Prior β-Blocker Treatment Decreases Leukocyte Responsiveness to Injury

Laurel A. Grisanti, PhD¹, Claudio de Lucia, MD², Toby P. Thomas, BS², Aron Stark, BS³, John T. Strony, BS³, Valerie D. Myers, PhD⁴, Remus Beretta, BS⁴, Daohai Yu, PhD⁵, Celestino Sardu, MD⁶, Raffaele Marfella, MD⁶, Erhe Gao, MD², Steven R. Houser, PhD⁴, Walter J. Koch, PhD², Eman A. Hamad, MD³ and Douglas G. Tilley, PhD²

¹Department of Biomedical Sciences, College of Veterinary Medicine, University of Missouri, Columbia, MO, USA, ²Center for Translational Medicine, ³Department of Medicine, ⁴Center for Cardiovascular Research, ⁵Department of Clinical Sciences, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, USA., and ⁶Department of Medical, Surgical, Neurological, Metabolic and Aging Sciences, University of Campania "Luigi Vanvitelli", Piazza Miraglia, 2, 80138, Naples, Italy

The authors have declared that no conflict of interest exists

**Corresponding Authors:**

Douglas G. Tilley, PhD
Room 945A MERB
Center for Translational Medicine
Lewis Katz School of Medicine, Temple University
3500 N. Broad St., Philadelphia, PA 19140
Tel.: 215-707-9758
Fax: 215-707-9890
E-mail: douglas.tilley@temple.edu

Laurel A. Grisanti, PhD
University of Missouri
College of Veterinary Medicine
W191 Veterinary Medicine
1600 E. Rollins St.
Columbia, MO 65211
Tel: 573-884-8852
E-mail: grisantil@missouri.edu
Abstract
Following injury, leukocytes are released from hematopoietic organs and migrate to the site of damage to regulate tissue inflammation and repair, however leukocytes lacking β2-adrenergic receptor (β2AR) expression have marked impairments in these processes. β-blockade is a common strategy for the treatment of many cardiovascular etiologies, therefore the objective of our study was to assess the impact of prior β-blocker treatment on baseline leukocyte parameters and their responsiveness to acute injury. In a temporal and βAR isoform-dependent manner, chronic β-blocker infusion increased splenic vascular cell adhesion molecule-1 (VCAM-1) expression and leukocyte accumulation (monocytes/macrophages, mast cells and neutrophils) and decreased chemokine receptor 2 (CCR2) expression, migration of bone marrow (BM) and peripheral blood leukocytes (PBL), as well as infiltration into the heart following acute cardiac injury. Further, CCR2 expression and migratory responsiveness was significantly reduced in the PBL of patients receiving β-blocker therapy compared to β-blocker-naïve patients. These results highlight the ability of chronic β-blocker treatment to alter baseline leukocyte characteristics that decrease their responsiveness to acute injury and suggest that prior β-blockade may act to reduce the severity of innate immune responses.
Introduction

Leukocytes are rapidly recruited to sites of acute injury where they regulate a wide variety of remodeling responses including inflammation, fibrosis and scar stabilization to promote wound healing(1-4). This early immune response includes the recruitment of inflammatory leukocytes, including granulocytes such as neutrophils and mast cells, as well as phagocytic monocytes, to attract further leukocyte recruitment and begin clearing the area of dead cells and debris(5, 6). Intense early and/or prolonged recruitment of inflammatory leukocytes can lead to maladaptive tissue remodeling/scar formation over time, with a negative impact on organ function, such as increased stiffness that reduces cardiac output following myocardial infarction (MI)(7, 8). Dampening leukocyte infiltration following injury has been shown capable of reducing subsequent maladaptive remodeling outcomes(8-12), therefore identifying therapeutics that reduce leukocyte responsiveness to acute injury is of great interest.

Acute injury events activate the sympathetic nervous system to regulate a host of immune system functions in part via activation of β-adrenergic receptors (βAR)(13), therefore understanding whether βAR-mediated regulation of immune cells impacts their response to injury may offer insight into how this process may be modulated. To this end, we previously developed chimeric mice lacking β2AR expression in cells of hematopoietic origin via transplantation of β2AR knockout (KO) bone marrow (BM) into wild-type (WT, C57Bl6/J) recipient mice and subjected them to MI. Strikingly, these chimeric mice displayed severe changes in several leukocyte parameters, including increased splenic vascular cell adhesion molecule-1 (VCAM-1) expression and leukocyte retention, as well as decreased bone marrow cell (BMC) expression of chemokine receptor 2 (CCR2) and post-injury leukocyte recruitment to the heart(14, 15). Collectively, these genetic studies suggested that since complete β2AR deletion negatively regulates leukocyte behavior both at baseline and following injury, clinically used β-blockers may also impact leukocyte responsiveness to acute injury.
β-blockers have been used successfully for decades to treat several pathologies, including hypertension, congestive heart failure and post-MI dysfunction (16). Many of the benefits of β-blockers are thought to be mediated via their direct actions on cardiac-expressed βAR to decrease cardiac output when administered after an event such as MI, or to decrease infarct size at the time of reperfusion as recently reported(17). However, the impact of prior β-blocker usage on immune cell function and responsiveness in human subjects is not well-documented. We previously observed that β-blocker treatment of human macrophages increased VCAM-1 expression, similar to macrophages derived from β2ARKO mice, and VCAM-1 expression was increased in the spleens of human subjects treated chronically with β-blocker(14). These results revealed that prior β-blocker treatment can alter the molecular signature of leukocytes, potentially impacting their function and responsiveness to acute injury. Thus, in our current study, we used mouse models and leukocytes from patients to examine whether chronic β-blocker treatment alters leukocyte splenic localization, chemokine receptor expression and migration to decrease their responsiveness to acute injury in vivo. Our results demonstrate that chronic treatment with β-blockers decreases leukocyte responsiveness in both murine models and human peripheral blood leukocytes (PBL), suggesting that β-blockers with β2AR selectivity are capable of fine-tuning innate immune responses to injury in vivo.
Results

\textbf{β-blocker infusion alters splenic leukocyte parameters over time in vivo.}

Our previous studies identified key molecular changes in leukocytes in response to hematopoietic cell-specific deletion of β2AR, including increased splenic VCAM-1 expression and leukocyte retention, as well as decreased leukocyte CCR2 expression in BM-derived leukocytes\(^{(14, 15)}\). Therefore, we aimed to determine whether prior treatment with β2AR-selective and/or clinically-used β-blockers would similarly alter these leukocyte parameters. To this end, WT C57BL/6 mice were administered via osmotic minipump either vehicle, ICI-118,551 (ICI, a β2AR-selective antagonist), carvedilol (Carv, a non-selective adrenergic receptor antagonist), a low dose of the β1AR-selective antagonist metoprolol (Met Low), which has been shown to be selective for β1AR\(^{(18-20)}\) and to block cardiac β1AR-mediated contractility (Supplemental Figure 1A), or a high non-selective dose of metoprolol (Met High) \(^{(21, 22)}\). Similar to changes in splenic parameters we reported in β2ARKO BMT mice, infusion of mice with ICI resulted in a temporally-dependent increase in spleen size and splenic VCAM-1 expression (Figure 1A-D), which peaked after 2 weeks of infusion. Using this timepoint, the impact of metoprolol and carvedilol were also tested, revealing that both Met High and Carv increased spleen size and splenic VCAM-1 expression, while Met Low did not impact these parameters (Figure 1E-H).

Previously, enhanced spleen size and VCAM-1 expression secondary to loss of leukocyte-expressed β2AR was shown to correlate with increased splenic retention of various leukocyte populations, including monocytes/macrophages, neutrophils and mast cells\(^{(14)}\). Thus, initially using immunohistochemical analysis we quantified the amount of these leukocyte populations within the splenic red pulp and white pulp (Supplemental Figure 1B) after β-blocker infusion. Similar to our data above, infusion with ICI enhanced the accumulation of monocyte/macrophage-(CD68\(^+\)), mast cell- (tryptase\(^+\)) and neutrophil- (MPO\(^+\)) lineage cells in a temporally-dependent
manner (Figure 2A-D, Supplemental Figure 2A-D). Infusion with either Met High or Carv for 2 weeks also resulted in the splenic accumulation of CD68+, tryptase+ and MPO+ leukocytes (Figure 2A-D), whereas Met Low had no significant impact on these parameters. In all cases, the changes in leukocyte parameters were observed in the red pulp, but not the white pulp, of the spleen (Supplemental Figure 2). In alignment with the immunohistochemistry results, flow cytometry analysis of cells isolated from the spleens of mice treated with ICI for 2 weeks also revealed enhanced accumulation of CD45+/CD68+ (hematopoiesis-derived monocyte/macrophage lineage), CD45+/CD117+ (hematopoiesis-derived mast cell lineage) and CD45+/Ly6G+ (hematopoiesis-derived neutrophil lineage) leukocytes (Figure 3A-F). These data support the concept wherein pharmacological inhibition of β2AR for as little as 2 weeks is sufficient to produce a detectable alteration in splenic leukocyte accumulation.

**Chronic β-blocker treatment reduces CCR2 expression and migration of bone marrow and peripheral blood leukocytes.**

CCR2 mediates the recruitment of leukocytes to sites of injury in response to its ligand CCL2 (8, 10, 23, 24). Since we had previously reported that complete loss of β2AR expression in leukocytes resulted in a marked reduction in CCR2 expression and BMC responsiveness to CCL2-mediated migration(15), we tested whether β-blocker infusion is also sufficient to alter these parameters. Indeed, infusion with ICI reduced BMC-expressed CCR2 in a temporally-dependent manner (Figure 4A), while infusion with Carv or Met High for 2 weeks also resulted in decreased BMC CCR2 expression and Met Low had no impact (Figure 4B). Via flow cytometry, decreased levels of CD45+/CCR2+ (hematopoiesis-derived CCR2+ cells) in BMC following 2 weeks of ICI infusion was also confirmed (Figure 4C-D). Molecular changes within the BM do not necessarily represent those that may occur in circulating leukocytes, thus we sought to determine whether the same responses to β-blockers we observed in isolated BMC could also be detected in PBL. To this end, we isolated buffy coats containing the PBL population from mice treated with each β-
blocker for 2 weeks and measured CCR2 expression. Similar to our observations in BMC, CCR2 expression was significantly decreased in PBL chronically exposed to ICI, Carv and Met High, but not Met low (Figure 4E). Since CCR2 expression was reduced in both BMC and PBL by β2AR-selective β-blockade, we tested whether these cells exhibited impaired migration toward CCL2. Responsiveness to CCL2 was almost completely abrogated in both BMC and PBL by prior infusion with ICI, Carv or Met High (Figure 5A-C). In contrast, CCL2-mediated migration was unaffected in either leukocyte population attained from Met Low-infused mice.

Although mice provide a useful in vivo model to explore the effects of β-blocker treatment on leukocytes, we aimed to determine whether our results translate to human leukocyte biology. Therefore, to determine whether a molecular readout of β-blocker-mediated changes in leukocytes can be also detected in human PBL, we analyzed CCR2 expression and responsiveness in buffy coat samples attained from patients treated chronically with or without β-blockers. Similar to our data attained in mice, CCR2 expression was significantly reduced in the PBL of patients who had been receiving chronic β-blocker therapy compared to β-blocker-naïve patients (Table 1). Further, PBL of patients having received chronic β-blocker treatment displayed reduced migratory responsiveness to CCL2 (Figure 5D-E). In all, these data highlight the translatability of our mouse model data toward clinical relevance in humans.

**Prior β-blocker infusion reduces leukocyte responsiveness to injury in vivo.**

We previously reported that mice with β2AR deletion specifically in cells of hematopoietic origin displayed reduced infiltration of leukocytes into the heart following acute myocardial infarction (MI)(14, 15). Since chronic infusion with β-blockers induced similar baseline changes in both splenic and BM leukocytes as observed in β2ARKO BMT mice, populations which are integral to post-injury innate immune responses(2, 5, 6, 25), we sought to determine whether chronic β-blocker treatment impacts leukocyte responsiveness to MI-induced injury. Therefore, mice were administered Veh, ICI, Carv, Met High or Met Low for 2 weeks, after which the pumps
were removed to allow drug washout for 1 day prior to sham or MI surgery. Infarct length, as assessed at both 4 and 21 days post-MI, was not different between Veh and any of the β-blocker treatment groups (Figure 6A-C). Via echocardiography, we also tracked the cardiac function of a cohort of mice infused with β-blockers prior to sham or MI surgery for up to 3 weeks post-MI. While all mice receiving MI experienced a significant reduction in contractility compared to sham-operated mice as estimated by ejection fraction (Supplemental Figure 3A), there were no significant post-MI differences between any of the β-blocker pretreatment groups. However, prior treatment with either ICI, Carv or Met high did significantly reduce survival by ~50-60% within the first week due to increased cardiac rupture events (Figure 6D) such that there were limited mice remaining in some groups with which to assess cardiac function. Met low did not significantly reduce survival when compared to Veh-infused mice.

Since β2ARKO BMT mice experienced 100% mortality following MI(14), but β-blocker-infused mice displayed variable mortality outcomes, these data suggest an intermediate effect of prior β-blocker treatment on leukocyte parameters. As such, we assessed splenic VCAM-1 and leukocyte levels, BMC CCR2 expression and cardiac leukocyte infiltration at 4 days post-MI. Although splenic VCAM-1 expression trended toward increased by ICI versus Veh in both sham and MI mice (Supplemental Figure 3B) post-procedural spleen weights (Supplemental Figure 3C) were not significantly altered by any of the β-blocker treatment regimens- versus Veh-treated mice (a total of 5 days following β-blocker cessation). Conversely, mice treated with ICI, Carv or Met High displayed decreased expression of CCR2 in their BMC, which was more apparent 4 days post-MI (Figure 6E), while Met Low had no effect. Correspondingly, cardiac infiltration of CD68+, tryptase+ and MPO+ cells into the border zone of post-MI hearts were each significantly decreased in mice that had received prior treatment with ICI, Carv or Met High compared with those that received Veh, while leukocyte infiltration was unaffected by prior treatment with Met Low (Figures 7A-D). None of the β-blocker regimens altered leukocyte numbers in sham-operated mouse hearts or the remote zone of hearts from MI mice (Supplemental Figure 4A-G). Flow cytometry
analysis of cardiac non-myocytes isolated 4 days post-MI from mice pretreated for 2 weeks with Veh or ICI prior to MI surgery showed significantly decreased accumulation of CD45+/CD68+ cells and a trend toward decreased CD45+/Ly6G+ cells within the heart 4 days post-MI, although no difference was observed for CD45+/CD117+ cells (Supplemental Figure 5A-F). These results suggest that despite recovery of splenic leukocyte parameters by 5 days post-MI, β-blocker-mediated changes in leukocyte CCR2 expression and responsiveness are more long-lasting, thereby diminishing leukocyte infiltration into the heart after acute cardiac injury. Collectively, our study highlights the ability of chronic β-blocker treatment to alter baseline leukocyte characteristics that decrease their responsiveness to acute injury and suggest that prior β-blockade may act, if properly titrated, to reduce the severity of immune responses to injury.
Discussion

We previously demonstrated that wild-type mice having received transplantation of β2ARKO BM, in which β2AR signaling was completely absent in leukocytes, were unable to achieve leukocyte egress from the spleen and recruitment to the heart following MI(14, 15). These data suggested that β-blockers, used extensively in the treatment of numerous clinical etiologies, could act to alter the molecular characteristics of leukocytes at baseline and reduce their responsiveness to acute injury. Therefore, in this study, we examined the impact of prior treatment with both β2AR-selective and clinically-used β-blockers on several leukocyte parameters, including splenic localization, CCR2-mediated migration and recruitment to the heart following injury. Indeed, we demonstrate that chronic treatment with β2AR-selective and non-selective β-blockers altered baseline splenic expression of VCAM-1 and leukocyte localization, as well as leukocyte-specific CCR2 expression and responsiveness to CCL2-mediated migration. Further, mice that underwent prior treatment with β2AR-selective and non-selective β-blockers displayed significantly impaired recruitment of leukocytes to the injured heart. While in accordance with our previous studies(14, 15), this study specifically highlights several important facets of the impact of prior β-blocker treatment on leukocyte responsiveness to injury.

First, β2AR selectivity is critical in the ability of a β-blocker to impart effects on splenic accumulation and leukocyte recruitment to the site of injury. Pretreatment of mice with ICI 118,551, a strong inverse β2AR agonist(26), carvedilol, a non-selective βAR antagonist, and a high non-selective dose of the neutral β1AR antagonist metoprolol(21), each increased splenic VCAM-1 expression and leukocyte accumulation while reducing leukocyte CCR2 expression and responsiveness to injury as well as post-MI survival. Most compellingly, metoprolol did not modulate leukocyte molecular parameters or responsiveness when infused at a low β1AR-selective dose, which still blocked catecholamine-induced cardiac contractility. These results suggest that both carvedilol and metoprolol, when used at a non-selective dose, allow β2AR antagonism that can alter the molecular blueprint of leukocytes and their resulting ability to
respond to acute injury. However, via allometric scaling from a human average weight of ~85kg in our study (Table 1), the dose of carvedilol used in this study (10mg/kg/day in mice) produces a human equivalent dose of ~70mg/day, which is greater than the maximal dose used in the SELECT trial (50mg/day)(21, 22). Similarly the Met High dose used in our study equates to ~350mg/day in an 85kg human, above the maximal dose of 200mg/day used in the SELECT trial, while the Met Low dose produces a human equivalent dose of ~7mg, well below the lowest dose of metoprolol (25mg/day) used in the SELECT trial(21, 22). Thus, it would be expected that the doses of Carv and Met High used in our study may cause more severe changes in leukocyte parameters than would be observed in humans receiving β-blockers within clinically-used ranges. Indeed, our transwell migration data is consistent with this notion, as prolonged infusion with either Carv or Met High completely ablated isolated mouse leukocyte migration (no fold change in response to CCL2), whereas PBL isolated from β-blocker treated humans retained some responsiveness to CCL2 (~2-fold change in migration). Therefore, while we provide a useful proof-of-concept study depicting the action of β2AR-selective β-blockers on in vivo leukocyte biology, further studies employing a range of doses of clinically-used β-blockers with varying β2AR-selectivities will be most informative in terms of defining thresholds of molecular changes produced by chronic β-blocker treatment. In fact, titration of β-blocker dosage could be achieved in which leukocyte responsiveness is partially, but not fully, diminished to promote a more restrained innate immune response that allows for tissue repair, but with reduced long-term inflammation.

Second, β-blocker treatment that precedes an injury may dampen leukocyte responsiveness to that injury, thereby reducing the inflammatory insult and potentially relaying better outcomes. Of note, while we observed that longer β-blocker infusion regimens (≥ 2 weeks) were required to significantly alter the molecular signatures of leukocytes at baseline or in response acute cardiac injury, we did not test the impact of β-blocker treatment on leukocyte parameters for less than 1 week. To this point, work by the Ibáñez group has demonstrated a
benefit of metoprolol infusion beginning just prior to reperfusion following MI in humans (METOCARD-CNIC trial patients) and pigs(17), which in a follow-up study was mechanistically linked with the direct negative regulation of neutrophil function and was associated with decreased myocardial injury in mice(27). Thus, the duration of β-blocker treatment necessary to dampen immune cell responses, but not negatively impact MI-induced scar formation and survival, is an open question, though this may be less of a concern than β-blocker dosage/selectivity.

Third, there is likely a critical washout period after cessation of β-blocker infusion in which full leukocyte responsiveness to acute injury may be restored. For instance, our initial experiments in which we established the impact of β-blocker infusion at baseline showed a marked increase in splenic hypertrophy and retention of leukocytes, while our subsequent studies in which we discontinued β-blocker infusion 1 day prior to sham or MI surgery and performed cellular and tissue analyses another 4 days later (5 days total following β-blocker cessation), splenic parameters were almost normalized even in the sham-operated mice. In contrast, our previous work showed that chimeric mice lacking β2AR expression in cells of hematopoietic origin exhibited baseline splenomegaly that increased in conjunction with leukocyte retention following cardiac injury, with correspondingly worsened leukocyte infiltration to the heart, which was partially relieved by splenectomy(14). Although splenic parameters were normalized after β-blocker discontinuation, the β-blocker-mediated decrease in leukocyte CCR2 expression and CCL2-mediated migration following injury were retained even 5 days following cessation of infusion, indicating a prolonged impact of β-blockade on CCR2 signaling in leukocytes. This observation points out that other β-blocker-sensitive cell types, including those in the heart, may still be altered at a molecular level similar to leukocytes, thereby contributing to the post-MI outcome. However, there were no differences in post-MI infarct size or cardiac function in the vehicle versus β-blocker treatment groups, suggesting that the initial response to acute MI was similar across all cohorts and that reduced immune cell infiltration into the heart by 4 days post-MI was specifically due to altered leukocyte CCR2 expression and responsiveness. The
intermediate phenotype of restored splenic leukocyte profile but reduced CCR2-mediated responsiveness could explain why β-blocker-treated mice experienced less post-MI mortality than we previously observed with leukocyte-specific genetic ablation of β2AR(14). It also suggests that a longer washout period between β-blocker cessation and injury is required to fully recover innate immune system responsiveness to injury.

While there are conflicting reports of the effects of the sympathetic nervous system on regulating the CCL2/CCR2 axis, with some showing no effect(28) and others showing decreased CCR2 in response to either non-selective AR activation(29) or β-blockade(30), previous findings in our laboratory have shown that deletion of hematopoietic β2AR almost completely ablates CCR2 expression(15). In accordance with our previous findings, this study demonstrates that chronic pharmacological inhibition in vivo, with increasing duration and selectivity toward β2AR, decreases CCR2 expression in both BMC and PBL. Further, our clinical data highlight the translational relevance of our studies in mice since CCR2 expression and CCL2 responsiveness were also decreased in the buffy coat samples of patients having taken a β-blocker chronically versus β-blocker naïve patients. Although interpretation of human data can be made difficult by variability between patient treatment and control groups, the alterations in CCR2 expression and responsiveness we observed in human peripheral blood leukocytes occurred irrespective of underlying conditions in the patient populations, since both β-blocker-treated and β-blocker naïve cohorts were comprised of patients with hypertension, diabetes mellitus, coronary artery disease and heart failure. Therefore, CCR2 expression levels in PBL may represent a molecular biomarker for predicting the potential impact/outcomes of chronic β-blocker treatment on leukocyte-dependent responsiveness to acute injury, which will require further assessment.

Beyond use as a biomarker in this regard, CCR2 has been explored more generally as a potential therapeutic target in injury and disease models(31). Classical inflammatory monocytes express high levels of CCR2, while non-classical reparative monocytes lack CCR2 expression(23). Previous studies have shown the importance of CCR2 in mediating classical
monocyte recruitment to the heart following MI, and more recently pressure overload, and the impact of deleting CCR2 expression genetically or via siRNA on preventing this response to improve outcomes in mice([10, 24, 32]). Thus, inhibition of CCR2 remains a viable strategy to dampen the inflammatory response after injury, though gene deletion or siRNA strategies to alter CCR2 expression in vivo remains more challenging than a pharmacologic approach. As such, selective targeting of inflammatory monocytes via β-blocker-mediated decreases in CCR2 expression may provide a strategy by which to decrease pathologic remodeling following tissue injury while not interfering with reparative monocyte/macrophage processes.

In summary, our study demonstrates that clinically relevant β-blockers modulate several leukocyte parameters at a molecular level, including VCAM-1 and CCR2 expression, thereby altering their splenic localization and migration, respectively, and reducing their responsiveness to injury. The use of clinical samples in this study suggests that chronic β-blocker treatment also alters leukocyte parameters in humans. Whether this impacts leukocyte responsiveness to acute injury events in humans and the subsequent remodeling outcomes remains to be tested vigorously. Thus, an expanded investigation into the impact of selectivity, dosage and duration of β-blocker usage on human leukocyte function would offer clinical insight and drive further translational research using preclinical models.
Methods

Experimental animals. Wild-type (WT) C57Bl/6 male mice aged 10-12 weeks, attained from The Jackson Laboratory, were used in this study to determine the impact of prior β-blocker infusion on parameters including splenic and cardiac accumulation of leukocytes before and after acute injury, alterations in expression of adhesion and chemokine receptors as well as leukocyte chemotaxis. WT mice were randomized into groups that were administered vehicle (Veh, sterile phosphate buffered saline (PBS) + 10% DMSO), ICI-118,551 (ICI, 0.1 mg/kg/d), metoprolol (either a low β1AR-selective dose of 1 mg/kg/d (Met Low) or a high non-selective dose of 50 mg/kg/d (Met High)) or carvedilol (Carv, 10 mg/kg/d) via osmotic minipumps (Alzet). For acute myocardial infarction experiments, infusion was discontinued 1 day prior to surgery to allow drug washout and the surgeon was blinded to the treatment groups.

Coronary Artery Occlusion Surgery. Myocardial infarction was induced as previously described(14, 15). In brief, mice were anesthetized with 2% isoflurane inhalation. A small skin incision was made and the pectoral muscles were dissected and retracted to expose the fourth intercostal space. A small hole was made and the heart popped out. The left coronary artery was sutured ~3 mm from its origin and the heart was placed back into the intrathoracic space and closure of muscle and skin. Animals received a single dose (0.3 mg/kg) of buprenorphine immediately following surgery. Injury level from myocardial infarction surgery was standardized by a predefined parameter as having an ejection fraction of <40% as measured by echocardiography 4 days post-MI, whereas animals having an ejection fraction of >40% were considered an un-successful surgery and excluded from the study.

Echocardiography. Cardiac function was performed at baseline, 7, 14 and 21 days post-MI via transthoracic two-dimensional echocardiography using a VisualSonics Vevo 2100 System with a 12 MHz probe on mice anesthetized with isoflurane (1.5%) and body temperature was maintained
on a heated table with embedded ECG leads to monitor heart rate, temperature of the animal, electrocardiogram and breathing. The parasternal short-axis views of the heart were obtained in B-mode by placing the transducer in the parasternal long-axis and rotating the transducer 90° in a clockwise fashion to find the parasternal short-axis. For measurements, M-mode echocardiography was performed in the parasternal short-axis view at the level of the papillary muscle to assess left ventricular (LV) fractional shortening and ejection fraction. Percent fractional shortening was calculated using the equation \( \frac{LVIDd - LVIDs}{LVIDd} \times 100\% \). Percent ejection fraction was calculated using the equation \( \frac{LVvold - LVvols}{LVvold} \times 100\% \). The assessor was blinded to the groups and the data was compiled into treatment and surgical groups only after the analyses were completed. To examine the ability of low dose metoprolol to block isoproterenol-induced cardiac contractility, echocardiography was performed following 2wk administration of vehicle (PBS+10% DMSO) or a low dose of metoprolol (1 mg/kg/day) at baseline and 5 min after 0.1 mg/kg (i.p.) isoproterenol injection.

**Histological Analysis.** Excised hearts and spleens were fixed in 4% paraformaldehyde, paraffin embedded and sectioned at 5 µm thickness, with the hearts subsequently stained with Masson trichrome (Sigma-Aldrich). Immunohistochemistry was performed on deparaffinized sections to examine infiltration of various immune cell types as previously described\(^{(14, 15)}\). Antigens were retrieved using a citrate-based antigen unmasking solution (Vector Laboratories; Burlingame, CA). Tissues were blocked (10% FBS/PBS), incubated with a 0.3% \( \text{H}_2\text{O}_2 \) solution was used to block endogenous peroxide activity followed by incubation with antibodies against CD68 (1:100; Abcam ab31360), mast cell tryptase (1:100; Abcam ab2378) or myeloperoxidase (MPO; 1:100, Santa Cruz, Dallas, TX). Washed slides were incubated with the appropriate secondary antibodies, anti-mouse-HRP (1:1000; GE Healthcare; Piscataway, NJ) or anti-goat-HRP (1:1000; Santa Cruz), followed by staining with hematoxylin. Positive controls for primary antibody detection of CD68 (spleen), tryptase (lung) and MPO (post-MI heart) and no secondary antibody
control staining are shown in Supplemental Figure 6. Stained tissues were developed using a DAB Substrate Kit (Vector Laboratories) and mounted using Permount™ Mounting Media (Thermo Scientific). Staining was visualized on a Nikon Eclipse microscope at 20X magnification and NIS Elements software was used for recording images and image analysis. Images were quantified as the number of positive cells per area. VCAM-1 immunofluorescence was detected as previously described(14).

**Immune Cell Isolation from the Heart, Spleen, Bone Marrow, and Blood**. BMC were isolated from the femurs of WT C57BL/6 mice as previously described(14, 15). PBL were isolated from blood collected from mice using a heparinized syringe and centrifuged for 10 min at 1000 revolutions per minute (rpm). The concentrated buffy coat layer was collected, washed with phosphate-buffered saline (PBS), and centrifuged for 5 min at 1000 rpm. De-identified human buffy coat samples were attained from Temple University Hospital and University of Campania "Luigi Vanvitelli" from patients who were naïve to β-blockers or were taking either metoprolol or carvedilol at the time of sample collection, inclusive of patients with hypertension, diabetes mellitus, coronary artery disease and heart failure (Table 1). Mouse spleens were isolated and underwent manual digestion in PBS using a syringe plunger until a single cell suspension was obtained. The cell slurry was then washed with PBS and centrifuged at 1000 rpm for 5 min. For each cell preparation, the supernatant was discarded, and the pellets resuspended in Ammonium-Chloride-Potassium (ACK) lysis buffer to lyse red blood cells. The cells then underwent additional washes with PBS and centrifugation at 1000 rpm for 5 min. The pellets were resuspended in PBS and strained through a primed 70 µm cell strainer prior to cell counting and use for RT-qPCR, cell migration or flow cytometry analyses.

Whole hearts were isolated and flushed with Hanks Buffer Salt Solution (HBSS) to remove the blood, then underwent manual digestion into 1 mm³ pieces followed by enzymatic digestion in a Collagenase II (150 U/mL) and Trypsin (0.6 mg/mL) solution. All digestion steps were carried
out in a rotating 37°C water bath. The Collagenase II/Trypsin solution was replaced after each digestion, and the cell-containing solution was placed in a 50 mL Falcon tube with 4 mL of fetal bovine serum (FBS). Subsequent digestions were carried out until the remaining tissue pieces were too small to separate from the digestion fluid. The cell suspension was centrifuged at 3000 rpm for 5 min and the supernatant was discarded, following which the pellet was resuspended in 4 mL of FBS and centrifuged at 300 rpm. The non-myocyte containing supernatant was transferred to a new tube and centrifuged at 3000 rpm for 5 min. The pellet was resuspended in Hanks balanced salt solution (HBSS) and stained through a primed 70 µm cell strainer prior to cell counting and use for flow cytometry analysis.

**Fluorescence-Activated Cell Sorting (FACS) Analysis.** Cells were stained in FACS Buffer (1% FBS in PBS) with the following antibodies: CD45-BUV395 (1:100, BD Biosciences 564279), CD68-PE (1:100, Biolegend 137014), CD117-APC-H7 (1:50, BD Biosciences 560185), Ly-6G-BV421 (1:100, BD Biosciences 562737), CCR2-FITC (1:100, Biolegend 150608), and LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (1:40, Invitrogen L34957). Isotype control antibodies that were used in conjunction with splenic leukocytes to validate cell staining (Supplemental Figure 7) were: FITC Rat IgG2bκ (1:100, Biolegend 400633), APC-H7 Rat IgG2bκ (1:50, BD Biosciences 560200), BUV395 Rat IgG2bκ (1:100, BD Biosciences 563560), BV421 Rat IgG2ak (1:100, BD Biosciences 562602), PE Rat IgG2ak (1:100, Biolegend 400507). Cells were stained for 30 minutes in the dark and on ice. After staining, the cells were washed twice with PBS and centrifuged for 5 minutes at 1000 rpm, after which the cells underwent resuspension in FACS buffer. Data were acquired on an LSR II flow cytometer and analyzed using FlowJo software. Splenic and bone marrow cells are expressed as % of CD45+ cells, while cardiac cells are expressed per 1x10^6 cells since CD45 cell populations differed between the Veh and ICI treatment groups in the heart only.
**Migration Assay.** Freshly isolated BMC or PBL were plated on 8 μm transmembrane inserts (Corning; Corning, NY) in a 24-well plate containing vehicle (Veh) or CCL2 (100 ng/mL), as previously described(15). Cells were stained with Hoechst and migration was imaged after 4h. Cells were visualized at 10X magnification using a Nikon Eclipse microscope and analyzed from 10 random fields per treatment.

**Reverse Transcription Quantitative PCR.** cDNA was synthesized from the total RNA of spleen, BMC or PBL samples using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Reverse transcription quantitative PCR (RT-qPCR) was performed to assess CCR2 and VCAM-1 expression with SYBR® Select Master Mix (Applied Biosystems) in triplicate for each sample using primers listed in Supplemental Table 1 at an annealing temperature of 60.1°C. All RT-qPCR data was analyzed using Applied Biosystems Comparative CT Method (ΔΔCT). Gene expression was normalized to translationally-controlled tumor protein-1 (TPT1), statistics performed on ΔCT values and results expressed as $2^{ΔΔCT}$ or relative quantitation (RQ) with RQ_{min/max} indicated for range.

**Statistical Analysis.** Data is presented in scatter dot plot format with mean ± standard error of the mean (SEM) indicated. Statistical comparisons of a continuous variable between different treatment groups were performed using the nonparametric Kruskal-Wallis test for three or more groups and exact Wilcoxon rank-sum tests with multiple pairwise comparison adjustments made using the formula: adjusted p-value = min ([#comparisons made] x [raw p-value], 1.00) to guard against possibly non-normally distributed data in small group sizes. Echocardiography data was analyzed using two-way repeated measures ANOVA. P-values and n (group size) values are reported in the figure legends, where p-values < 0.05 were considered statistically significant. All statistical analyses were performed using SAS version 9.4 software (SAS Institute Inc., Cary, NC). Each assay was performed in a minimum of 3 independent experiments.
Study approval. All animal procedures and experiments were carried out according to the National Institutes of Health Guidelines on the Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Human buffy coat samples and data were not collected specifically for our study and no one on our study team has access to the subject identifiers linked to the specimens or data, thus our study is not considered human subjects research and did not require institutional review board approval.

Author Contributions: L.A.G. and D.G.T. wrote the manuscript and designed the experiments. L.A.G. conducted most of the experiments and analyzed the data, with contributions from T.P.T. and A.S.. E.G. performed the sham and MI surgeries. D.Y. performed statistical analyses. C.d.L., J.T.S, V.D.M, R.B., C.S. and R.M. processed human buffy coat samples and/or provided de-identified patient demographics. S.R.H., W.J.K., A.M.F., E.H. and D.G.T. provided intellectual guidance and manuscript revision.

Acknowledgements: This work was supported by NIH grants R01 HL105414 (D.G.T.), R01 HL139522 (D.G.T.) and P01 HL091799 (W.J.K., S.R.H.) and by American Heart Association Scientific Development Grant 17SDG33400114 (L.A.G.) and Postdoctoral Fellowship 17POST33660942 (C.d.L.).
References


### Table 1. Patient demographics and CCR2 expression in human PBL

<table>
<thead>
<tr>
<th></th>
<th>β-blocker Naïve (n=27)</th>
<th>β-Blocker Treated (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years ± SEM (range)</td>
<td>56.3±2.9 (24-77)</td>
<td>58.5±2.0 (25-80)</td>
</tr>
<tr>
<td>Mean weight in kg ± SEM (range)</td>
<td>80.2±3.5 (41-109)</td>
<td>88.0±3.6 (53-138)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>11 (40.7)</td>
<td>26 (68.4)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>15 (55.6)</td>
<td>12 (31.6)</td>
</tr>
<tr>
<td>Not reported (%)</td>
<td>1 (3.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (%)</td>
<td>11 (40.7)</td>
<td>20 (52.6)</td>
</tr>
<tr>
<td>Black or African-American (%)</td>
<td>6 (22.2)</td>
<td>11 (28.9)</td>
</tr>
<tr>
<td>Not reported (%)</td>
<td>10 (37.0)</td>
<td>7 (18.4)</td>
</tr>
<tr>
<td>Cardiovascular Disease Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTN (% within treatment group)</td>
<td>12 (44.4)</td>
<td>22 (57.9)</td>
</tr>
<tr>
<td>DM (% within treatment group)</td>
<td>6 (22.2)</td>
<td>14 (36.8)</td>
</tr>
<tr>
<td>CAD (% within treatment group)</td>
<td>8 (29.6)</td>
<td>11 (28.9)</td>
</tr>
<tr>
<td>HF (% within treatment group)</td>
<td>10 (37.0)</td>
<td>21 (55.3)</td>
</tr>
<tr>
<td>NYHA Class for HF Patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (% within treatment group)</td>
<td>1 (10)</td>
<td>1 (4.76)</td>
</tr>
<tr>
<td>2 to 3 (% within treatment group)</td>
<td>2 (20)</td>
<td>13 (61.9)</td>
</tr>
<tr>
<td>3 to 4 (% within treatment group)</td>
<td>7 (70)</td>
<td>6 (28.6)</td>
</tr>
<tr>
<td>CCR2 expression as RQ relative to Control</td>
<td>1.01 (0.85-1.17)</td>
<td>0.44* (0.33-0.55)</td>
</tr>
</tbody>
</table>

Abbreviations: HTN (Hypertension), DM (Diabetes Mellitus), CAD (Coronary Artery Disease), HF (Heart Failure), NYHA (New York Heart Association), CCR2 (Chemokine Receptor 2), RQ (Relative Quantitation). * p < 0.05 vs β-blocker Naïve, Exact Wilcoxon rank-sum test.
**Fig. 1: β-blocker infusion alters baseline splenic parameters.** Spleen size was quantified by gravimetric analysis (SW) and normalized to tibia length (TL) (A, E), RT-qPCR was used to measure splenic VCAM-1 expression (B, F) and immunohistochemistry was used to detect splenic VCAM-1 expression (C, D, G, H) from mice infused with Veh or ICI for 1-4 weeks (A-D) or Carv, Met Low or Met High for 2 weeks (E-F) via osmotic minipumps. Overall Kruskal-Wallis test p values: (A) p=0.0001, (B) p=0.0031, (D) p=0.0008, (E) p=0.0004, (F) p=0.0116, (H) p<0.0001. (A, B): n=7 for Veh, n=9 for 1 wk ICI, n=8 for 2 wk ICI, n=8 for 4 wk ICI; (D): n=6 for Veh, n=9 for 1 wk ICI, n=8 for 2 wk ICI, n=8 for 4 wk ICI; (E, F, H): n=6 for Veh, n=9 for Carv, n=7 for Met Low, n=7 for Met High. Results of Exact Wilcoxon rank-sum tests with multiple comparison adjustment (3 comparisons, timepoints vs Veh (A-D) or β-blocker vs Veh (E-H)) are indicated in scatter dot plots, * p<0.05, † p<0.01, ‡ p<0.001 vs Veh. Scale bar = 200 µm.
**Fig. 2: β-blocker infusion alters baseline splenic leukocyte localization.** (A) Representative CD68, tryptase and MPO staining (black arrowheads) within the spleens of mice that were administered Veh, ICI, Carv, Met Low or Met High for 2 weeks. Scale bar = 200 µm. Quantification of CD68⁺ (B), tryptase⁺ (C) and MPO⁺ (D) cells within the splenic red pulp from (A). Overall Kruskal-Wallis test p values: (B) p<0.0001, (C) p=0.0001, (D) p<0.0001. n=13 for Veh, n=8 ICI, n=9 for Carv, n=7 for Met Low, n=7 for Met High. Results of Exact Wilcoxon rank-sum tests with multiple comparison adjustment (4 comparisons, β-blockers vs Veh) are indicated in scatter dot plots, † p<0.01, ‡ p<0.001 vs Veh.
Fig. 3: Flow cytometry analysis of splenic leukocyte accumulation following infusion of ICI 118,551. Via flow cytometry, CD45+/CD68+ (A, B), CD45+/CD117+ (C, D) and CD45+/Ly6G+ (E, F) leukocytes were quantified from the spleens of mice infused for 2 weeks with Veh or ICI (unstained controls in left panels, isotype controls are shown in Supplemental Figure 7). n=4 Veh and n=5 ICI, * p<0.05, Exact Wilcoxon rank-sum test.
**Fig. 4: β-blocker infusion decreases CCR2 expression in BMC and PBL.** RT-qPCR was used to measure BMC CCR2 expression from WT C57BL/6 mice treated with Veh versus ICI for increasing timepoints (A) or Veh versus Carv, Met Low or Met High for 2 weeks (B) via osmotic minipumps. Overall Kruskal-Wallis test p values: (A) \( p<0.0001 \), (B) \( p=0.0006 \). (A): \text{n}=8 \text{ for Veh, n}=9 \text{ for 1 wk, n}=8 \text{ for 2 wk, n}=8 \text{ for 4 wk}; (B): \text{n}=6 \text{ for Veh, n}=9 \text{ for Carv, n}=7 \text{ for Met Low, n}=7 \text{ for Met High.} \) Results of Exact Wilcoxon rank-sum tests with multiple comparison adjustment (3 comparisons, timepoints vs Veh (A) or β-blocker vs Veh (B)) are indicated in scatter dot plots, * \( p<0.05 \), † \( p<0.01 \), ‡ \( p<0.001 \) vs Veh. (C, D) Via flow cytometry, CD45+/CCR2+ BMC were quantified from mice infused for 2 weeks with Veh or ICI (unstained controls in left panels, isotype controls are shown in Supplemental Figure 7). \text{n}=4 \text{ Veh and n}=5 \text{ ICI, * p<0.05, Exact Wilcoxon rank-sum test. (E) RT-qPCR was used to measure PBL CCR2 expression from WT C57BL/6 mice treated with Veh versus ICI, Carv, Met Low or Met High for 2 weeks via osmotic minipumps. Overall Kruskal-Wallis test p value: \( p=0.0001 \). \text{n}=8 \text{ for Veh, n}=6 \text{ for ICI, n}=11 \text{ for Carv, n}=12 \text{ for Met Low, n}=9 \text{ for Met High. Results of Exact Wilcoxon rank-sum tests with multiple comparison adjustment (4 comparisons, β-blocker vs Veh) are indicated in scatter dot plots, * p<0.05, † p<0.01 vs Veh.}
**Fig. 5: Prior β-blocker infusion decreases mouse and human leukocyte migration.** (A) Representative Hoechst staining from transwell migration assays of BMC or PBL isolated from WT mice infused for 2 weeks with Veh, ICI, Carv, Met Low or Met High and treated with Veh or CCL2 for 4 hr. Scale bar = 200 µm. Quantification of migration assay results for BMC (B) or PBL (C). Values are expressed as fold over Veh-stimulated migration. Overall Kruskal-Wallis test p values: (B) p<0.0001, (C) p=0.0001. (B): n=10 for Veh, n=8 for ICI, n=9 for Carv, n=12 for Met Low, n=7 for Met High; (C): n=13 for Veh, n=6 for ICI, n=11 for Carv, n=12 for Met Low, n=9 for Met High. Results of Exact Wilcoxon rank-sum tests with multiple comparison adjustment (4 comparisons, β-blockers vs Veh) are indicated in scatter dot plots, † p<0.01, ‡ p<0.001 vs Veh. (D) Representative Hoechst staining from transwell migration assays of human PBL isolated patients taking β-blockers or β-blocker naïve patients and treated with Veh or CCL2 for 4 hr. Scale bar = 200 µm. (E) Quantification of migration assay results from (D), where values are expressed as fold over Veh-stimulated migration. n=5 naïve patients, n=9 β-blocker patients, * p<0.05, Exact Wilcoxon rank-sum test.
Fig 6: Chronic β-blocker infusion prior to myocardial infarction does not impact infarct size but does alter survival and CCR2 expression. (A) Representative Masson trichrome staining of infarcted hearts, 4 and 21 days post-MI, from mice treated for 2 weeks with Veh, ICI, Carv, Met Low or Met High prior to surgery. For 4 days: Veh (n=11), ICI (n=7), Carv (n=6), Met low (n=5) or Met high (n=4), for 21 days: Veh (n=8), ICI (n=2), Carv (n=5), Met low (n=8) or Met high (n=5). Scale bar = 2 mm. (B, C) Quantification of infarct lengths from (A). (D) Survival of mice that had received infusion of Veh, ICI, Carv, Met Low or Met High for 2 weeks prior to sham or MI surgery, as monitored daily for 21 days post-MI. Log-rank test, * p <0.05, † p < 0.01 vs Veh MI. Starting n=10 for Veh Sham, n=5 for ICI Sham, n=4 for Carv Sham, Met High Sham and Met Low Sham, n=26 for Veh MI, n=13 for ICI MI, Carv MI, Met High MI and Met Low MI. (E) RT-pPCR was used to quantify BMC CCR2 expression from sham and 4 day post-MI mice that were administered Veh, ICI, Carv, Met Low or Met High for 2 weeks prior to surgery. Overall Kruskal-Wallis test p value: p<0.0001. n=8 for Veh Sham, n=11 for Veh MI, n=7 for ICI Sham, n=7 for ICI MI, n=4 for Carv Sham, n=6 for Carv MI, n=4 for Met Low Sham, n=5 for Met Low MI, n=3 for Met High Sham, n=4 for Met high MI. Results of Exact Wilcoxon rank-sum tests with multiple comparison adjustment (13 comparisons; Sham β-blockers vs MI β-blockers, Sham β-blockers vs Sham Veh and MI β-blockers vs MI Veh) are indicated in scatter dot plots, * p<0.05, † p<0.01.
Fig. 7: Chronic β-blocker infusion prior to myocardial infarction decreases leukocyte infiltration into the heart. (A) Representative CD68, tryptase and MPO staining (black arrowheads) within the border zone of hearts from WT C57BL/6 mice that were administered Veh, ICl, Carv, Met Low or Met High 2 weeks prior to MI surgery. Scale bar = 200 µm. Quantification of CD68\(^+\) (C, overall \(p=0.0001\), Kruskal-Wallis test), tryptase\(^+\) (C, overall \(p=0.0002\), Kruskal-Wallis test) and MPO\(^+\) (D, overall \(p=0.0001\), Kruskal-Wallis test) cell infiltration into the heart from (A). n=11 for Veh, n=7 for ICl, n=6 for Carv, n=5 for Met Low, n=4 for Met high. Results of Exact Wilcoxon rank-sum tests with multiple comparison adjustment (4 comparisons, MI β-blockers vs MI Veh) are indicated in scatter dot plots, † \(p<0.01\), ‡ \(p<0.001\) vs Veh.