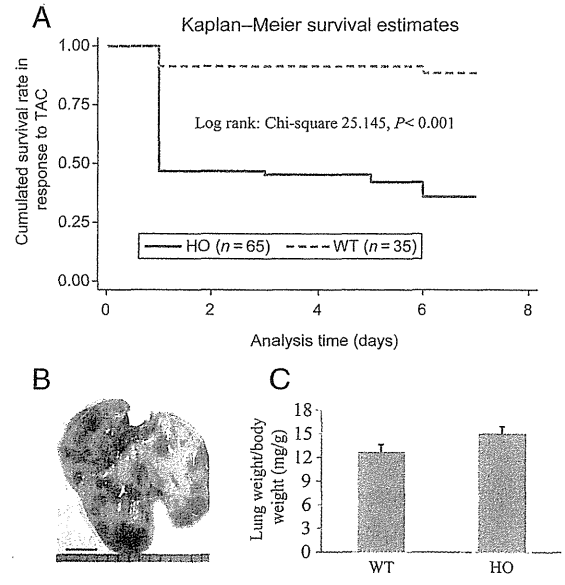

Figure 2 Effect of type one cannabinoid receptor deficiency on 1-week survival and cause of death in response to transverse aortic constriction. (*A*) Kaplan–Meier survival curves for mice subjected to transverse aortic constriction. $N = 35$ and $n = 65$ in the type one cannabinoid receptor wild-type and type one cannabinoid receptor knockout groups, respectively ($P < 0.001$, Log-rank test). (*B*) Representative picture of lung with pulmonary oedema and haemorrhage. Scale bar = 3 mm. (*C*) The lung weight/body weight ratio for both the wild-type ($n = 4$) and knockout ($n = 42$) mice died of acute heart failure.

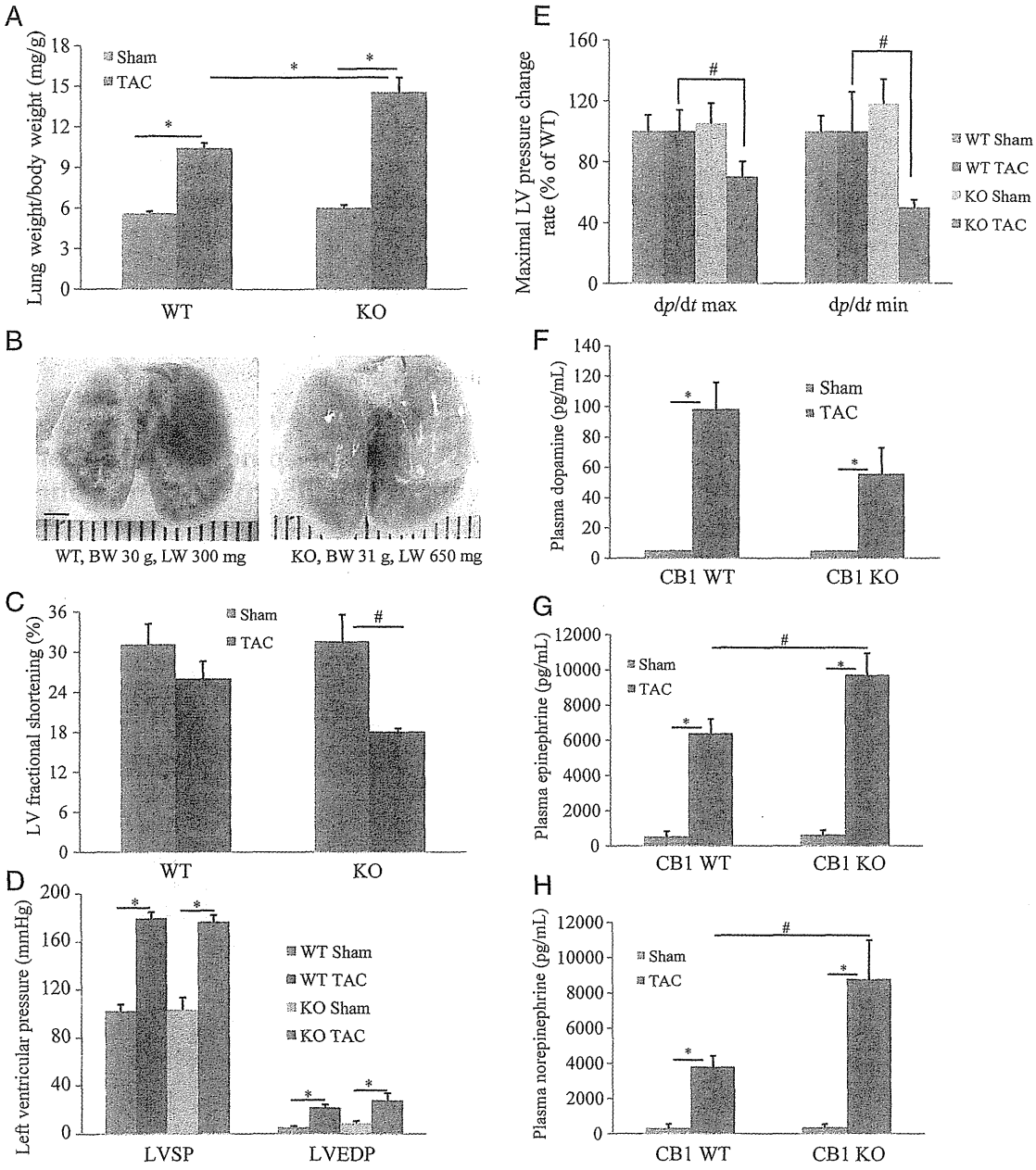

Figure 3 Effects of type one cannabinoid receptor deficiency on pulmonary oedema, cardiac function, and circulatory catecholamine in response to transverse aortic constriction. (*A*) Lung weight/body weight ratio at 1 h after transverse aortic constriction. $n = 16$, 9, 16, and 14 in the wild-type sham, wild-type transverse aortic constriction, knockout sham, and knockout transverse aortic constriction group, respectively. (*B*) Examples of congestive lungs from two wild-type and knockout mice with similar body weight subjected to transverse aortic constriction. Scale bar = 2 mm. (*C*) Left ventricular fractional shortening measured under anaesthesia at 10 min after surgery ($n = 5$ per group). (*D*) Left ventricular systolic and end-diastolic pressure. (*E*) Maximal left ventricular pressure change rate [data were transformed to percentage of the corresponding wild-type groups. $n = 5$, 4, 8, and 8 in wild-type sham, wild-type transverse aortic constriction, knockout sham, and knockout transverse aortic constriction groups, respectively for both (*C*) and (*D*)]. Plasma levels of dopamine (*F*), epinephrine (*G*), and norepinephrine (*H*) at 1 h after transverse aortic constriction were measured. $N = 5$, 6, 7, and 12 in the Sham knockout, transverse aortic constriction knockout, Sham WT, and transverse aortic constriction WT group, respectively. $^{\#}P < 0.05$, $^{*}P < 0.01$.

(*Figure 2A*). By daily observation of the mice and performing autopsy of the dead animals, we found that acute pulmonary oedema was the major cause of death, as indicated by the presence of pulmonary haemorrhage/oedema (*Figure 2B*). The LW/BW ratio was usually higher than 12 mg/g (more than two folds of the normal value) in mice that died of AHF. These results indicate that CB1 activation is crucial to protect the heart from acute pulmonary oedema.

Acute cardiac effects of type one cannabinoid receptor inactivation or activation

When the mice were subjected to TAC and sacrificed after 1 h, we found that the CB1 KO mice developed more severe pulmonary oedema. There was a significant difference of LW/BW ratio between the KO and WT mice (nearly 40% larger in KO group than in WT group, *Figure 3A* and *B*), suggesting that CB1 plays an important role in pulmonary oedema.

We then examined echocardiograph and LV haemodynamic parameters of cardiac function. At 10 min after TAC, CB1 KO mice showed a significant decrease of LV fractional shortening (LV dimensions and wall thickness are showed in Supplementary material online, *Figure S1*), whereas no significant change was observed in WT mice (*Figure 3C*). At 30 min after TAC, the extent of pressure overload (i.e. the LVSP) was similar between CB1 KO mice and WT mice (*Figure 3D*), but, CB1 KO mice had a lower LV d*p*/d*t* max (reduced by 30%) and d*p*/d*t* min (reduced by 50%), a lower contractility index, and a larger tau (*Figure 3E*). These data indicated that CB1 inactivation was detrimental to both systolic and diastolic cardiac function in the context of pressure overload.

Type one cannabinoid receptor deficiency promotes catecholamine release in response to transverse aortic constriction

At one hour after TAC, catecholamine was elevated significantly in both KO and WT mice (*Figure 3F–H*), but CB1 KO mice had much higher epinephrine (152% of WT) and norepinephrine (231% of WT) levels than WT mice (*Figure 3G* and *H*). The decrease in CB1 activity dependent dopamine release may be one of the reasons for the lower plasma concentration of dopamine following TAC in CB1 KO mice than in WT mice, which was in agreement with previous studies.[13] It was plausible that the extremely high epinephrine and norepinephrine levels in CB1 KO mice might have contributed to their high mortality.

Influence of epinephrine on left ventricular haemodynamic and pulmonary oedema

In both normal and TAC wild-type mice, LVEDP was significantly elevated after intraperitoneal injection of epinephrine at 2 mg/kg (*Figure 4B*). Epinephrine increased the LVSP, heart rate, and LV contractility index more markedly in normal mice than in TAC

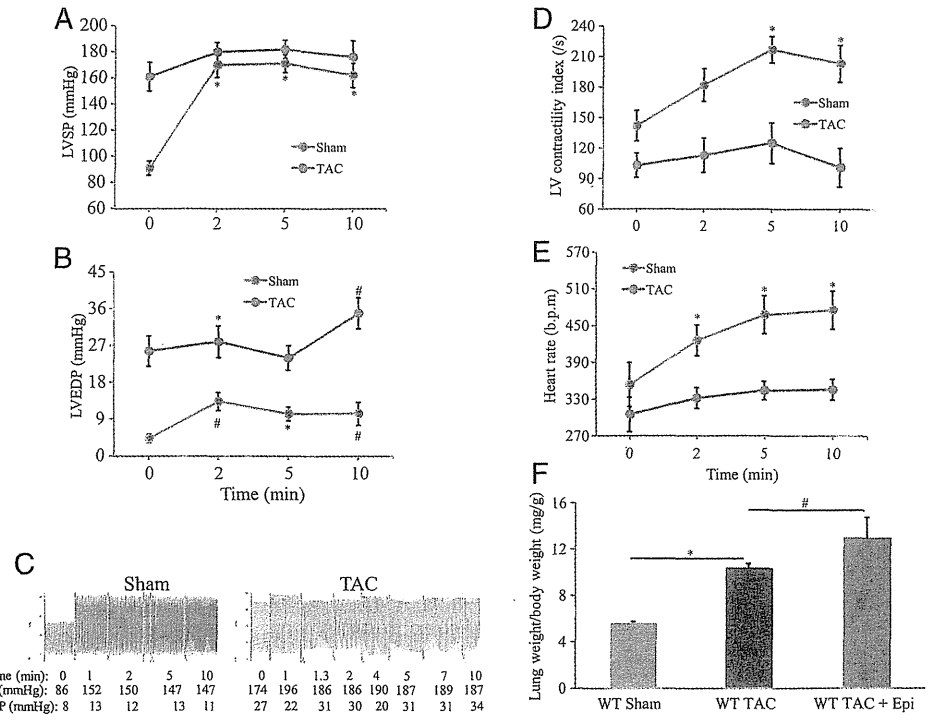

Figure 4 Acute effect of epinephrine on left ventricular haemodynamic and pulmonary oedema. (*A*) Left ventricular systolic pressure. (*B*) Left ventricular end-diastolic pressure. (*C*) Examples of left ventricular pressure recording. (*D*) The left ventricular contractility index. (*E*) Heart rate. $^{\#}P < 0.05$, $^{*}P < 0.01$, compared with the respective baseline values (0). $N = 7$ in each group. Epinephrine (2 mg/kg) was intraperitoneal injected. (*F*) Epinephrine (2 mg/kg, i.p.) significantly increased lung weight to body weight ratio at 1 h after transverse aortic constriction ($n = 16$, 9, and 7 in the Sham, transverse aortic constriction, and transverse aortic constriction + epinephrine group). $^{\#}P < 0.05$, $^{*}P < 0.01$.

mice (*Figure 4A* and *C–E*), suggesting that excessive sympathetic activation led to suppression of cardiac diastolic function. We further confirmed that epinephrine exaggerated acute pulmonary oedema in TAC mice indicated by a 25% increase of LW/BW (*Figure 4F*).

Effects of type one cannabinoid receptor inactivation on haemodynamic, blood cell counts and endocannabinoids system

Genotyping results are showed in *Figure 5A*. Intraperitoneal injection of CB1 agonist WIN exerted no effect on systolic blood pressure in CB1 KO mice while it decreased blood pressure markedly in WT mice (*Figure 5B*), and greater suppression of heart rate was also noted in WT mice (*Figure 5C*), indicating a vascular dilatory effect and a negative chronotropic character of CB1 activation.

No significant differences on peripheral blood cell counts were found between WT and KO mice in either sham or TAC groups (*Figure 5D*). Cardiac FAAH expression level was higher in WT sham group than in KO sham group ($P < 0.05$), which was down-regulated in WT TAC group ($P < 0.05$), whereas there was no significant difference between KO sham and KO TAC groups (*Figure 5E*). Pulmonary FAAH expression level was a little lower but with statistical significance in WT TAC mice than in WT sham mice ($P < 0.05$) (*Figure 5F*). Monoacylglycerol lipase gene expression in hearts of WT TAC mice was significantly lower

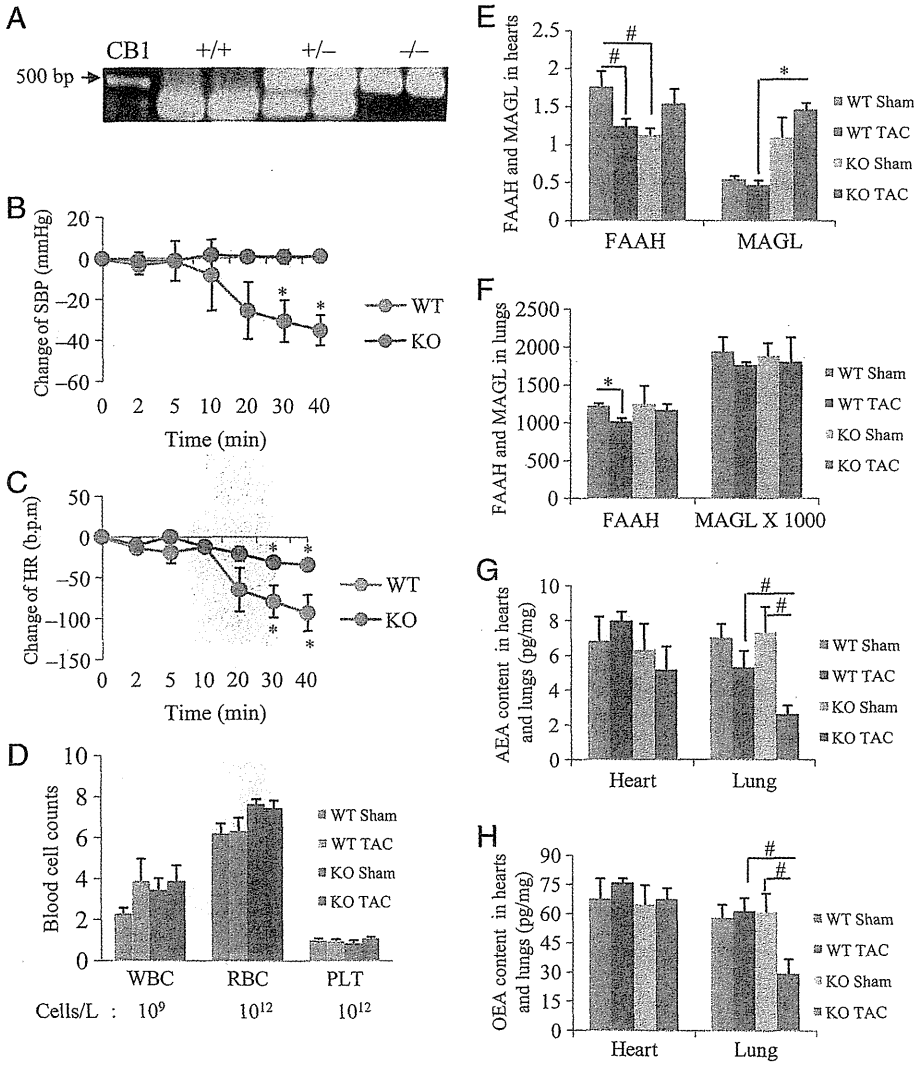

Figure 5 Effects of type one cannabinoid receptor deficiency on haemodynamic, blood cell counts, and endocannabinoid system. (*A*) Genotyping results. Effect of intraperitoneal injection of CB1 agonist WIN (Win 55, 212–2, 0.2 mg/kg) on systolic blood pressure (*B*) and heart rate (*C*) in CB1 knockout and wild-type mice. $^{*}P < 0.05$ vs. their corresponding baselines (0 min); $n = 3$ and 5 for knockout and wild-type mice, respectively. (*D*) Blood cell counts, WBC, white blood cell; RBC, red blood cell, PLT, platelet; $n = 4$ in each group. Real-time PCR results for fatty acid amide hydrolase and the monoacylglycerol lipase in hearts (*E*) and lungs (*F*), $n = 6$ per group. Anandamide (*G*) and *N*-oleoylethanolamine (*H*) contents in hearts and lungs (pg/mg), $n = 4$ per group. $^{*}P < 0.01$, $^{\#}P < 0.05$.

than in KO TAC mice ($P < 0.01$) (*Figure 5E*), while no significant differences were found on pulmonary MAGL expression among the four groups (*Figure 5F*) (also see Supplementary material online, *Figure S2*).

We further measured endogenous cannabinoids in heart and lung samples and found that there were no significant differences on AEA and OEA contents between WT and KO sham mice. Different from WT mice, both AEA and OEA contents in lung tissues were significantly decreased in KO mice after TAC ($P < 0.05$) (*Figure 5G* and *H*).

Type one cannabinoid receptor agonists rescue acute heart failure

We next tested the effects of two CB1 agonists WIN and 2-AG and a selective CB1 antagonist (AM251) on acute pulmonary oedema. These drugs were administrated before the performance of TAC. One hour after TAC, we sacrificed the mice and evaluated the LW/BW ratio. Treatment with either WIN (0.2 mg/kg) or 2-AG (5 mg/kg) significantly reduced LW/BW by ~30 and 40%, respectively ($P < 0.05$ and 0.01), while the administration of AM251 (0.3 mg/kg) increased it by 10% (*Figure 6A*). We also evaluated the effect of CB1 activation on catecholamine and LV function. In WT TAC mice, WIN did not suppress cardiac systolic function (*Figure 6B*), but caused a significant decrease in LVEDP by ~60% ($P < 0.01$), an index of pulmonary congestion, and also slowed the heart rate (*Figure 6C* and *D*).

In addition to the preventive use of CB1 agonists, we further evaluated their therapeutic effect on pulmonary congestion. One hour after the TAC, WIN, 2-AG, or AM251 was given to the mice at the same dosage as above described and LW/BW was

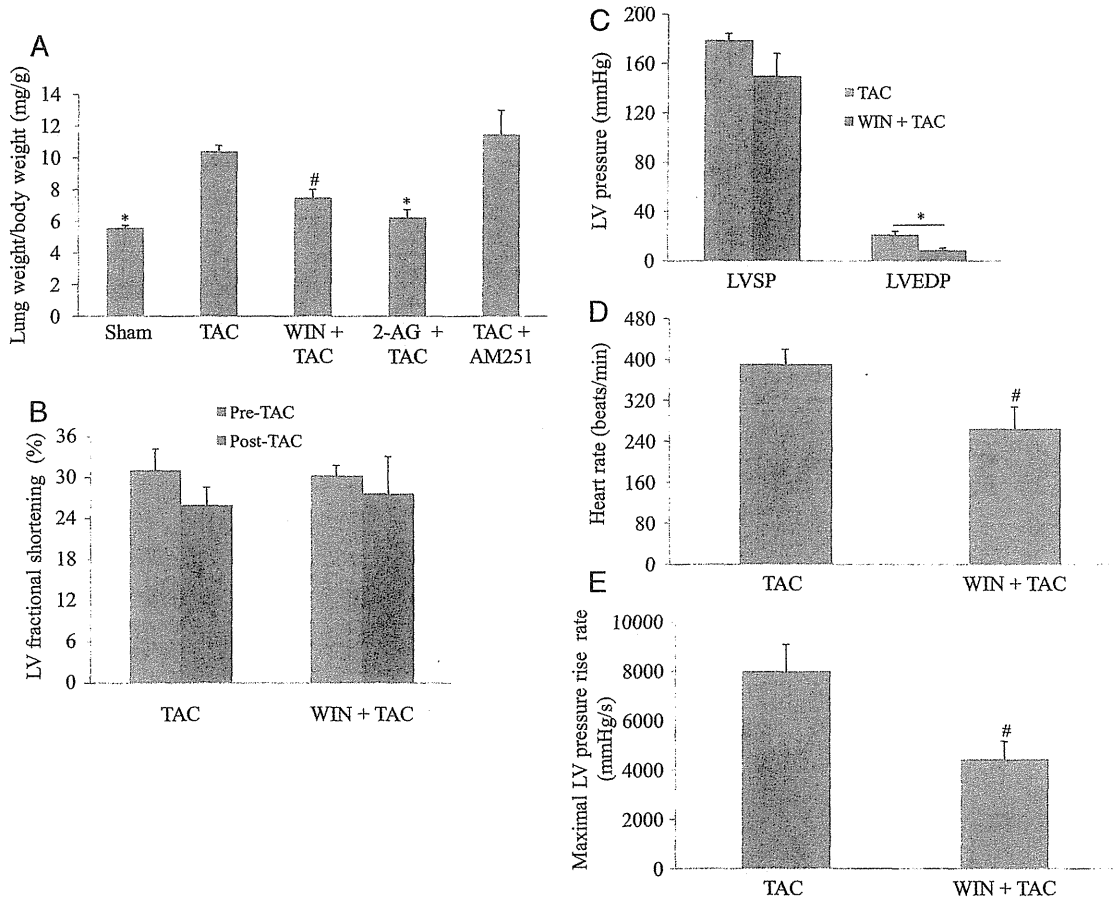

Figure 6 Effect of type one cannabinoid receptor activation on pulmonary oedema and heart function. (*A*) WIN 55, 212-2 (WIN) (0.2 mg/kg, i.p.) or 2-arachidonyl glycerol (5 mg/kg, i.p.) significantly reduced the lung weight to body weight ratio at 1 h after transverse aortic constriction. The type one cannabinoid receptor antagonist AM251 (0.3 mg/kg, i.p.) tended to increase pulmonary oedema. $n = 16$, 9, 5, 6, and 6 in the Sham, transverse aortic constriction, transverse aortic constriction + WIN, transverse aortic constriction + 2-arachidonyl glycerol, and transverse aortic constriction + AM251 group, respectively. (*B*) At 10 min after transverse aortic constriction, no significant difference on left ventricular fractional shortening was found between transverse aortic constriction and WIN + transverse aortic constriction groups. (*C*) Effects of pre-treatment with WIN on left ventricular systolic pressure and left ventricular end-diastolic pressure. (*D*) WIN treatment decreased the heart rate. (*E*) WIN treatment decreased maximal LV pressure rise rate. $^{\#}P < 0.05$, $^{*}P < 0.01$, compared with the transverse aortic constriction group, $n = 5$ in each group.

measured 24 h later. We noted that WIN and 2-AG reduced LW/BW by ~28 and 30%, respectively ($P < 0.05$), while the administration of AM251 increased it by 13% ($P > 0.05$. Supplementary material online, *Figure S4*).

Effects of type one cannabinoid receptor activation on plasma catecholamine and AMP-activated protein kinase activity

WIN reduced circulatory epinephrine by 27% and norepinephrine by 37% in wild-type TAC mice ($P < 0.01$, *Figure 7A*). In cultured cardiomyocytes, co-culture with WIN for 30–60 min significantly enhanced AMPK activity (*Figure 7B*), and this effect was abrogated by knockdown of CB1 (*Figure 7C*). Similarly, phosphorylation of AMPK was also increased in WIN-treated WT sham and TAC mice (*Figure 7D* and *E*).

Discussion

In this study, we found that CB1 plays a previously unrecognized role in acute pulmonary oedema resulting from LV pressure overload. Type one cannabinoid receptor KO mice with pressure overload displayed high levels of catecholamine, severe acute pulmonary oedema, and a high acute mortality rate, while administration of epinephrine to WT mice increased LVEDP and exaggerated pulmonary oedema, indicating that excessive sympathetic activation contributed to the worsening of AHF. Since inhibition of catecholamine release is a well-recognized effect of CB1 agonists[8,14] and pressure overload-induced stress stimulates

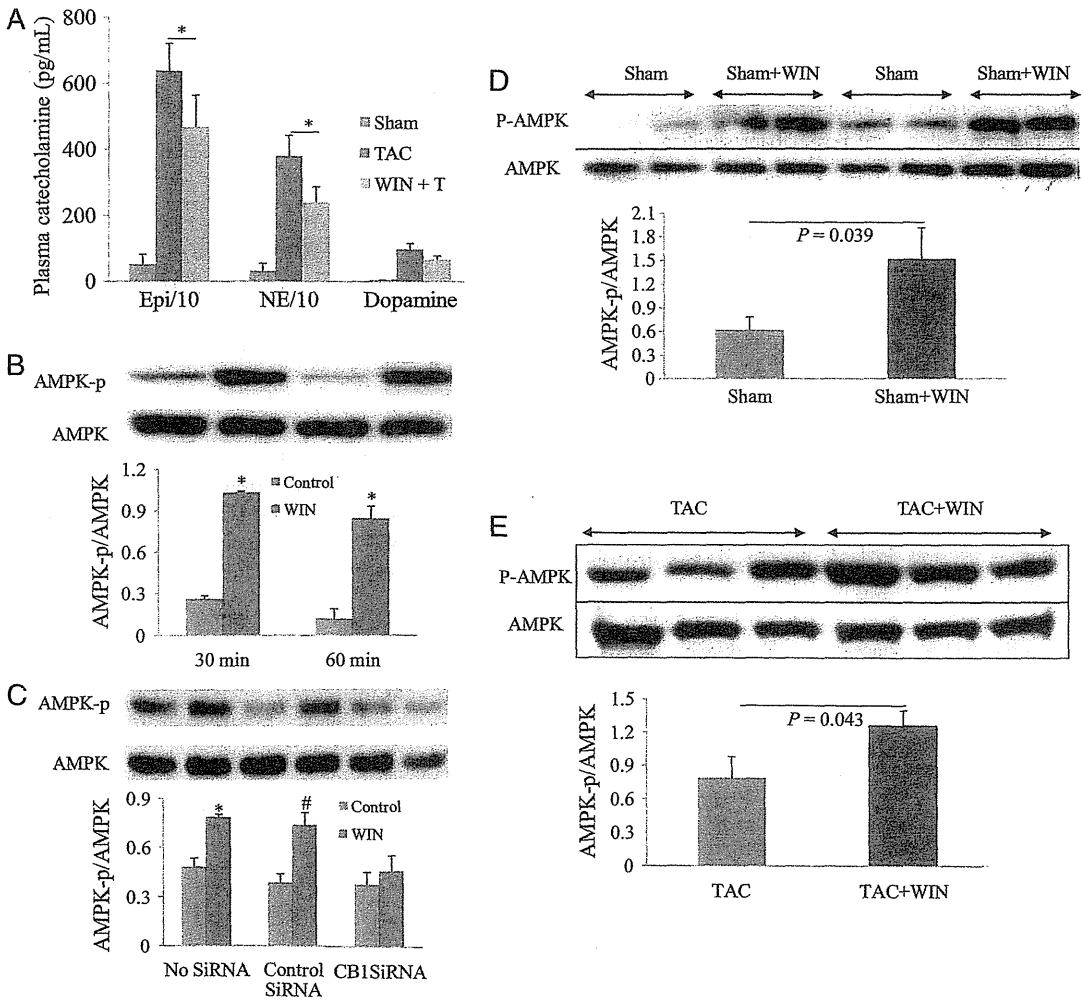

Figure 7 Effects of type one cannabinoid receptor activation on catecholamine levels and AMP-activated protein kinase activity. (*A*) Effects of pretreatment with WIN on plasma levels of epinephrine, norepinephrine, and dopamine. $N = 6, 12, 9$ in sham, transverse aortic constriction, and WIN + transverse aortic constriction groups, respectively, $^{*}P < 0.01$. (*B*) Effect of WIN on AMP-activated protein kinase activity in cultured neonatal rat cardiomyocytes. (*C*) Effect of type one cannabinoid receptor knockdown on AMP-activated protein kinase activity. AMP-activated protein kinase phosphorylation in the hearts of sham (*D*) and transverse aortic constriction mice (*E*) was also examined. Experiments in (*B*) and (*C*) were repeated for three to four times, $^{\#}P < 0.05$, $^{*}P < 0.01$, compared with the responding control group.

sympathetic activity, it seems reasonable that high catecholamine levels were found in CB1 KO mice after TAC. Notably, the decrease in pulmonary endogenous cannabinoids following pressure overload might have also contributed to the severer pulmonary oedema in KO mice, suggesting a cardioprotection conferred by endocannabinoids.

Most patients with AHFS present with pulmonary oedema and a normal-to-high systolic blood pressure,[15] which is similar to our murine model of AHF. Currently available vasodilators used to treat AHFS include nitroglycerin, nitroprusside, and nesiritide, which were reported to ameliorate symptoms and improve clinical status, but did not decrease the mortality rate.[16–18] Our results in the present study that mortality of AHF was increased in CB1 deficient mice and pulmonary oedema was improved by CB1 agonists in WT mice would raise new hope for the treatment of acute heart failure.

Beneficial effects of cannabinoids have been observed in various critical cardiovascular diseases such as septic shock, myocardial ischaemia/reperfusion injury, or myocardial infarction.[11,19–23] A wealth of data have shown that cardiovascular beneficial effects could be mediated by activation of either CB2 or CB1,[24–27] however, deleterious actions of CB1 activation on doxorubicin-induced cardiac dysfunction have also been reported.[26,28] Emerging evidence shows that selective CB1-receptor blockade with rimonabant significantly promotes reduction in weight and favourable changes in cardiometabolic risk factors,[29,30] which are at least in part attributable to blocking the role of cannabinoids on appetite stimulation.[31] Several recent published reports from almost the same laboratories have suggested that CB1 antagonist rimonabant is protective in some non-classical forms of heart failure induced by hepatic cirrhosis and doxorubicin.[28,32,33] It should keep in mind that those forms of heart failure are largely different from the classical types. On the other hand, similar to beta 1 blocker, CB1 agonists have negative inotropic effect, thus it is not surprising that CB1 activation reduced myocardial contractility in hepatic cirrhosis or doxrubicine-induced heart failure.[28,32,33] Lim *et al.*[34] reported that rimonabant reduced infarct size in wild-type mice, but in untreated mice, infarct size was similar between CB1 KO and wild-type groups, suggesting that rimonabant has CB1-independent cardioprotective role. Paradoxically, a recent published large clinical trial (RESCENDO) showed that rimonabant did not improve major vascular event-free survival in obesity patients with previously manifest or increased risk of vascular disease at a mean follow-up of 14 months,[35] which is by coincidence in agreement with previous experimental studies.[11,23] It seems plausible that agents for reducing cardiovascular risk factors are not necessarily effective for treatment of overt cardiovascular disease. Noticeably, a recent review of endocannabinoid effects on the heart by Hiley[26] shows that it is not possible yet to define CB1 activation simply as protective or injurious to the cardiovascular system, though the balance does seem to favour a protective role. Therefore, extensively studies to address this issue should be encouraged.

Pulmonary congestion is one of the main reasons for admission and readmission in patients with AHFS. The pathophysiology may be dominated by hypertension and neurohormonal activation resulting in increased LV filling pressure. Our murine model of AHF was characterized by hypertension and neurohormonal activation as well as pulmonary oedema. This study implicated that endogenous activation of CB1 seems to meet at least three ideal properties for an AHFS therapy[36]: (i) improve pulmonary congestion, (ii) improve haemodynamic, and (iii) improve the neurohormonal profile. Other pharmacological properties of CB1 activation may also favour AHFS, for example, limiting damage to the heart,[20,23] stimulating AMP-activated protein kinase activity in cardiomyocytes as showed in this study and elsewhere,[9] reinforcing properties of morphine,[2] a long-standing therapy for AHFS recommended by Practice Guidelines.[37] For patients with AHFS, in addition to optimal therapy, metabolic modulators are an attractive choice of co-treatment. AMP-activated protein kinase activation is an important preventative therapeutic target for the progression of heart failure.[38,39] We postulate that CB1 agonists may exert additional benefit for the failing heart by improving energy metabolism through enhancement of AMPK activity.

Accumulated evidence shows that endocannabinoids seem to be produced 'on demand'.[40] Our findings in this study suggest that endocannabinoids fulfill a protective role in AHF. Therefore, pharmacologically manipulation of endocannabinoid levels might be a possible therapeutic approach for AHFS.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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Activation of Natural Killer T Cells Ameliorates Postinfarct Cardiac Remodeling and Failure in Mice

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Rationale: **Chronic inflammation in the myocardium is involved in the development of left ventricular (LV) remodeling and failure after myocardial infarction (MI). Invariant natural killer T (iNKT) cells have been shown to produce inflammatory cytokines and orchestrate tissue inflammation. However, no previous studies have determined the pathophysiological role of iNKT cells in post-MI LV remodeling.**

Objective: **The purpose of this study was to examine whether the activation of iNKT cells might affect the development of LV remodeling and failure.**

Methods and Results: **After creation of MI, mice received the injection of either α-galactosylceramide (αGC; n=27), the activator of iNKT cells, or phosphate-buffered saline (n=31) 1 and 4 days after surgery, and were followed during 28 days. Survival rate was significantly higher in MI+αGC than MI+PBS (59% versus 32%, $P<0.05$). LV cavity dilatation and dysfunction were significantly attenuated in MI+αGC, despite comparable infarct size, accompanied by a decrease in myocyte hypertrophy, interstitial fibrosis, and apoptosis. The infiltration of iNKT cells were increased during early phase in noninfarcted LV from MI and αGC further enhanced them. It also enhanced LV interleukin (IL)-10 gene expression at 7 days, which persisted until 28 days. AntienIL-10 receptor antibody abrogated these protective effects of αGC on MI remodeling. The administration of αGC into iNKT cell-deficient $J\alpha18^{-/-}$ mice had no such effects, suggesting that αGC was a specific activator of iNKT cells.**

Conclusions: **iNKT cells play a protective role against post-MI LV remodeling and failure through the enhanced expression of cardioprotective cytokines such as IL-10. (*Circ Res.* 2012; 111:1037-1047.)**

Key Words: natural killer T cells ■ myocardial infarction ■ inflammation ■ heart failure ■ cytokines

Myocardial infarction (MI) leads to the development of heart failure (HF), which is the major cause of death in post-MI patients. The changes in left ventricular (LV) geometry, such as cavity dilatation associated with myocyte hypertrophy and interstitial fibrosis, referred to as remodeling, contribute to the development of depressed cardiac function in HF after MI.[1] It has been reported that monocytes and lymphocytes are infiltrated in noninfarcted area as well as infarcted area of LV after MI.[2,3] Chemokines, monocyte chemoattractant protein-1 (MCP-1), and RANTES (regulated on activation normally T-cell expressed and secreted), are essential factors in the recruitment and activation of monocyte and lymphocyte. These chemokines are also increased in noninfarcted LV after MI and contribute to local inflammation through the release of inflammatory cytokines including tumor necrosis factor-α (TNF-α).[2,4] Targeted deletion of CC chemokine receptor 2 or anti–MCP-1 gene therapy has been shown to attenuate LV remodeling after MI.[2,5] Thus, chronic tissue inflammation plays an important role in LV remodeling process.

Invariant natural killer T (iNKT) cells are innate-like T-lymphocyte population coexpressing NK markers and an αβ T-cell receptor that recognize glycolipid antigens. They can rapidly and robustly produce a mixture of T-helper type 1 (T_H1) and T_H2 cytokines, such as TNF-α, interferon-γ (IFN-γ), interleukin (IL)-10, and IL-4, and also a vast array of chemokines in shaping subsequent adaptive immune response.[6] Thus, iNKT cells can function as a bridge between the innate and adaptive immune systems, and orchestrate

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Non-standard Abbreviations and Acronyms	
αGC	α-galactosylceramide
HF	heart failure
IFN-γ	interferon-γ
IL	interleukin
iNKT	invariant natural killer T
LV	left ventricle
MCP-1	monocyte chemoattractant protein-1
MI	myocardial infarction
MMP	matrix metalloproteinase
NK	natural killer
PBS	phosphate-buffered saline
qRT-PCR	quantitative reverse transcriptase–polymerase chain reaction
RANTES	regulated on activation normally T cell expressed and secreted
T_H1	T-helper type 1
T_H2	T-helper type 2
TNF-α	tumor necrosis factor-α

tissue inflammation. Indeed, we have shown that iNKT cells activate vascular wall inflammation in atherogenesis and adipose tissue inflammation in obesity-induced glucose intolerance.[7,8] On the other hand, iNKT cells play a protective role against autoimmune and inflammatory diseases such as type 1 diabetes,[9,10] allergic encephalomyelitis,[9,11] and rheumatoid arthritis.[12] These findings suggest that iNKT cells may have bidirectional effects on tissue inflammation. However, no previous studies have examined the changes of iNKT cells and their pathophysiological role in LV remodeling and failure after MI.

Therefore, the purpose of the present study was to determine whether iNKT cells might affect the development of LV remodeling and failure after MI. We demonstrated that the activation of iNKT cells by α-galactosylceramide (αGC), a specific activator for iNKT cells,[13] attenuated the development of LV remodeling and failure after MI in mice. The enhanced gene expression of IL-10 might be involved in these beneficial effects of iNKT cells on this disease process.

Methods

All procedures and animal care were approved by our institutional animal research committee and conformed to the animal care guideline for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine.

Experiment 1: Time-Dependent Changes of iNKT Cell Receptors in Post-MI Hearts

Animal Models

MI was created in male C57BL/6J mice, 6 to 8 weeks old and 20 to 25 g body weight, by ligating the left coronary artery as described previously.[14] Sham operation without ligating the coronary artery was also performed as control. MI mice were euthanized and the hearts were excised at days 3, 7, 14, and 28 for quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) measurements.

Quantitative RT-PCR

Quantitative PCR for Vα14Jα18 (a specific marker of iNKT cells) was performed, as described previously.[8]

Experiment 2: Effects of iNKT Cell Activation on Post-MI Heart Animal Models

Sham and MI mice were created in male C57BL/6J as described in experiment 1. Each group of mice was randomly divided into 2 groups; either αGC (0.1 μg/g body weight; Funakoshi Company, Ltd, Tokyo, Japan), the activator of iNKT cells, or phosphate-buffered saline (PBS) was administered via intraperitoneal injection 1 and 4 days after surgery. The concentration of αGC was chosen based on the previous study of its efficacy.[8] Thus, the experiment was performed in the following 4 groups of mice; sham+PBS (n=10), sham+αGC (n=10), MI+PBS (n=31), and MI+αGC (n=27).

Survival

The survival analysis was performed in all 4 groups of mice. During the study period, the cages were inspected daily for dead animals. All dead mice were examined for the presence of MI as well as pleural effusion and cardiac rupture.

Echocardiographic and Hemodynamic Measurements

Echocardiographic and hemodynamic measurements were performed under light anesthesia with tribromoethanol/amylene hydrate (avertin; 2.5% wt/vol, 8 μL/g ip), as described previously.[14]

Myocardial Histopathology, Infarct Size, Myocardial Apoptosis, and Matrix Metalloproteinase Zymography

Myocyte cross-sectional area, collagen volume fraction, infarct size, myocardial apoptosis, and zymographic matrix metalloproteinase (MMP) levels were determined as described previously.[14,15]

Isolation of Cardiac Mononuclear Cell and Flow Cytometry

Cardiac mononuclear cells from 3 mice were isolated, pooled, and subjected to flow cytometric analysis as previously described.[7,16]

Quantitative RT-PCR

Quantitative PCR for Vα14Jα18, CD11c (a marker of M1 macrophages), arginase-1 (a marker of M2 macrophages), MCP-1, RANTES, IFN-γ, IL-4, IL-6, TNF-α, and IL-10 was performed, as described previously.[8]

Immunohistochemistry

LV sections were immunostained with antibody against mouse MAC3 (a macrophage marker), mouse CD3 (a T-cell marker), or mouse myeloperoxidase (a leukocyte marker), followed by counterstaining with hematoxylin.

Plasma Cytokine Concentration

Plasma IL-10, TNF-α, IFN-γ, IL-6, and IL-4 levels were measured by commercially available ELISA kit (R&D systems, Inc) in all groups.

Experiment 3: Effects of IL-10 Neutralization on αGC-Treated Post-MI Hearts

MI mice were divided into the following 3 groups; MI+αGC (n=18), MI+anti–IL-10 receptor antibody (n=12), and MI+αGC+anti–IL-10 receptor antibody (n=19). αGC was administered identically as in experiment 2. Anti–IL-10 receptor antibody (500 μg/mouse, BD Pharmingen, San Diego, CA) was administered via

intraperitoneal injection 1, 4, and 14 days after surgery. The concentration of anti–IL-10 receptor antibody was chosen based on the previous study of its efficacy.[12] Four weeks after surgery, echocardiographic and hemodynamic measurements were performed. Separate groups of mice were used in the MI+αGC group in experiment 2.

Experiment 4: Specificity of αGC for NKT Cells

Vα14$^+$ NKT cell-deficient Jα18$^{-/-}$ (Jα18 KO) mice were provided by Dr M. Taniguchi (RIKEN, Yokohama, Japan) and back-crossed 10 times to C57BL/6J.[17] Sham and MI mice were created in male Jα18 KO mice as described in experiment 1. Each group of mice was treated identically to experiment 2. Thus, the experiment was performed in the following 4 groups; KO+sham+PBS, KO+sham+αGC, KO+MI+PBS, and KO+MI+αGC. One week after surgery, all mice (n=9 for each group) were euthanized and used for immunohistochemistry (n=3 for each group) and for qRT-PCR (n=6 for each group). These analyses were performed as described in experiment 2.

Statistical Analysis

Data are expressed as mean±SEM. Survival analysis was performed by the Kaplan-Meier method, and between-group differences in survival were tested by the log-rank test. A between-group comparison of means was performed by 1-way ANOVA, followed by *t* test. The Bonferroni correction was applied for multiple comparisons of means. $P<0.05$ was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors had read and agreed to the manuscript as written.

Results

Experiment 1: Time-Dependent Changes of iNKT Cell Receptors in Post-MI Hearts

The quantification of iNKT cells by Vα14/Jα18 gene expression demonstrated that iNKT cell infiltration into the noninfarcted LV was significantly enhanced at 7 days (1.7±0.2-fold changes from baseline, $P<0.05$ versus baseline) after MI and returned to baseline at 14 and 28 days after MI (1.0±0.2- and 1.1±0.1-fold changes from baseline, respectively). In the infarcted LV, its gene expression was significantly elevated 7 days and remained elevated 28 days after MI (data not shown).

Experiment 2: Effects of iNKT Cell Activation on Post-MI Hearts

By using flow cytometric analysis, iNKT cells were detected in LV from all groups of mice (Figure 1A). αGC injection increased iNKT cells infiltration into the noninfarcted LV both in sham+αGC and MI+αGC mice after 7 days (Figure 1A). Moreover, it remained enhanced at 28 days in MI+αGC (Figure 1A).

Quantitative RT-PCR also demonstrated that gene expression of Vα14/Jα18, a marker of iNKT cell infiltration, was significantly elevated in the noninfarcted LV from sham+αGC and MI+αGC mice after 7 days (Figure 1B). Interestingly, it remained significantly increased at 28 days only in MI+αGC (Figure 1B).

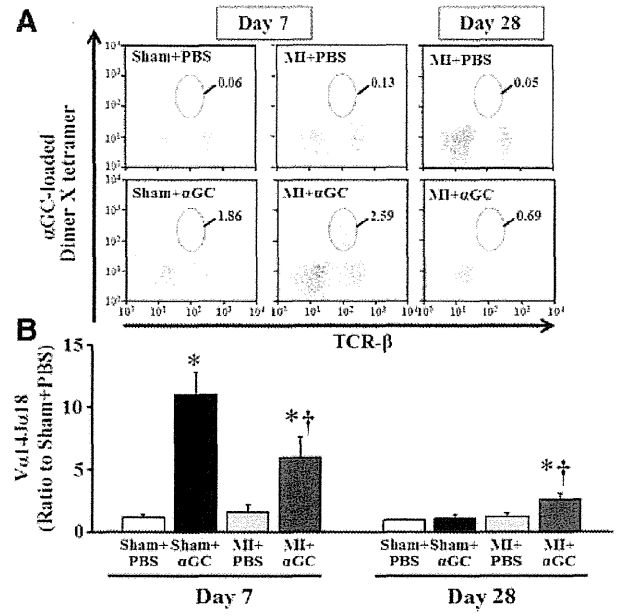

Figure 1. A, Representative flow cytometric assessment of cardiac mononuclear cells obtained from sham+PBS, sham+αGC, MI+PBS, and MI+αGC at days 7 and 28. Cardiac mononuclear cells from 5 different mice for each group were pooled and analyzed. The experiments were performed 3 times. iNKT cells were gated as the αGC-loaded dimer X tetramer$^+$TCR-β$^+$ population. The **inset** numbers are a percentage of the gated region of the samples. **B**, Gene expression of Vα14/Jα18 in noninfarcted LV from sham+PBS, sham+αGC, MI+PBS, and MI+αGC 7 days (n=6) and 28 days (n=4) after surgery. They were normalized to GAPDH gene expression and expressed as ratio to sham+PBS values. Data are expressed as mean±SEM. *$P<0.05$ versus sham+PBS, †$P<0.05$ versus MI+PBS.

Survival

There were no deaths in sham-operated groups. The survival rate during 28 days was significantly higher in MI+αGC compared with MI+PBS mice (59% versus 32%; $P<0.05$; Figure 2A). Thirteen MI+PBS (42%) and 8 MI+αGC (30%) mice died of LV rupture (P=NS).

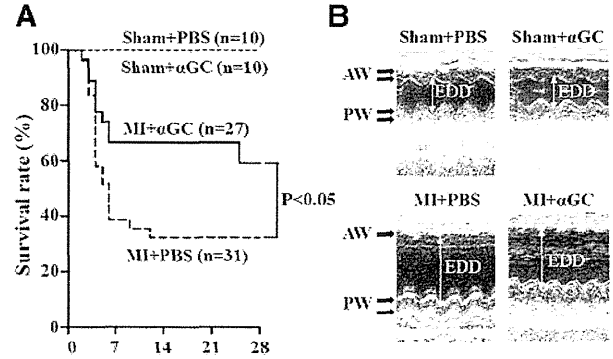

Figure 2. A, Percent survival of sham+PBS (n=10), sham+αGC (n=10), MI+PBS (n=31), and MI+αGC (n=27) mice shown by Kaplan-Meier method. B, Representative M-mode echocardiographic images obtained from sham+PBS, sham+αGC, MI+PBS, and MI+αGC. AW indicates anterior wall; PW, posterior wall; EDD, end-diastolic diameter.

Table 1. Echocardiography, Hemodynamics, and Organ Weights in Experiment 2

	Sham+PBS (n=10)	Sham+αGC (n=10)	MI+PBS (n=10)	MI+αGC (n=16)
Echocardiography				
Heart rate, bpm	522±10	522±12	531±16	520±13
LVEDD, mm	3.4±0.1	3.4±0.04	5.4±0.1*	5.0±0.1*†
LVESD, mm	2.1±0.03	2.1±0.04	4.5±0.1*	4.1±0.1*†
FS, %	38.2±0.7	38.3±0.6	16.5±0.6*	18.8±0.6*†
AWT, mm	0.63±0.01	0.62±0.01	0.31±0.01*	0.30±0.01*
PWT, mm	0.68±0.02	0.68±0.01	0.97±0.01*	0.96±0.02*
Hemodynamics				
Heart rate, min	507±9	499±9	485±23	495±11
Mean AoP, mm Hg	78.1±2	77.7±2	75.0±3	79.3±1
LVEDP, mm Hg	1.7±0.3	2.3±0.1	10.7±1.1*	6.6±0.6*†
LV +dP/dt, mm Hg/s	15 625±623	14 972±398	7352±697*	9386±476*†
LV –dP/dt, mm Hg/s	9983±697	9130±691	5045±482*	5861±286*
Organ weights				
Body wt, g	25.1±0.3	24.9±0.2	24.5±0.4	24.8±0.3
Heart wt/body wt, mg/g	4.6±0.1	4.5±0.1	6.8±0.2*	6.1±0.1*†
Lung wt/body wt, mg/g	5.2±0.03	5.2±0.1	7.2±0.7*	5.9±0.2†
Infarct size, %	...	...	56±2	55±1

LVEDD indicates left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness; AoP, aortic pressure; LVEDP, left ventricular end-diastolic pressure; wt, weight. Data are mean±SEM.

*P<0.05 versus sham+PBS.

†P<0.05 versus MI+PBS.

Echocardiography and Hemodynamics

The echocardiographic and hemodynamic data from 4 groups of survived mice at 28 days are shown in Figure 2B and Table 1. There were no significant differences in either echocardiographic or hemodynamic parameters between sham+PBS and sham+αGC mice. LV diameters were significantly greater and LV fractional shortening was significantly lower in MI mice than sham mice. These changes were ameliorated by the treatment of MI mice with αGC. There were no significant differences in heart rate or aortic blood pressure among groups. LV end-diastolic pressure (LVEDP) was significantly increased, and LV +dP/dt and LV –dP/dt were significantly decreased in MI compared with sham, which was ameliorated by the treatment of MI mice with αGC.

Organ Weights, Infarct Size, and Histology

There were no significant differences in heart weight/body weight and lung weight/body weight between sham+PBS and sham+αGC mice (Table 1). In agreement with LVEDP, heart weight/body weight and lung weight/body weight were increased in MI mice, and these increases were significantly attenuated in MI+αGC (Table 1).

Infarct size measured by the morphometric analysis was comparable (56±2% versus 55±1%; P=NS) between MI+PBS (n=6) and MI+αGC (n=6) groups (Table 1).

Histomorphometric analysis of noninfarcted LV sections showed that myocyte cross-sectional area was increased in MI+PBS compared with sham mice and was significantly attenuated in MI+αGC (Figure 3A). Collagen volume fraction was also increased in MI+PBS compared with sham mice and was significantly attenuated in MI+αGC (Figure 3A).

There were rare TUNEL-positive nuclei in both sham and sham+αGC mice. The number of TUNEL-positive myocytes in the noninfarcted LV was increased in MI+PBS and was significantly decreased in MI+αGC (Figure 3B).

Myocardial MMP Activity

Representative gelatin zymography of the noninfarcted LV tissue at day 7 from 4 groups of mice was shown in Figure 4A. There were no zymographic MMP-2 and 9 levels in the sham+PBS and sham+αGC. Zymographic MMP-2 level was significantly increased in MI+PBS mice compared with sham mice at day 7. αGC injection significantly decreased this after MI (Figure 4B). Zymographic MMP-9 level was also increased in MI+PBS mice compared with sham mice at day 7, which, however, was not affected by αGC (Figure 4C).

Zymographic MMP-2 level was increased in MI+PBS mice also at day 28, and αGC injection tended to decrease it (3.7±1.1 versus 2.1±0.8 in ratio to sham, P=0.08).

Inflammatory and Cytokine Gene Expression

Immunohistochemical stainings for MAC3 and CD3 were increased in MI+PBS compared with sham+PBS and were further increased by αGC at day 7 (Figure 5). MPO-positive cells were not detected in the LV tissue from either group of mice (data not shown).

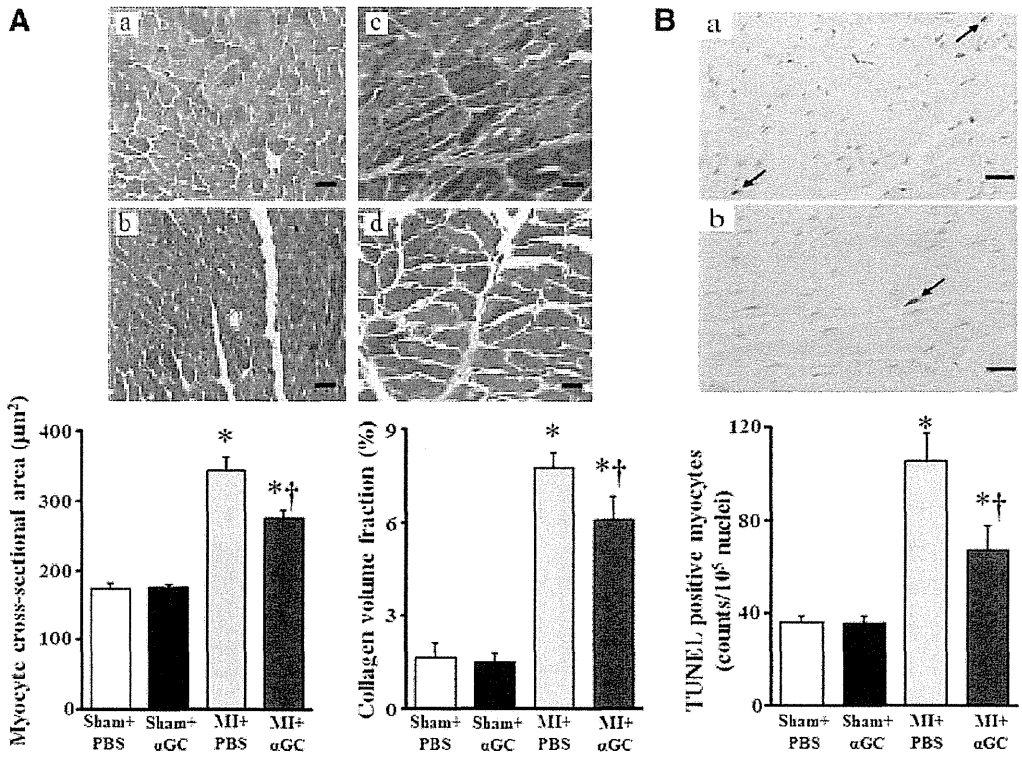

Figure 3. A, Representative high-power photomicrographs of LV cross sections stained with Masson trichrome from sham+PBS (a), sham+αGC (b), MI+PBS (c), and MI+αGC (d) and summary data of myocyte cross-sectional area and collagen volume fraction in 4 groups of mice (n=6). Scale bar, 20 μm. **B,** Representative photomicrographs TUNEL staining of LV sections from MI+PBS **(a)** and MI+αGC **(b)** and summary data for the number of TUNEL-positive cells in the noninfarcted LV (n=6). Scale bar, 20 μm. Data are expressed as mean±SEM. *P<0.05 versus sham+PBS, †P<0.05 versus MI+PBS.

CD11c (a marker of M1 macrophage) and arginase 1 (a marker of M2 macrophage) gene expressions were significantly increased in noninfarcted LV from MI+PBS compared with sham+PBS at day 7 (Figure 6A and 6B). αGC significantly increased their expressions in both sham and MI animals at day 7. Arginase 1 but not CD11c was increased in noninfarcted LV from MI+PBS and MI+αGC at day 28. There was no significant difference in arginase 1 between these 2 groups. MCP-1 and RANTES gene expressions were increased in noninfarcted LV from MI+PBS compared with sham+PBS at day 7 (Figure 6C and 6D). αGC significantly increased their expressions in both sham and MI animals at day 7. In contrast, there was no significant difference in their expressions among all groups at day 28.

IFN-γ, TNF-α, IL-6, and IL-10 gene expression levels were significantly increased in sham and MI mice by αGC at

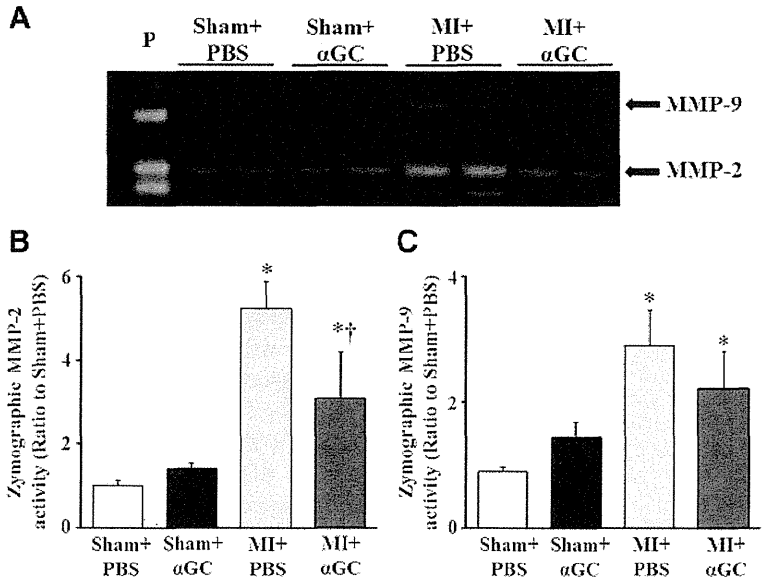

Figure 4. Representative LV zymographic MMP-2 and MMP-9 activities in noninfarcted LV at 7 days after surgery (A) and their densitometric analysis (B and C; n=5 for each). P indicates positive control. Data are expressed as mean±SEM. *P<0.05 versus sham+PBS, †P<0.05 versus MI+PBS.

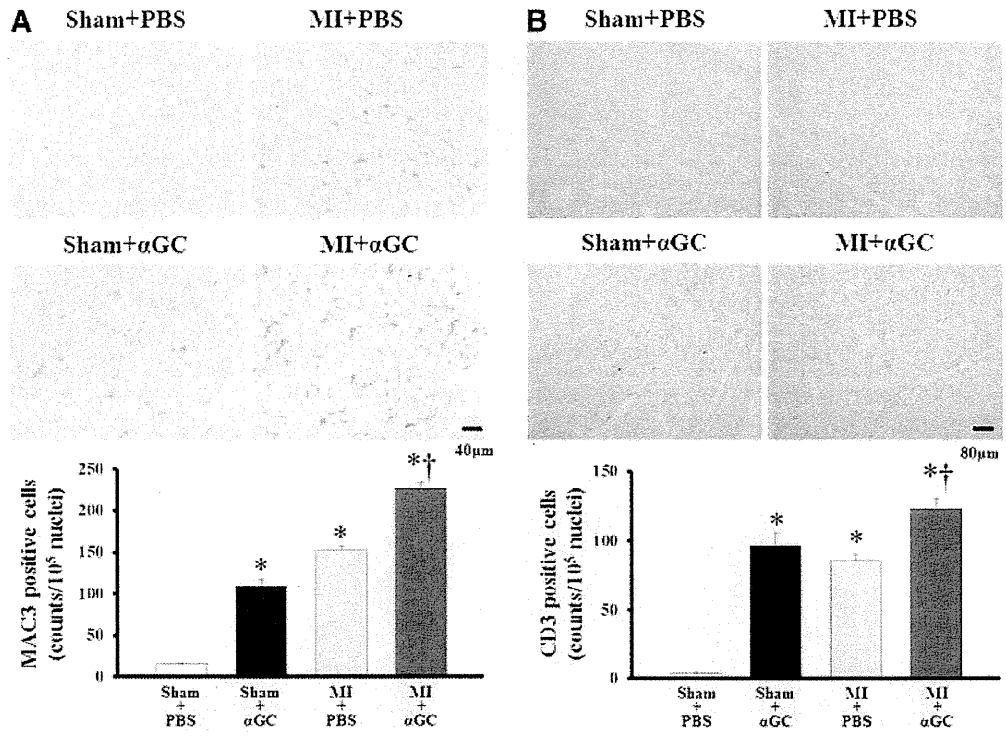

Figure 5. Representative photomicrographs of LV cross sections stained with (A, upper panel) anti-MAC3 and (B, upper panel), anti-CD3 in sham+PBS, sham+αGC, MI+PBS, and MI+αGC. Summary data of the numbers of **(A, lower panel)** MAC3 and **(B, lower panel)** CD3-positive cells in the LV (n=4–8 for each). Data are mean±SEM. *P<0.05 versus sham+PBS, †P<0.05 versus MI+PBS.

day 7 (Figure 6E through 6H). IL-10 gene expression alone significantly elevated up to 2.6-fold in the noninfarcted LV from MI+αGC mice at day 28 (Figure 6H). These time-dependent and αGC-mediated changes in IL-10 gene expression (Figure 6H) in the LV were matched with those in NKT cell infiltration (Figure 1B). IL-4 was not detected in either group.

Plasma Cytokine Concentration

Plasma IL-10 level was similar among sham+PBS, sham+αGC, and MI+PBS groups (9.0±0.5 versus 9.8±2.3 versus 10.6±2.3 pg/mL). However, in parallel to IL-10 gene expression in the LV, it significantly increased up to 2-fold in MI+αGC (21.1±2.3 pg/mL) compared with sham and MI+PBS mice (P<0.05). Plasma IFN-γ level was similar among 4 groups of mice (1.4±0.3 versus 1.7±0.3 versus 0.9±0.2 versus 1.0±0.2 pg/mL, P=NS). Plasma TNF-α, IL-6, and IL-4 levels were not detected in either group.

Experiment 3: Effects of IL-10 Neutralization on αGC-Treated Post-MI Heart Survival

The survival rate during 28 days tended to be higher in MI+αGC than in MI+anti–IL-10 receptor antibody and MI+αGC+anti–IL-10 receptor antibody (66.7% versus 44.4% and 42.1%, P=0.4).

Echocardiography and Hemodynamics

The echocardiographic and hemodynamic data from 3 groups of surviving mice are shown in Table 2. IL-10 receptor antibody injection significantly increased LV diameters, LVEDP, and decreased LV fractional shortening in αGC-treated MI mice. In contrast, there were no differences in these parameters between MI+anti–IL-10 receptor antibody and MI+αGC+anti–IL-10 receptor antibody. There was no significant difference in heart rate and aortic blood pressure among 3 groups.

Organ Weights and Infarct Size

In agreement with LVEDP, lung weight/body weight ratio was significantly increased in MI+αGC+anti–IL-10 receptor antibody compared with MI+αGC (Table 2). There were also no differences in these parameters between MI+anti–IL-10 receptor antibody and MI+αGC+anti–IL-10 receptor antibody.

Infarct size was comparable (56±2%, 54±2%, and 56±4%; P=NS) among MI+αGC (n=8), MI+anti–IL-10 antibody (n=8), and MI+αGC+anti–IL-10 receptor antibody (n=8) groups.

Experiment 4: Specificity of αGC for iNKT Cells

Immunohistochemical stainings for MAC3 and CD3 were increased in KO+MI+PBS compared with KO+sham+PBS. In contrast to the results from wild-type (Figure 5), αGC did not alter them (Online Figure I). MPO-positive cells were not detected in the LV tissue from either group of mice (data not shown). MCP-1 and RANTES were increased in KO+MI+PBS compared with KO+sham+PBS and were not affected by αGC (Online Figure IIA and B). There was

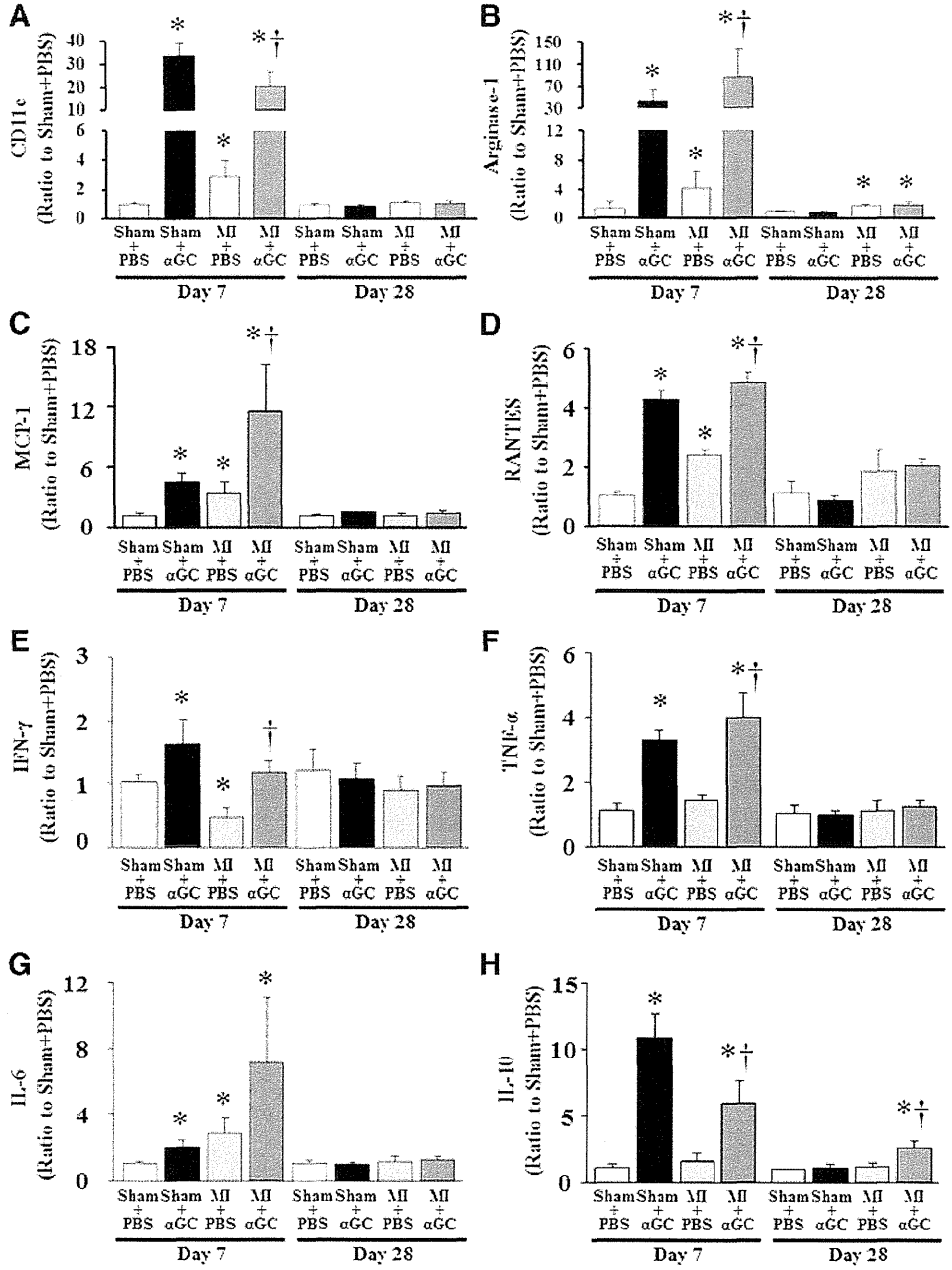

Figure 6. Quantitative analysis of gene expression of CD11c (A), arginase (B), MCP-1 (C), RANTES (D), IFN-γ (E), TNF-α (F), IL-6 (G), and IL-10 (H) in the noninfarcted LV at day 7 (n=6) and day 28 (n=4) after surgery. Gene expression was normalized to GAPDH and depicted as the ratio to sham+PBS. Data are expressed as mean±SEM. *P<0.05 versus sham+PBS, †P<0.05 versus MI+PBS.

no difference in TNF-α and IL-10 in the LV tissue from either group of mice (Online Figure IIC and D). These data suggest that αGC did not directly activate other inflammatory cell, induce chemokines, or produce inflammatory cytokines.

Discussion

The present study demonstrated that the activation of iNKT cells by αGC improved survival and ameliorated LV remodeling and failure after MI in mice, accompanied by the decreases in interstitial fibrosis, cardiomyocyte hypertrophy, and apoptosis. Furthermore, the enhanced expression of IL-10 by αGC is involved in these effects. This is the first report to provide direct evidence for increased iNKT cells in MI and the inhibitory effects of their activation on the development of post-MI HF.

Chronic Infiltration of Inflammatory Cells Including iNKT Cells in Post-MI Heart

In the setting of acute MI, the infiltration of inflammatory cells such as neutrophils, macrophages, and lymphocytes is a physiological repair process and beneficial removing dead cardiomyocytes and leading to the repair and scar formation of infarcted area.[18] However, the chronic inflammatory response in the noninfarcted area causes the further myocardial damage and fibrosis, leading to the progressive impairment of cardiac function.[19] We have previously demonstrated that anti–MCP-1 gene therapy improved survival and attenuated LV dilation and contractile dysfunction, which was associated with the decreases in macrophage infiltration and gene expression of myocardial inflammatory cytokines.[2] Therefore, chronic myocardial inflammation plays a crucial role on