

β 2-Adrenergic receptor-dependent chemokine receptor 2 expression regulates leukocyte recruitment to the heart following acute injury

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Following cardiac injury, early immune cell responses are essential for initiating cardiac remodeling and tissue repair. We previously demonstrated the importance of β 2-adrenergic receptors (β 2ARs) in the regulation of immune cell localization following acute cardiac injury, with deficient leukocyte infiltration into the damaged heart. The purpose of this study was to investigate the mechanism by which immune cell-expressed β 2ARs regulate leukocyte recruitment to the heart following acute cardiac injury. Chemokine receptor 2 (CCR2) expression and responsiveness to C-C motif chemokine ligand 2 (CCL2)-mediated migration were abolished in β 2AR knockout (KO) bone marrow (BM), both of which were rescued by β 2AR reexpression. Chimeric mice lacking immune cell-specific CCR2 expression, as well as wild-type mice administered a CCR2 antagonist, recapitulated the loss of monocyte/macrophage and neutrophil recruitment to the heart following myocardial infarction (MI) observed in mice with immune cell-specific β 2AR deletion. Conversely to β 2AR ablation, β 2AR stimulation increased CCR2 expression and migratory responsiveness to CCL2 in BM. Mechanistically, G protein-dependent β 2AR signaling was dispensable for these effects, whereas β -arrestin2-biased β 2AR signaling was required for the regulation of CCR2 expression. Additionally, activator protein 1 (AP-1) was shown to be essential in mediating CCR2 expression in response to β 2AR stimulation in both murine BM and human monocytes. Finally, reconstitution of β 2ARKO BM with rescued expression of a β -arrestin-biased β 2AR in vivo restored BM CCR2 expression as well as cardiac leukocyte infiltration following MI. These results demonstrate the critical role of β -arrestin2/AP-1-dependent β 2AR signaling in the regulation of CCR2 expression and recruitment of leukocytes to the heart following injury.

β 2-adrenergic receptor | leukocyte | C-chemokine receptor 2 | cardiac injury | β -arrestin

Healing following ischemic cardiac injury is highly regulated by immune responses, with impairments or exacerbations in inflammation leading to alterations in infarct expansion, remodeling, and ultimately cardiac function (1). Cells of the innate immune system including monocytes/macrophages, mast cells, and neutrophils play critical roles in infarct healing through tissue phagocytosis and activation of reparative responses. Recruitment and trafficking of these leukocytes to the heart following acute injury occur through the action of chemokines on their receptors to promote their migration to the site of injury (2), and have been the focus of much research in recent years (1, 3).

Sympathetic activity is important for regulating immune responses, primarily through the β 2-adrenergic receptor (β 2AR) subtype (4–6). Recently, we showed that immune cell-expressed β 2AR is required for leukocyte recruitment to the heart following acute myocardial infarction (MI), without which the heart cannot mount a repair response, ultimately undergoing rupture (7). Because chemokine receptors play a critical role in migration and infiltration of leukocyte populations, we hypothesized that immune cell-expressed chemokine receptor activity and/or expression may be altered in the absence of β 2AR, thereby impairing leukocyte migration to the injured heart.

The impact of immune cell-specific β 2AR expression on chemokine receptor expression and leukocyte infiltration following MI was investigated through the use of chimeric mice, wherein bone marrow transplant (BMT) recipient mice received bone marrow from β 2ARKO donor mice. Through the use of these chimeric mice, we demonstrate that β 2AR is critical in regulating chemokine receptor 2 (CCR2) expression, and responsiveness to its ligand C-C motif chemokine ligand 2 (CCL2), via a β -arrestin2 (β ARR2)-biased signaling pathway involving activator protein 1 (AP-1). These results highlight the importance of β 2AR in regulating immune cell expression of CCR2, thereby impacting the ability of leukocytes to respond to acute cardiac injury.

Results

CCR2 Expression and Migratory Responsiveness Are Abolished in β 2ARKO BM. We recently observed decreased leukocyte infiltration into the hearts of chimeric mice lacking immune cell-expressed β 2AR following MI (7). Chemokines produced following injury are important for recruitment of immune cells, through their action on chemokine receptors. Thus, to assess whether differences in chemokine receptor expression could contribute to alterations in leukocyte infiltration in β 2ARKO BMT mouse hearts post-MI, reverse transcription-quantitative PCR (RT-qPCR) was used to examine those known to play an important role in immune cell migration following acute cardiac injury (Table 1 and Table S1). β 2ARKO BM had significantly decreased expression of CCR2 and C-X-C motif chemokine receptor 4 (CXCR4) compared with WT BM. To test the impact of

Significance

The sympathetic nervous system influences various immune cell functions, in particular via β 2-adrenergic receptor (β 2AR) signaling. Although immune cell recruitment is critical for cardiac repair following ischemia, the impact of β 2AR on this process is unclear. We describe how immune cell-specific β 2AR depletion ablates chemokine receptor 2 (CCR2) expression and leukocyte recruitment to the heart postischemia. Reciprocally, β 2AR activation increases CCR2 expression and responsiveness in a β -arrestin-dependent manner, and expression of a β -arrestin-biased β 2AR in β 2AR-depleted immune cells restores CCR2 levels and leukocyte recruitment to the postischemic heart. These results highlight the potential utility of next-generation β -arrestin-biased β 2AR ligands to selectively modulate leukocyte responsiveness, and suggest that β -blockers, used commonly in peri/postischemic patients, may impact leukocyte-mediated repair mechanisms.

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Table 1. Effects of β 2ARKO on chemokine receptor expression

Chemokine receptor	WT BMT	β 2ARKO BMT
CCR1	1.00 \pm 0.20	1.06 \pm 0.13
CCR2	1.00 \pm 0.21	0.27 \pm 0.07*
CCR5	1.00 \pm 0.16	1.32 \pm 0.18
CXCR1	1.00 \pm 0.54	1.71 \pm 0.56
CXCR2	1.00 \pm 0.51	1.63 \pm 0.62
CXCR4	1.00 \pm 0.06	0.32 \pm 0.02*
CXCR7	1.00 \pm 0.03	0.90 \pm 0.04
CXC3CR1	1.00 \pm 0.12	1.09 \pm 0.14
CD45	1.00 \pm 0.22	0.90 \pm 0.30

RT-qPCR analysis of changes in expression of chemokine receptor transcripts in reconstituted WT or β 2ARKO BM from transplanted mice. $n = 4-8$.

* $P < 0.001$ vs. WT BMT, two-tailed unpaired t test.

these altered chemokine receptor levels, we performed in vitro migration assays, wherein β 2ARKO BM displayed decreased migration toward CCL2 (MCP-1), the ligand for CCR2, with no difference in migratory responses to CCL3 or C-X-C motif chemokine ligand 12 (CXCL12), a CXCR4 ligand (Fig. 1*A* and *B*). Lentiviral-mediated restoration of β 2AR expression in β 2ARKO BM restored CCR2 expression to endogenous levels (Fig. 1*C*) as well as the migratory response to CCL2, without affecting migration to CCL3 or CXCL12 (Fig. 1*A* and *B*). CCR2 antagonism blocked migration toward CCL2 following β 2AR rescue but had no effect on CCL3 and CXCL12 responses, confirming that this response was CCR2-dependent.

Specific Ablation of CCR2 Reduces Leukocyte Recruitment to the Heart Following MI. Based on our in vitro assessment of the impact of β 2AR deletion on CCR2 expression and BM cell migration, we sought to determine whether CCR2 inhibition in vivo, either pharmacologically or genetically, could recapitulate the impaired leukocyte post-MI infiltration phenotype observed in β 2ARKO BMT mice (7). To assess this, WT mice underwent sham or MI surgery followed by daily injections with vehicle or CCR2 antagonist (2 mg·kg⁻¹·d⁻¹), or underwent irradiation and received WT, β 2ARKO, or CCR2KO BM 1 mo before surgery (Fig. S1). Analysis of infarct size 4 d post-MI showed no differences between groups, confirming similar surgical conditions for all groups of animals (Fig. S2). Immunohistochemistry was performed on heart sections 4 d postsurgery to quantify infiltration of immune cell populations in sham hearts and the remote (Fig. S3), border (Fig. 2 and Fig. S2*E-H*), and infarct (Fig. S4) zones of MI hearts. Both pharmacological CCR2 antagonism (Fig. S2*E* and *F*) and genetic CCR2 deletion (Fig. 2*A* and *B*) significantly reduced the infiltration of monocytes/macrophages (CD68⁺ cells) and neutrophils [myeloperoxidase (MPO)⁺ cells; Fig. 2*A* and *D* and Fig. S2*E* and *H*] to the border and infarct (Fig. S4) zones of the heart following MI. These data recapitulate those attained in β 2ARKO BMT mice (Fig. 2*A*), where decreased infiltration of monocytes/macrophages (Fig. 2*B*) and neutrophils (Fig. 2*D*) into the border and infarct (Fig. S4) zones were observed 4 d following MI. Interestingly, unlike in the β 2ARKO BMT mice, CCR2 inhibition did not impact mast cell infiltration (tryptase⁺ cells; Fig. 2*A* and *C* and Fig. S2*E* and *G*). Further, post-MI survival, infarct size, and contractility did not differ between WT BMT and CCR2KO BMT mice (Fig. S5*A* and Table S2), although CCR2KO BMT mice had slightly less dilation following MI than WT BMT mice. These results suggest that altered CCR2 expression may be a major contributing, but not sole, factor to the decreased leukocyte recruitment response to the injured heart in mice lacking immune cell-expressed β 2AR.

β 2AR Stimulation Alters CCR2 Expression in a β -Arrestin2-Dependent Manner. Because β 2ARKO BM has decreased expression of CCR2 compared with WT, we next sought to determine whether pharmacological activation of β 2AR reciprocally increases CCR2 expression. Thus, BM was isolated from WT C57BL/6 mice and

treated with the β 2AR-selective agonist salbutamol (Sal). Expression of CCR2 was quantified by RT-qPCR following Sal treatment over time. CCR2 levels were increased 6 and 24 h following β 2AR activation, demonstrating the ability to pharmacologically alter CCR2 levels using β 2AR ligands (Fig. 3*A*). Salbutamol-induced CCR2 expression observed in WT BM was not observed in β 2ARKO BM (Fig. 3*B*), confirming the specificity of the response. Migration assays were performed to determine whether β 2AR-dependent increases in CCR2 expression result in an enhanced functional response to CCL2-mediated migration. Indeed, CCL2-induced migration of WT BM was augmented with Sal pretreatment (Fig. 3*C* and *D*), whereas β 2ARKO BM did not migrate in response to CCL2, and Sal treatment had no effect on this response (Fig. 3*E* and *F*).

Because β 2AR stimulation engages both G protein- and β -arrestin (β ARR)-dependent signaling cascades, either of which may regulate downstream gene expression (8), we next sought to determine the proximal mechanism through which β 2AR stimulation increases CCR2 expression. Thus, β 2ARKO BM was infected with lentiviral constructs encoding either WT β 2AR (versus a GFP control

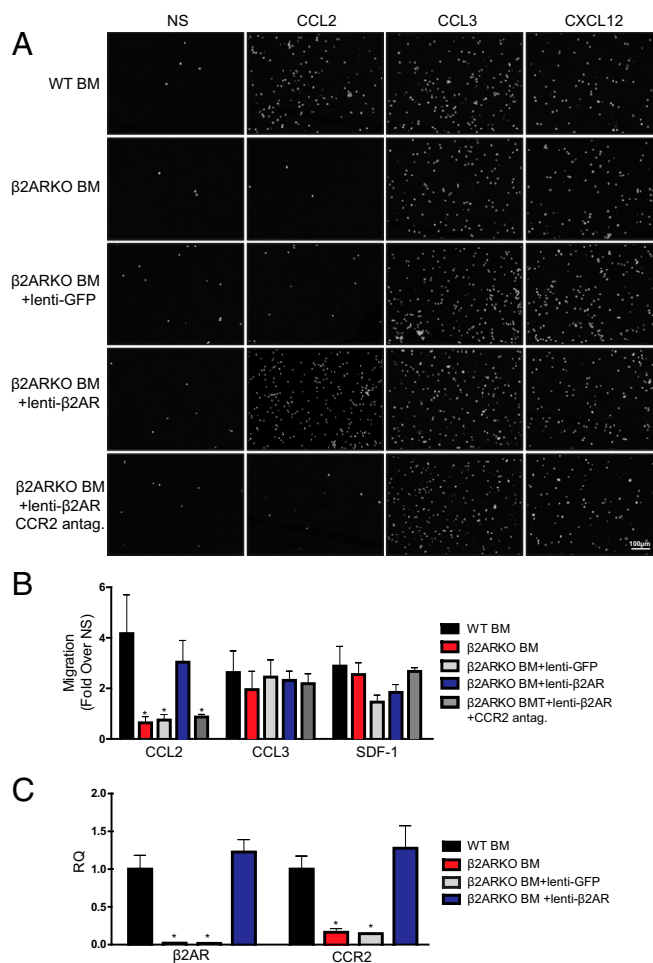


Fig. 1. Effects of β 2ARKO on BM migration in response to chemokines. (A) Representative Hoechst staining (white) from a 4-h migration assay of WT BM, β 2ARKO BM, β 2ARKO BM+lenti-GFP, and β 2ARKO BM+lenti- β 2AR in response to CCL2 (100 ng/mL), CCL3 (100 ng/mL), or CXCL12 (10 ng/mL). A 1-h pretreatment with a CCR2 antagonist (10 nM) was used to inhibit CCR2-mediated migration. (B) Quantification of migration assay results. Values are expressed as fold over vehicle-stimulated migration. $n = 4-8$; one-way ANOVA, * $P < 0.05$ vs. WT BMT. (C) RT-qPCR was used to measure β 2AR and CCR2 expression in WT and β 2ARKO BM and β 2ARKO BM that had been transduced with either a GFP or β 2AR lentivirus. $n = 4-8$; one-way ANOVA, * $P < 0.05$ vs. WT BMT. Data are expressed as mean \pm SEM. NS, nonstimulated; RQ, relative quantitation.

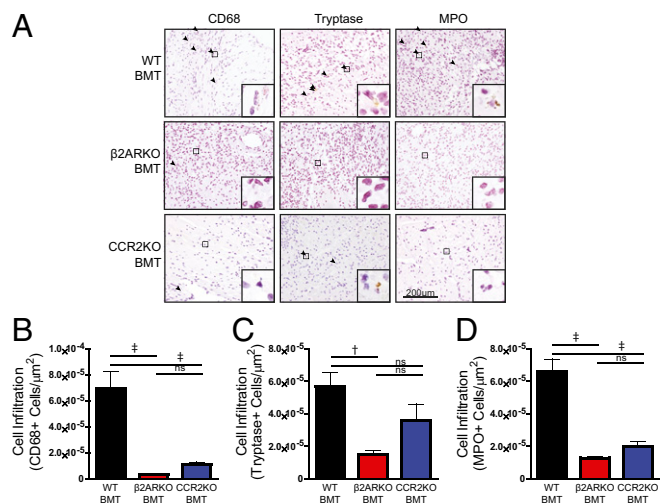


Fig. 2. CCR2KO BMT reduces leukocyte infiltration into the heart following MI. (A) Representative CD68, tryptase, and MPO staining for the border zone of hearts from WT C57BL/6 mice receiving WT, β 2ARKO, or CCR2KO BMT that underwent MI surgery. Arrowheads indicate positive staining. *Insets* show higher magnification at 250 \times . (B–D) Quantification of CD68 (B), tryptase (C), and MPO (D) staining for the border zone of 4-d post-MI hearts from WT, β 2ARKO, and CCR2KO BMT mice. $n = 4$ –8; one-way ANOVA, $^{\dagger}P < 0.01$ vs. WT BMT, $^{\ddagger}P < 0.001$ vs. WT BMT; ns, not significant. Data are expressed as mean \pm SEM.

lentivirus), β 2AR^{TY} [lacking stimulatory G alpha subunit ($G\alpha_s$) coupling (9)], or β 2AR^{GRK-} [deficient in G protein-coupled receptor kinase (GRK)-mediated phosphorylation and β ARR recruitment (10)], and CCR2 expression was measured. Each lentiviral construct induced β 2AR expression in β 2ARKO BM to levels similar to WT BM, whereas GFP had no effect (Fig. S6A). Flag and GFP expression was also assessed by immunoblot to confirm transgene expression (Fig. S6B). WT β 2AR and β 2AR^{TY} restored CCR2 expression in β 2ARKO BM, whereas neither GFP nor β 2AR^{GRK-} altered CCR2 expression (Fig. 4A). Functionally, migration in response to vehicle was unchanged by expression of any β 2AR construct (Fig. 4B and C). However, corresponding to changes in CCR2 expression, β 2AR^{GRK-} did not alter CCL2-mediated migration, whereas both WT β 2AR and β 2AR^{TY} had enhanced migration in response to CCL2 (Fig. 4D and F). These results indicate that β 2AR-mediated changes in CCR2 are dependent proximally upon β ARR-dependent signaling. To confirm these results, BM was isolated from β ARR1KO and β ARR2KO BM, and CCR2 expression and migration responses were examined following treatment with Sal. As was observed in WT BM, Sal treatment increased CCR2 expression in β ARR1KO BM (Fig. 5A) and resulted in enhanced migration in response to CCL2 (Fig. 5B and C). Conversely, Sal treatment of β ARR2KO BM was unable to increase CCR2 expression (Fig. 5A) or CCL2-mediated migration (Fig. 5D and E). Thus, β ARR2-dependent β 2AR signaling increases CCR2 expression, thereby enhancing immune cell responsiveness to CCL2-mediated migration.

To further define the mechanism through which β 2AR regulates CCR2 expression, transcription factor activation was examined using EMSAs. We assessed DNA binding of transcription factors reported to have putative binding sites in the CCR2 promoter and/or to regulate CCR2 expression [AP-1 (11), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (12), and nuclear factor of activated T cells (NFAT) (13)], as well as cAMP response element binding protein (CREB) as a positive control, because it is known to be regulated downstream of β AR but with minimal impact on CCR2 transcription (14–16). AP-1 (Fig. 6A) and CREB (Fig. S7A) transcription factor binding were both decreased in BM from β 2ARKO mice when compared with WT BM, whereas NF- κ B (Fig. S7B) and NFAT (Fig. S7C) binding were unaltered between groups. We subsequently tested whether rescue of β 2AR expression would restore transcription factor

binding in the β 2ARKO BM and, as expected, canonical β AR-sensitive CREB DNA binding was restored upon reexpression of β 2AR (Fig. S7D). Similarly, whereas GFP-infected β 2ARKO BM still displayed reduced AP-1 DNA binding similar to β 2ARKO alone (Fig. 6B), reexpression of WT β 2AR restored AP-1 binding to WT levels. To determine the importance of AP-1 activation in the induction of CCR2 transcription, WT BM was pretreated with the AP-1 inhibitor SR11302 before Sal treatment. Increased CCR2 expression in response to Sal treatment was blocked by pretreatment with SR11302 (Fig. 6C). Human monocytes were also treated with Sal \pm SR11302, yielding identical results to those attained in mouse BM cells, highlighting the potential human relevance of our findings (Fig. 6D).

To determine whether β ARR-dependent β 2AR signaling is involved in the control of AP-1-dependent CCR2 expression, as suggested from our results above, β 2ARKO BM was infected with the WT β 2AR, β 2AR^{TY}, and β 2AR^{GRK-} lentiviral constructs, or GFP control, and CCR2 expression was assessed. With restoration of WT β 2AR expression, Sal increased CCR2,

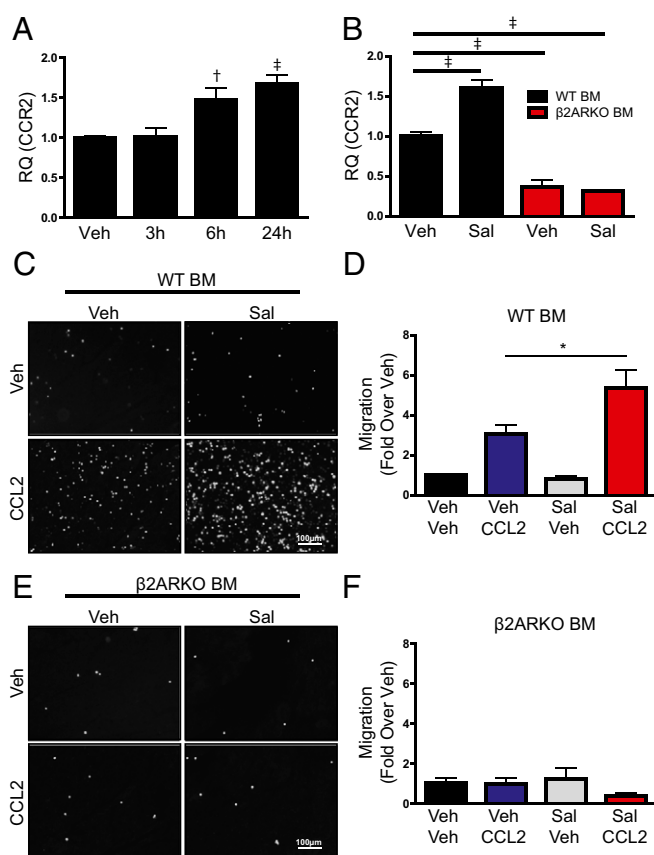


Fig. 3. β 2AR stimulation increases CCR2 expression and migration. (A) RT-qPCR was used to measure CCR2 expression in WT BM treated with vehicle (Veh) control or 1 μ M Sal over time (3 to 24 h). $n = 3$ –8; one-way ANOVA, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$ vs. Veh. (B) RT-qPCR was used to measure CCR2 expression in WT and β 2ARKO BM treated with Sal. $n = 6$; one-way ANOVA, $^{\ddagger}P < 0.001$ vs. Veh. (C) Representative Hoechst staining (white) from a 4-h migration assay of WT BM pretreated with vehicle or Sal and allowed to migrate in response to CCL2 (100 ng/mL). (D) Quantification of WT BM migration assay results. Values are expressed as fold over WT vehicle-stimulated migration. $n = 7$ –8; one-way ANOVA, $^*P < 0.05$. (E) Representative Hoechst staining (white) from a 4-h migration assay of β 2ARKO BM pretreated with vehicle or Sal and allowed to migrate in response to CCL2 (100 ng/mL). (F) Quantification of β 2ARKO BM migration assay results. Values are expressed as fold over WT vehicle-stimulated migration. $n = 7$ –8. Data are expressed as mean \pm SEM.

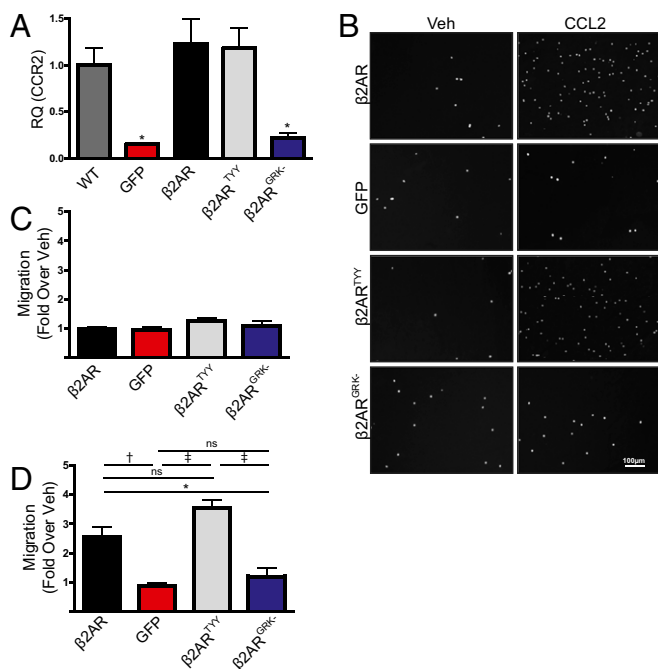


Fig. 4. Restoration of β 2AR expression reverses impairments in migration through β ARR-dependent mechanisms. (A) RT-qPCR was used to measure CCR2 expression in BM from WT or β 2ARKO BM transduced with GFP, β 2AR, β 2AR^{TYY}, or β 2AR^{GRK-/-} lentivirus. $n = 4-8$; one-way ANOVA, $*P < 0.05$ vs. WT. (B) Representative Hoechst staining (white) from a 4-h migration assay in response to vehicle or CCL2 for β 2ARKO BM transduced with lentiviral constructs for GFP, β 2AR, β 2AR^{TYY}, or β 2AR^{GRK-/-}. (C and D) Quantification of migration assay results. Values are expressed as fold over β 2ARKO+ β 2AR Veh. $n = 4-8$; one-way ANOVA, $*P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$. Data are expressed as mean \pm SEM.

as previously seen in WT BM, which could be prevented with SR11302 pretreatment (Fig. 6E). GFP-infected cells were unresponsive to Sal \pm SR11302 (Fig. S7E). Similar to the WT β 2AR rescue, β 2AR^{TYY}-infected β 2ARKO BM had increased CCR2 expression following Sal treatment, which was blocked by SR11302 (Fig. 6F); however, β 2AR^{GRK-/-}-infected β 2ARKO BM showed no alteration in CCR2 expression with either Sal or SR11302 (Fig. S7F). These findings confirm that β ARR2-dependent β 2AR signaling controls CCR2 expression via regulation of AP-1 in hematopoietic cells.

Restoration of β 2AR-Mediated β ARR Signaling in Vivo Reverses Leukocyte Dysfunction Following MI. To determine whether restoration of β 2AR expression, in particular β 2AR-mediated β ARR signaling, in β 2ARKO BM could rescue the impaired cardiac leukocyte recruitment observed in post-MI β 2AR BMT mice, β 2ARKO BM was transduced with either the WT β 2AR, β 2AR^{TYY}, β 2AR^{GRK-/-}, or GFP control lentiviral constructs before BMT. β 2AR transcript expression in β 2ARKO BM following reconstitution was approximately that of endogenous levels for WT and mutant β 2AR (Fig. S6C), which corresponded to restoration of β AR membrane expression (Fig. S6D). Flag and GFP expression was assessed by immunoblot to confirm transgene expression in reconstituted BM (Fig. S6E). CCR2 expression was restored with the reexpression of WT β 2AR as well as with β 2AR^{TYY}, but not in either GFP- or β 2AR^{GRK-/-}-transduced BM (Fig. 7A). Analysis of the infarct size of animals showed similar surgical conditions between groups (Fig. S8). Leukocyte levels in the remote region (Fig. S9) were unchanged following MI; however, correlating with restoration of CCR2 expression, immunohistochemistry for monocyte/macrophages, mast cell and neutrophil infiltration to the border zone (Fig. 7B-E), and infarct (Fig. S10) of the injured myocardium of WT β 2AR and

β 2AR^{TYY} were rescued compared with those of β 2ARKO+GFP and β 2AR^{GRK-/-} BMT mice.

Discussion

Inflammatory processes are activated following acute cardiac injury, including chemokine-induced recruitment of immune cells to the site of injury, which are essential for mounting a reparative response and allowing subsequent healing (1). Sympathetic activity is known to regulate inflammation, with β 2AR being the predominant adrenergic receptor subtype involved in immunomodulation (4-6). Recent findings from our laboratory have identified a critical role for hematopoietically expressed β 2AR in survival following MI, wherein a lack of β 2AR on immune cells resulted in decreased leukocyte infiltration to the heart, failed scar formation, and cardiac rupture (7). Although splenic retention of leukocytes played a role in the phenotype, whether the diminished leukocyte recruitment to the injured heart in the absence of immune cell-expressed β 2AR involved an alteration in the response to promigratory chemokines was not determined. Thus, the purpose of this study was to determine whether immune cell-expressed β 2AR plays a key role in regulating chemokine responsiveness and leukocyte infiltration following acute cardiac injury.

Because trafficking of immune cells to sites of inflammation occurs through various chemokine receptors (2), we initially surveyed the expression levels of a number of the receptors in WT versus β 2ARKO BM, finding that CCR2 and CXCR4 levels in particular were significantly decreased. However, only CCR2 deficiency had a negative effect on BM cell migration in response to its ligand CCL2. This was in the absence of deficiencies in egress of cells from BM (7) that occur with global depletion of CCR2 (17, 18). Although the importance of CCL2 as a major chemoattractant of mononuclear cells to the ischemic heart has been extensively studied, the results are often in conflict, with both administration and inhibition of CCL2 showing improvements and detrimental effects in the remodeling following MI (19, 20). These discrepancies may be a result of the timing of administration or inhibition, because short-term elevations in CCL2 are protective whereas sustained elevations in CCL2 contribute to an enhanced progression toward heart failure (21-24). Indeed, studies examining the involvement of CCR2 following cardiac injury have shown beneficial effects with CCR2 inhibition (25-27), wherein both global and monocyte-directed RNAi-mediated deletion of CCR2 in mice that underwent MI surgery resulted in improved left ventricular remodeling (25, 26).

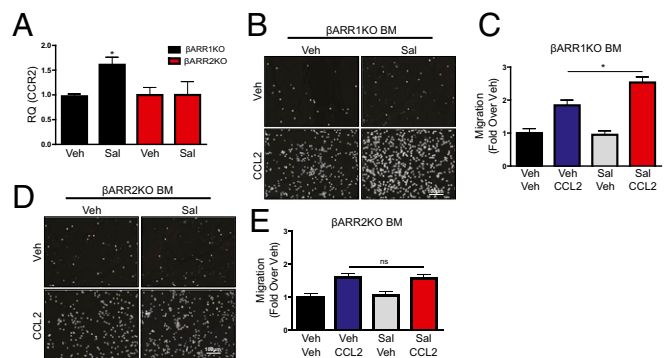


Fig. 5. β 2AR stimulation increases CCR2 expression and migration through β ARR2. (A) RT-qPCR was used to measure CCR2 expression in β ARR1KO and β ARR2KO treated with vehicle control or Sal. One-way ANOVA, $*P < 0.05$ vs. Veh. (B-E) Representative Hoechst staining (white) and quantified results from a 4-h migration assay of β ARR1KO (B and C) and β ARR2KO BM (D and E) pretreated with Veh or Sal (1 μ M; 24 h) before migration in response to Veh or CCL2 (100 ng/mL). Values are expressed as fold over Veh. $n = 4-8$; one-way ANOVA, $*P < 0.05$. Data are expressed as mean \pm SEM.

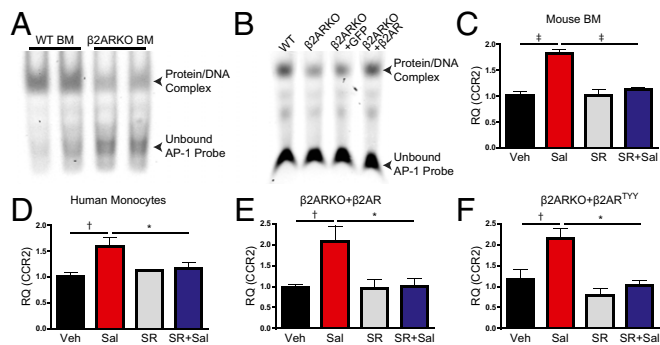


Fig. 6. AP-1 activation is decreased in β 2ARKO BM. (A) Representative EMSAs from nuclear WT and β 2ARKO BM extracts incubated with an AP-1-specific probe. (B) A representative EMSA from nuclear WT and β 2ARKO BM extracts or β 2ARKO BM infected with lentiviral-expressed GFP or β 2AR and incubated with an AP-1-specific probe. (C) CCR2 expression was quantified in WT BM treated with vehicle, Sal \pm SR11302, or SR11302 alone. $n = 4-8$; one-way ANOVA, $^{\dagger}P < 0.001$. (D) Human monocytes were treated with vehicle, Sal \pm SR11302, or SR11302 alone. CCR2 expression was measured using RT-qPCR. $n = 6-12$; one-way ANOVA, $*P < 0.05$, $^{\dagger}P < 0.01$. (E and F) β 2ARKO BM was infected with lentiviral-expressed WT β 2AR (E) or lenti- β 2AR^{TYY} (F) and treated with vehicle, Sal \pm SR11302, or SR11302 alone. CCR2 expression was examined by RT-qPCR. $n = 4-6$; one-way ANOVA, $*P < 0.05$, $^{\dagger}P < 0.01$. Data are expressed as mean \pm SEM.

CCR2 is highly expressed on proinflammatory monocyte populations and, although β 2AR has been shown to modulate chemotaxis, our findings are novel in that they directly link decreased hematopoietic cell β 2AR expression with a corresponding reduction in CCR2 levels and CCL2-dependent migration. Previous studies have shown either no effect of sympathetic nervous system (SNS) stimulation on CCR2 expression in peripheral blood mononuclear cells (28) or decreased CCR2 expression in BM with SNS stimulation (29). These differences may be due to the lack of

specificity of norepinephrine and epinephrine treatment and activation of multiple adrenergic receptor subtypes. Further, β 2AR stimulation was previously shown in THP-1 human monocytic cells to increase CCR2 expression and migration through an undefined mechanism (30), whereas another study demonstrated that treatment of THP-1 cells with cAMP-elevating agents, including PDE3 inhibitors and dibutyl cAMP, decreased both CCR2 expression and CCL2-mediated migration (31). These results may suggest that G_{α_s} protein-dependent β 2AR signaling acts to repress CCR2 expression.

Consistent with these reports, using β 2AR mutants lacking the ability to either engage G_{α_s} protein signaling (β 2AR^{TYY}) or to be phosphorylated by GRK (β 2AR^{GRK-}), we identified GRK-dependent β 2AR signaling as the mechanism by which β 2AR stimulation enhances CCR2 expression. Additionally, using BM cells from β ARRKO mice, we demonstrated that β ARR2, but not β ARR1, is specifically required for this effect. β ARR2 has been shown to regulate inflammatory responses in MI (32), as β ARR2KO mice had decreased survival after MI and decreased infiltration of macrophages to the infarct following MI, similar to our findings. β ARRs are known to be involved, either directly or indirectly, in the regulation of a number of transcription factors that could regulate CCR2 gene transcription, including AP-1 and NF- κ B (33-37). Although the role of AP-1 has been minimally studied following MI (38, 39), it plays a well-established role in inflammation (40). We have demonstrated that β ARR2-dependent β 2AR signaling via AP-1 is required for CCR2 up-regulation in response to sympathetic stimulation, and that restoration of GRK/ β ARR-dependent β 2AR signaling in immune cells rescues CCR2 expression, migration in response to CCL2, and cardiac infiltration following MI *in vivo*.

Although we show in our study that CCR2 deficiency in immune cells reduces their capacity for chemotaxis and infiltration to the heart post-MI, this deficiency was limited to the monocyte/macrophage and neutrophil populations *in vivo*, whereas mast cell infiltration was unchanged. This is in contrast to our previously reported study in which β 2ARKO chimeric mice had impairment in cardiac recruitment of all three cell types (7).

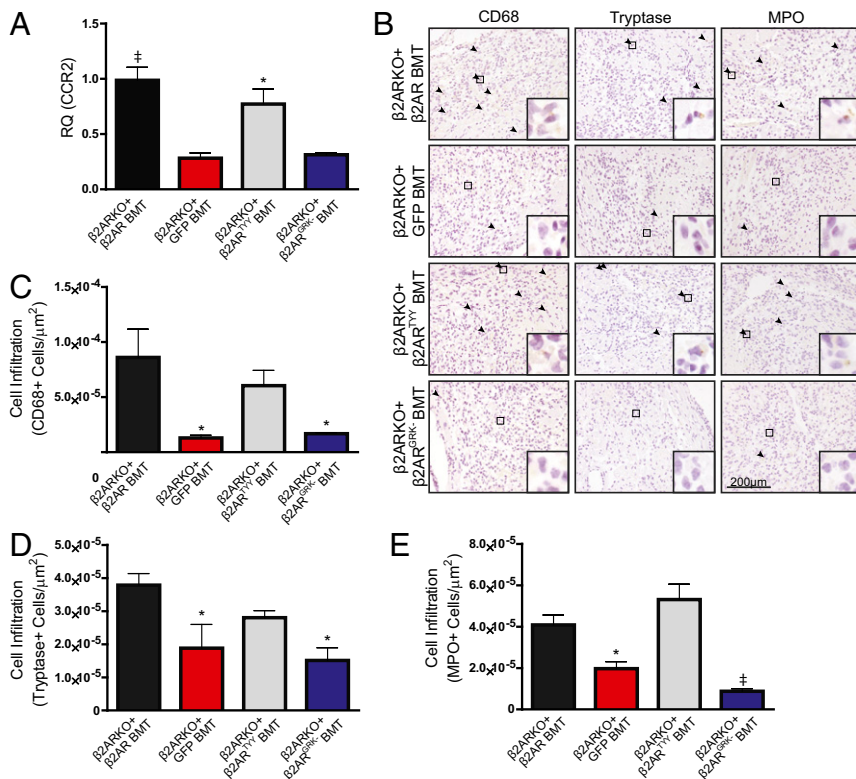


Fig. 7. Restoration of β 2AR expression in β 2ARKO BM reverses immune dysfunction following MI. (A) RT-qPCR was used to quantify CCR2 expression in reconstituted BM from mice that had GFP, WT β 2AR, β 2AR^{TYY}, or β 2AR^{GRK-} transduced into β 2ARKO BM by lentivirus before transplantation. Values are expressed relative to WT BMT. $n = 4-8$; one-way ANOVA, $*P < 0.05$, $^{\dagger}P < 0.001$ vs. β 2ARKO+ β 2AR BMT. (B) Representative CD68, tryptase, and MPO staining for the border zone of hearts from WT C57BL/6 mice receiving β 2ARKO BM with GFP, WT β 2AR, β 2AR^{TYY}, or β 2AR^{GRK-} transduced before transplantation that underwent MI surgery. Arrowheads indicate positive staining. Insets show higher magnification at 250 \times . (C-E) Quantification of CD68 (C), tryptase (D), and MPO (E) staining for the border zone of 4-d post-MI hearts from GFP, WT β 2AR, β 2AR^{TYY}, or β 2AR^{GRK-} BMT mice. Arrowheads indicate positive staining. $n = 4-8$; one-way ANOVA, $*P < 0.05$, $^{\dagger}P < 0.001$ vs. β 2ARKO+ β 2AR BMT. Data are expressed as mean \pm SEM.

Further, in our current study, we did not observe an altered progression toward heart failure in CCR2KO BMT mice, which, coupled with the positive outcome of CCR2 inhibition in the aforementioned studies (25, 26) versus the catastrophic impact of immune cell-specific β 2AR deletion on cardiac remodeling following MI we previously reported (7), suggests that β 2ARKO chimeric mice likely have additional factors that contribute to their observed post-MI phenotype. For instance, we previously showed that enhanced vascular cell adhesion molecule 1 expression was associated with splenic retention of leukocytes in β 2ARKO BMT mice and that splenectomy partially restored cardiac leukocyte infiltration following MI (7), demonstrating that even with diminished CCR2 expression and responsiveness, β 2AR-deficient leukocytes retain some capacity to traffic to sites of injury in vivo. Thus, it may be beneficial to further investigate the role of CXCR4 expression, as well as a larger number of chemokine receptors and secreted factors from individual immune cell populations, to more fully elucidate how β 2AR controls these processes. The potential existence of multiple β 2AR-dependent mechanisms that could influence distinct immune cell populations suggests a widespread impact of β 2AR modulation on the regulation of early immune responses that could be targeted to alter post-MI recovery.

In summary, we have identified a role for β 2AR in the regulation of immune cell-specific CCR2 expression, where a lack of β 2AR expression in leukocytes results in decreased CCR2 expression, impaired migration to CCL2 in vitro, and decreased monocyte/macrophage and neutrophil cardiac infiltration following MI in vivo. Lentiviral-mediated reexpression of β 2AR in

β 2ARKO BM before transplantation restored CCR2 expression and BM migration through a β ARR2-dependent pathway. These results demonstrate an immunomodulatory role for β ARR-biased β 2AR signaling in early immune responses following MI, which could be targeted to promote reparative processes while preventing chronic inflammatory events that are detrimental to healing. Further, because β -blockers are commonly used in patients around the time of acute cardiac ischemia, our results suggest they could impact the leukocyte-mediated repair response, warranting further investigation.

Materials and Methods

Surgery and Assays. Detailed descriptions of coronary artery occlusion surgery, echocardiography, human monocyte cell culture, reverse transcription-quantitative PCR, migration assay, histological analysis, radioligand binding, immunoblot analysis, bone marrow transplant, bone marrow isolation, and lentiviral infection are provided in *SI Materials and Methods*. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Temple University School of Medicine.

Statistical Analysis. Data presented are expressed as mean \pm SEM. Statistical analysis was performed using unpaired Student *t* tests, one-way ANOVA with a Tukey's multiple comparison test, or two-way repeated-measures ANOVA where appropriate using Prism 5.0 software (GraphPad Software), with *P* values indicated in the figure legends.

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