

Optogenetic vagal nerve stimulation attenuates heart failure by limiting the generation of monocyte-derived inflammatory CCR2⁺ macrophages

Highlights

- Vagal stimulation prevents pressure-overload-induced HF via the brain-heart axis
- Vagal stimulation inhibits Ccr12⁺ macrophages via the α 7nAChR-NRF2 pathway
- Ccr12⁺ macrophages derive from monocytes with pro-hypertrophic/fibrotic/TNF- α traits

Authors

Guoqi Li, Congcong Zhang, Yang Li, ..., Zhuofeng Lin, Ping Li, Yulin Li

Correspondence

zlin@gdmu.edu.cn (Z.L.),
doctorliping7511@sohu.com (P.L.),
lyllyl_1111@163.com (Y.L.)

In brief

Parasympathetic neuronal dysfunction is associated with heart failure, yet the underlying mechanism is poorly understood. Here, Li et al. demonstrate that optogenetic activation of the vagus nerve prevents the progression of heart failure by suppressing a distinct Ccr12⁺ macrophage subset in an α 7nAChR-dependent manner. Deletion of Ccr12⁺ macrophages or activation of α 7nAChR rescues heart failure.

Article

Optogenetic vagal nerve stimulation attenuates heart failure by limiting the generation of monocyte-derived inflammatory CCR2⁺ macrophages

Guoqi Li,^{1,2,7} Congcong Zhang,^{1,2,7} Yang Li,^{1,2,7} Jie Yang,^{1,2} Jianing Wu,^{1,2} Yihui Shao,^{1,2} Ke Ma,^{1,2} Xinyu Zhang,^{1,2} Shuolin Zhu,^{1,2} Jie Du,^{1,2} Xin-Liang Ma,³ Liping Wang,⁴ Zhuofeng Lin,^{5,6,*} Ping Li,^{1,*} and Yulin Li^{1,2,8,*}

¹Beijing Anzhen Hospital of Capital Medical University, Beijing, China

²Beijing Institute of Heart, Lung and Blood Vessel Diseases, Beijing, China

³Department of Emergency Medicine, Thomas Jefferson University, Philadelphia, PA, USA

⁴Shenzhen Institute of Advanced Technology, Shenzhen, China

⁵Innovation Center of Cardiometabolic Diseases, Guangdong medical university, Dongguan, China

⁶The Affiliated Dongguan Songshan Lake Central Hospital, Dongguan, China

⁷These authors contributed equally

⁸Lead contact

*Correspondence: zlin@gdmu.edu.cn (Z.L.), doctorliping7511@sohu.com (P.L.), lyllyl_1111@163.com (Y.L.)

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SUMMARY

Parasympathetic neuronal dysfunction is associated with heart failure (HF), yet the underlying mechanism is poorly understood. Here, we report that targeted vagal nerve stimulation (VNS) using optogenetics attenuated cardiac remodeling and HF induced by pressure overload. Unbiased approaches revealed that VNS decreased the proportion of Ccr2⁺ macrophages, which were derived from myeloid monocytes and exhibited a distinct tumor necrosis factor alpha (TNF- α) cytokine-responsive, pro-hypertrophic, and profibrotic signature. Elimination of Ccr2⁺ macrophages prevented cardiac remodeling and HF. Ccr2⁺-macrophage-specific overexpression or global genetic loss of $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) highlighted their crucial contribution to VNS-mediated cardioprotection. Activation of $\alpha 7$ nAChR inhibited Ccr2⁺ macrophages' TNF- α responsiveness through increased expression of the transcription factor NRF2. Cardiac Ccr2⁺ macrophages and TNF- α -responsive proteins positively correlated with cardiac remodeling and dysfunction in humans. An $\alpha 7$ nAChR agonist effectively blocked the development of HF. These results suggest that the vagal neuroimmune axis modulates HF and is a promising target for treatment.

INTRODUCTION

Heart failure (HF) is the leading cause of morbidity and mortality worldwide.¹ Despite improvements in medical therapy and device-based treatments, HF continues to pose an enormous burden on patients and healthcare systems worldwide. The balance in the autonomic nervous system is disrupted in HF, as evidenced by the withdrawal of the parasympathetic tone and overactivation of the sympathetic tone.² Autonomic nervous system imbalances are positively associated with mortality risk in patients with HF.³ Patients who receive vagal nerve stimulation (VNS) show improvements in HF symptoms and quality of life.^{4,5} Although the findings of investigative clinical studies suggest the enhancement of vagal tone as a new avenue for HF treatment, its clinical application remains limited owing to major side effects and surgically associated injuries.⁶

Pharmacological interventions that increase parasympathetic tone or modulate downstream molecules may be more feasible and less invasive than VNS treatment. However, data on the mo-

lecular mechanisms responsible for the cardioprotective effects of VNS are limited; therefore, effective drugs that mimic VNS are lacking. The most basic research on VNS involves electrical stimulation of the cervical vagus nerve.⁷ The cervical vagus nerve contains afferent and efferent vagal fibers in an approximate ratio of 4:1. Cervical electrical stimulation preferentially activates afferent fibers owing to their lower activation threshold than efferent fibers.⁸ Afferent fibers end in the nucleus tractus solitarius (NTS), where they activate the central autonomic network. This network activates both parasympathetic and sympathetic tones through descending pathways, targeting the pre-ganglionic neurons of vagal efferents originating in the dorsal motor nucleus of the vagus (DMV) and pre-ganglionic sympathetic neurons of the intermediolateral cell column in the spinal cord, respectively.⁹ The simultaneous activation of parasympathetic and sympathetic tones through electrical vagal afferents produces significant side effects and makes precise mechanistic investigations impossible. Developing a technique that specifically activates vagal neurons is optimal to avoid the side effects

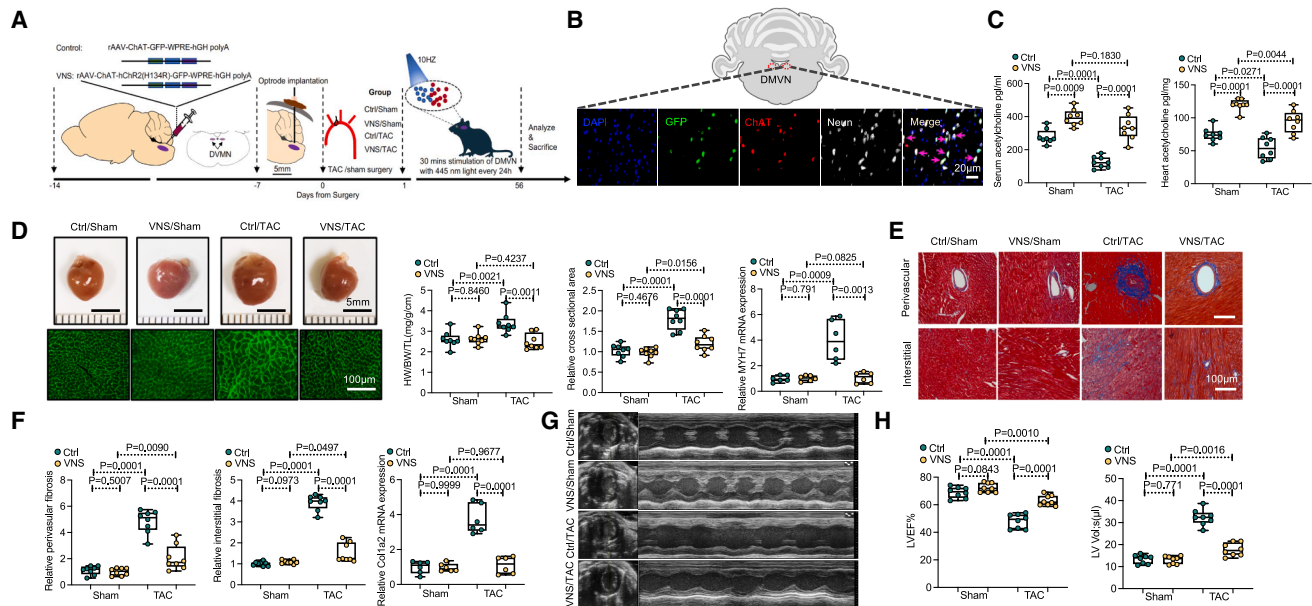


Figure 1. Optogenetic VNS suppresses HF in mice

(A) The experimental timeline, study protocols, and schematic illustration of the key methods. The mouse brain is drawn in sagittal and coronal projections to illustrate the anatomical position of the DVMN of the vagus nerve.

(B) GFP and ChAT⁺ neurons in the caudal region of the DVMN at 2 weeks after rAAV injection.

(C) Serum and cardiac ACh concentration at 8 weeks after sham surgery or TAC. *n* = 8 per group in biologically independent experiments.

(D) Representative images of gross heart morphology and heart sections immunostained with wheat germ agglutinin (WGA). Quantification of the heart weight/body weight/tibia length ratio (HW/BW/TL), cross-sectional area of CMs, and *Myh7* mRNA expression. *n* = 6–8 per group in biologically independent experiments.

(E) Representative images of Masson trichrome staining in the perivascular and interstitial areas of hearts.

(F) Quantification of perivascular, interstitial fibrosis, and *Col1a2* mRNA expression in the hearts. *n* = 6–8 per group in biologically independent experiments.

(G and H) Representative short axis and M-mode echocardiograms and corresponding values for LVEF and LV vol. *n* = 8 per group in biologically independent experiments.

Statistical significance was determined using two-way ANOVA, followed by Tukey's multiple-comparison test (C, E, G, and H). All data are presented as mean ± standard error of the mean (SEM). This figure is also related to [Figure S1](#).

of VNS and clarify the underlying mechanism of VNS. Optogenetics has been applied as a cell-specific approach for precise modulation of neural functions *in vivo*.¹⁰

This study aimed to investigate the mechanism of action of VNS in HF using optogenetics and explore potential pharmacological strategies. We developed an optogenetic approach to activate vagal neurons and determined whether optogenetic VNS regulates HF development. Using a combination of optogenetics and *in vivo*, *in vitro*, and *in silico* transcriptomic analyses, we uncovered the underlying mechanisms and validated these findings in patients with HF. Finally, we used an animal model to test the potential of the pharmacological therapies for HF.

RESULTS

Selective optogenetic stimulation of vagus neurons protects against HF

We evaluated vagus activity in human and mouse models. The general characteristics of HF patients and healthy controls (HCs) are presented ([Tables S1](#) and [S2](#)). Serum acetylcholine (ACh) concentrations were decreased in HF patients compared with HCs and were positively correlated with the ejection fraction (EF) ([Figures S1A](#) and [S1B](#)). Choline acetyltransferase (ChAT)⁺ protein gene product 9.5 (PGP9.5)⁺ neurons were decreased in

the hearts of HF patients compared with HCs ([Figure S1C](#)). ACh concentrations were low in both the plasma and heart tissues of mice subjected to transverse aortic constriction (TAC), which correlated with left ventricular ejection fraction (LVEF), and ChAT⁺ neurons was decreased in the hearts of TAC mice ([Figures S1D–S1G](#)). Excitation of ChAT⁺ neurons in the dorsal vagal motor nucleus (DVMN) was decreased. The excitation of TH⁺ neurons was significantly increased in the rostral ventrolateral medulla (RVLM) and NTS and slightly increased in the locus coeruleus after TAC ([Figures S1H](#) and [S1I](#)), indicating a parasympathetic/sympathetic imbalance during HF.

We used optogenetics to specifically activate vagus pre-ganglionic neurons in the DVMN and evaluated the development of HF. A recombinant adeno-associated virus (rAAV) carrying the activator channel rhodopsin (ChR2) (H134R)-enhanced GFP under the control of the ChAT promoter (rAAV-ChAT-hChR2 [H134R]-GFP) was injected into the DVMN of mice. Mice that received rAAV-ChAT-GFP served as the control (Ctrl). Magnetic resonance confirmed the placement of optical fiber in the DVMN area. rAAV was efficiently transfected into the DVMN by immunofluorescence (IF) ([Figure S1J](#)). The mice were subjected to TAC and received 30 min of light stimulation at 8 pm (zeitgeber time 12) every 24 h for up to 8 weeks ([Figure 1A](#)). GFP⁺ immunoreactivity was mostly present in ChAT⁺ neurons but not in

sympathetic or dopaminergic neurons in the DVMN (Figures 1B, S1K, and S1L). We selected a moderate stimulus intensity (10 Hz, 10 mA) (Figure S1M). Nerve recordings revealed robust light-locked electrical activity across the vagal fibers (Figure S1N). Heart rate variability decreased after TAC but increased in mice with VNS (Figure S1O), indicating successful vagus activation. After 8 weeks of TAC, wild-type (WT) mice that received VNS showed increased ACh content in both plasma and heart tissues compared with Ctrl mice (Figure 1C). VNS reduced pressure-induced cardiac hypertrophy, as evidenced by reduced heart weight/body weight/tibia length ratio (HW/BW/TL), cardiomyocyte (CM) cross-sectional area, and hypertrophic gene *Myh7* expression, as well as cardiac fibrosis, as evidenced by reduced collagen fiber and fibrotic gene *Col1a2* expression in VNS-TAC mice compared with those in Ctrl-TAC mice (Figures 1D–1F). HF was significantly inhibited by VNS, as characterized by decreased left ventricular end-systolic volume (LV vol;s) and increased LVEF in TAC-VNS mice (Figures 1G and 1H). We confirmed the role of the optogenetic manipulation of the vagus nerve in HF. rAAV carrying the Cre-dependent ChR2 or control vector was injected into the DVMN of ChAT-Cre mice (Figure S1P). Numerous EYFP⁺ signals were colocalized with ChAT⁺ neurons in the DVMN (Figure S1Q). VNS elevated ACh concentrations in the serum and heart compared with the Ctrl (Figure S1R). VNS significantly reduced cardiac hypertrophy, fibrosis, and dysfunction elicited by TAC in ChAT-Cre mice (Figures S1S–S1W). These data demonstrate that optogenetic VNS diminishes cardiac remodeling and HF in response to pressure overload.

VNS suppresses the Ccr2⁺ macrophage subset in HF via unbiased approaches

We performed bulk RNA sequencing (RNA-seq) and single-cell RNA sequencing (scRNA-seq) 8 weeks after TAC. Bulk RNA-seq analysis of heart tissues identified 873 differentially expressed genes (DEGs) between Ctrl-TAC and VNS-TAC mice, and the significant pathways in the VNS-TAC group were mapped to 16 categories, most of which were involved in the immune response (Figures S2A–S2C). Bulk RNA-seq results from both groups confirmed attenuated cardiac remodeling and indicated that the downregulated immune response was most noticeable in the VNS-TAC group. scRNA-seq showed that the median number of genes detected per cell was 2,739 and 2,250 for the Ctrl-TAC and VNS-TAC samples, respectively. Unsupervised Seurat clustering of the pool of CD45⁺ leukocytes from the two samples identified 10 clusters (Figures 2A, S2D, and S2E). Macrophages represented 66% of CD45⁺ leukocytes in both groups. Among the 10 clusters, the proportion of C-C chemokine receptor-like 2 (Ccr2)⁺ macrophages were markedly decreased in CD45⁺ leukocytes from VNS-TAC compared with that in CD45⁺ leukocytes from Ctrl-TAC. The number of DEGs between the two groups was highest in Ccr2⁺ macrophages (Figures 2B and 2C). Ccr2⁺ macrophages were enriched for multiple proinflammatory pathways; the most significant was the tumor necrosis factor alpha (TNF- α) signaling pathway (Figure S2F). To explore this phenomenon, we isolated four macrophage subsets from the hearts of Ctrl-TAC mice and performed bulk transcriptome profiling (Figure 2D). The selected proteins and sorting strategies are shown (Figures S2G and S2H). Sorted Ccr2⁺ mac-

rophages exhibited high expression of CD83 and Ccr2 and low expression of Lyve1, CD74, and Trem2 (Figure 2E). There were 5,726, 7,409, and 2,401 DEGs in Ccr2⁺ macrophages compared with Lyve1⁺, CD74⁺, and Trem2⁺ macrophages, respectively. Individual comparisons of the Ccr2⁺ macrophages with the other subsets revealed that the most significant pathway was TNF- α signaling (Figure 2F). The mRNA of multiple TNF- α signaling components was upregulated in Ccr2⁺ macrophages, including TNF- α signal transducers (Tnfrsf4, Fos, and Junb), pro-apoptotic factors (Ripk1), and chemokines (CCL2/5 and CXCL1/10) (Figure 2G). Having established the presence and signature of Ccr2⁺ macrophages in the heart after TAC, we verified whether VNS suppressed Ccr2⁺ macrophages. Flow cytometry analyses revealed a significant decrease in the number of CD83⁺Ccr2⁺ macrophages, but not in Lyve1⁺CD163⁺, Trem2⁺CD9⁺, or CD74⁺H2-Ab1⁺ macrophages, in the hearts of VNS-TAC mice (Figures 2H and 2I). There were more Ccr2⁺ cells in left ventricular wall (LVW) than in the interventricular septum and the right ventricular wall (RVW) (Figure S3A). Compared with sham operation, a greater quantity and closer distance of Ccr2⁺ cells were observed near to the PGP9.5⁺Chat⁺ neuron in the hearts of TAC mice (Figure S3B). Among the four clusters, Ccr2⁺ macrophages resided near the ChAT⁺ neurons (Figure 2J). We confirmed the adjacent distribution of vagal terminals and macrophages using AAV-mediated anterograde transsynaptic tagging tools.¹¹ Chat-Cre mice received a stereotaxic injection of an AAV2/1-hSyn-FLEX-EGFP-WPRE-pA in the bilateral DVMN, which can be transported to downstream neurons. IF staining showed effective labeling of Chat⁺ neurons with GFP in the DVMN area (Figure S3C). In particular, Ccr2⁺ cells located in the proximity of GFP⁺PGP9.5⁺ neurons in the heart after TAC (Figure S3D), indicating the vagus projections from DMVN near to Ccr2 macrophages. These results indicate that VNS inhibits a distinctive Ccr2⁺ macrophage subset with a TNF- α -responsive signature.

Ccr2⁺ macrophages are derived from myeloid monocytes

scRNA analysis showed that Ccr2⁺ macrophages exhibited elevated mRNA of recruitment macrophage markers (C-C chemokine receptor type 2 (Ccr2) and interleukin [IL]-1 β), while demonstrating reduced expression of resident macrophage markers (Tim4 and Fcrl2) (Figure S4A). To explore the developmental trajectory of Ccr2⁺ macrophages in TAC-induced HF, we performed a pseudotime analysis of the four macrophage subpopulations and monocytes in the heart. We employed CytoTRACE methodology to prognosticate and assign scores to cellular stemness. The immature state of cell subpopulations followed a sequential pattern of monocyte-Trem2-Lyve1-Ccr2-CD74 macrophages (Figures S4B and S4C). Monocle 2 analysis displayed that Ccr2 subtypes were positioned along the segment associated with monocytes, whereas the Trem2 and Lyve1 subtypes were predominantly located at the opposite end of the trajectory (Figures S4D and S4E). This positioning suggests that the Ccr2 subtypes primarily originate from monocytes and undergo differentiation along this pathway. To confirm whether Ccr2 macrophages were derived from myeloid cells, LysM-Cre mice were crossed with mTmG reporter mice in which LysM-expressing macrophages fluoresced green but all other lineages fluoresced red. Ccr2 colocalized with LysM-GFP⁺ myeloid cells in

exhibited high expression of adhesion molecules (*Vcam1*) and chemokines (*Ccl2*, *Ccl5*, etc.), facilitating monocyte infiltration and recruitment. Therefore, VNS plays double roles in suppressing monocyte production and the proinflammatory function of CCR2⁺ macrophages, eventually leading to decreased CCR2⁺ macrophages in the heart.

CCR2⁺ macrophages have profibrotic and prohypertrophic functions

Longitudinal analysis revealed that an increase in CCR2⁺ macrophages occurred prior to the deterioration of cardiac function (Figure S5A). CCR2⁺-cell-enriched areas showed more obvious collagen deposition, hypertrophy, and apoptosis in the heart. The degree of these cardiac pathologies was positively correlated with the abundance of CCR2⁺ cells (Figures S5B and S5C). We investigated whether CCR2⁺ macrophages are pathophysiologically important in HF using a diphtheria toxin (DT)-based system to deplete CCR2⁺ macrophages. CCR2 mRNA was highly expressed in CCR2⁺ macrophages, and CCR2 signals did not colocalize with Trem2, Lyve1, or CD74 macrophages (Figures S5D–S5F). CCR2-Cre mice were constructed and crossed with induced DT receptor (iDTR) mice to establish a system that allowed for specific depletion of CCR2⁺ macrophages (Figure S5G). CCR2^{Cre}/iDTR mice were administered DT every 5 days and subjected to TAC (Figure 3A). DT-treated CCR2^{Cre}/iDTR mice were used as controls. The numbers of CMP and circulating Ly6C^{hi} monocytes were similar in CCR2^{Cre}/iDTR and CCR2^{Cre}/iDTR mice after sham operation or TAC (Figures S5H and S5I). DT-treated CCR2^{Cre}/iDTR mice exhibited a significant reduction in both the number of CCR2⁺ macrophages and the mRNAs of TNF- α -responsive genes in cardiac tissue, whereas other macrophage populations remained unchanged (Figures 3B–3D and S6G), indicating that only CCR2⁺ macrophages were efficiently ablated. Pressure-overload-induced increases in gross heart size, HW/BW/TL, CM cross-sectional area, perivascular and interstitial fibrosis, and hypertrophic and fibrotic gene expression decreased in CCR2^{Cre}/iDTR mice (Figures 3E and 3F). Echocardiography showed that CCR2^{Cre}/iDTR mice had a higher LVEF than CCR2^{Cre}/iDTR mice (Figure 3G). Deletion of CCR2⁺ macrophages in a non-pressure overload state (sham operation group) did not affect cardiac phenotype and function.

We confirmed the function of CCR2⁺ macrophages through *in vitro* co-culture of primary CMs and cardiac fibroblasts (CFs) with CCR2⁺ macrophages or CCR2[−] macrophages (Figure 3H). Compared with the gene expression profiles of CMs co-cultured with CCR2[−] macrophages, the gene expression profiles of CMs co-cultured with CCR2⁺ macrophages were characterized by an enrichment of focal adhesion, extracellular matrix-receptor interaction, Hippo signaling, Wnt signaling, etc., which were closely related to hypertrophic and apoptotic responses in the heart^{15–17} (Figure 3I). CFs co-cultured with CCR2⁺ macrophages showed proinflammatory and proliferative gene expression profiles, as evidenced by the enrichment of multiple inflammation- and cell-cycle-related pathways (Figure 3J). CCR2⁺ macrophages promoted CM hypertrophy, CF proliferation, and myofibroblast differentiation (Figure 3K). These data indicate that CCR2⁺ macrophages exacerbate myocardial injury and remodeling and have a detrimental effect on heart function.

α 7nAChR activation restrains CCR2⁺ macrophages

We evaluated genetic associations of 19 muscarinic (mAChR) and nicotinic ACh receptor (nAChR) genes in HF patients and assessed their expression profiles in splenic tissue and peripheral blood. Cholinergic receptor nicotinic alpha (CHRNA4), CHRNA7, and cholinergic receptor muscarinic (CHRM5) had significant expression quantitative trait loci (eQTLs) for the spleen, whereas CHRNA2, CHRNA10, and CHRNB2 had eQTL ($p < 0.001$) for blood. Genome-wide association study (GWAS) revealed that the eQTL for CHRNA7 and CHRNB2 were significantly associated with the occurrence of HF (Figure 4A). Twenty-five eQTLs associated with decreased CHRNB2 expression and 14 associated with increased CHRNA7 expression were negatively associated with HF (Figure 4B; Table S3). LocusZoom plots of the eQTL and GWAS signals at the target loci for CHRNA7 are displayed (Figure 4C). *Chrna7* mRNA (encoding α 7nAChR) was the highest in sorted CCR2⁺ macrophages (Figure 4D). α 7nAChR was higher in CCR2⁺ macrophages and was located near ChAT⁺ neurons (Figures 4E and 4F).

We explored the role and molecular mechanism of α 7nAChR on CCR2⁺ macrophages. Transcription factors (TFs) were identified in CCR2⁺ macrophages using single-cell regulatory network inference analysis.^{1,8} Four candidate TFs were identified: *Nfe2l2* (encoding nuclear factor erythroid 2-related factor 2 [NRF2]), *Fos* (encoding activator protein 1 [AP-1] subunit c-Fos), *Cebpb* (encoding c/EBP β), and *Rel* (encoding nuclear factor-kappa B [NF- κ B] subunit c-Rel) (Figures 4G and S6A). Immunoblot analysis showed that NRF2 protein expression was significantly decreased in cardiac tissue after TAC but increased in response to VNS (Figure 4H). The mRNA expression of TNF- α -responsive genes was increased following TNF- α stimulation, but the increase was attenuated when NRF2 was overexpressed (Figures 4I and S6B). Next, we investigated whether α 7nAChR modulates TNF- α responsiveness through NRF2. Treatment with the selective α 7nAChR agonist PNU-282987 significantly upregulated NRF2 protein levels, whereas the selective α 7nAChR antagonist methyllycaconitine (MLA) downregulated NRF2 expression in macrophages (Figures 4J and 4K). Consistent with the changes in NRF2 expression, PNU-282987 inhibited the expression of TNF- α -responsive genes but MLA enhanced their expression (Figure 4L). Moreover, the inhibition of TNF- α responsiveness conferred by PNU-282987 was abolished by small interfering RNA (siRNA) knockdown of NRF2 (Figures 4L and S6C). Therefore, α 7nAChR blocked the TNF- α -induced positive feedback loop of CCR2⁺ macrophages by activating NRF2.

α 7nAChR accounts for the cardioprotective effect of VNS

To test the role of CCR2⁺-macrophage-specific α 7nAChR in HF development, we generated α 7nAChR^{flox/flox} overexpression (α 7^{fl/fl} OE) mice and crossed them with CCR2^{Cre} mice (Figure S7A). In CCR2^{Cre}/ α 7^{fl/fl} OE mice, expression of α 7nAChR proteins was increased in the heart and CCR2⁺ macrophages (Figures S7B and S7C). The number of CCR2⁺ macrophages and expression of TNF- α -responsive genes significantly decreased in the hearts of CCR2^{Cre}/ α 7^{fl/fl} OE mice (Figures S7D and S7E). The mean fluorescence intensity (MFI) levels of NRF2 protein were increased in CCR2^{Cre}/ α 7^{fl/fl} OE mice, and NRF2 was localized in CCR2⁺ macrophages (Figures S7F and S7G). TAC-induced gross

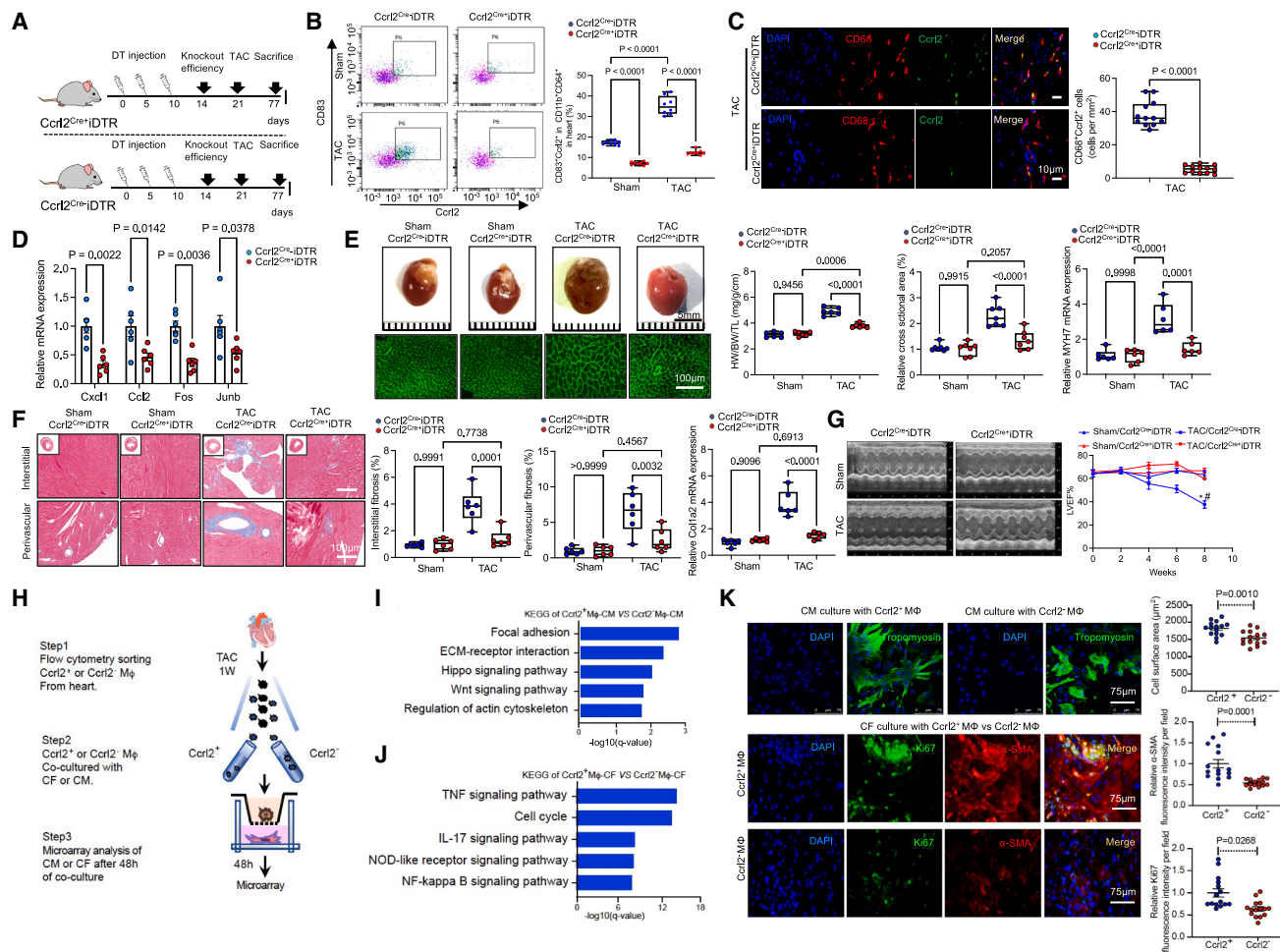


Figure 3. Ablation of $Ccr12^{+}$ macrophages reduces HF

(A) Detailed experimental schedule. $Ccr12^{Cre+}iDTR$ and $Ccr12^{Cre-}iDTR$ mice were included in the study. The administration of DT occurred on day 0 and was repeated every 5 days when performing TAC operation. Knockout efficiency of $Ccr12^{+}$ cells was measured after injection of DT at 21 days.

(B) Flow cytometry analysis and quantification of $CD83^{+}Ccr12^{+}$ macrophages after sham or TAC. $n = 8$ per group in biologically independent experiments.

(C) Representative images and quantification of $Ccr12^{+}CD68^{+}$ M ϕ in heart after TAC.

(D) Relative mRNA expression of TNF- α -responsive genes (*Cxcl1*, *Ccl2*, *Fos*, and *Junb*) in hearts after TAC. $n = 6$ per group in biologically independent experiments.

(E) Representative images of gross heart morphology and WGA staining. Quantification of HW/BW/TL; cross-sectional area of CMs, and *Myh7* mRNA expression in hearts. $n = 6-8$ per group in biologically independent experiments.

(F) Representative images of Masson's trichrome staining and quantification of perivascular and interstitial fibrosis and *Col1a2* mRNA expression in the hearts.

(G) Representative M-mode echocardiogram and longitudinal echocardiogram data: #significantly between TAC/ $Ccr12^{Cre-}iDTR$ and sham/ $Ccr12^{Cre-}iDTR$ ($p < 0.05$), *significantly between TAC/ $Ccr12^{Cre-}iDTR$ and TAC/ $Ccr12^{Cre+}iDTR$ treatment ($p < 0.05$). $n = 6$ per group in biologically independent experiments.

(H) Workflow for co-culture of M ϕ and CMs/CFs.

(I and J) Top five upregulated KEGG pathways in CMs or CFs based on DEGs between co-culture with $Ccr12^{+}M\phi$ and $Ccr12^{-}M\phi$.

(K) Representative IF images of tropomyosin, Ki67, and alpha smooth muscle actin (α -SMA) (left). Quantification of cell surface area in CMs and MFI of α -SMA and Ki-67 in CFs.

Statistical significance was determined using the unpaired Mann-Whitney U test (B), unpaired Student's t test (C, D, and K), and two-way ANOVA followed by Tukey's multiple-comparisons test (E-G). All data are presented as mean $\pm$ SEM. This figure is also related to [Figures S4](#) and [S5](#).

and microscopic changes were significantly attenuated in $Ccr12^{Cre+}\alpha7^{fl/fl}$ OE mice, specifically for HW/BW/TL, CM cross-sectional area, cardiac fibrosis, and expression of hypertrophic and fibrotic genes ([Figures 5A](#) and [5B](#)). Finally, cardiac function was significantly improved in $Ccr12^{Cre+}\alpha7^{fl/fl}$ OE mice subjected to TAC ([Figure 5C](#)). The basal cardiac phenotype of $Ccr12^{Cre+}\alpha7^{fl/fl}$ OE mice was similar to that of $Ccr12^{Cre-}\alpha7^{fl/fl}$ OE mice. These data demonstrate the cardioprotection conferred by

$\alpha7nAChR$ expression in $Ccr12^{+}$ macrophages. We further evaluated whether the anti-HF role of VNS is dependent on $\alpha7nAChR$. Global *Chrna7* $^{-/-}$ and *Chrna7* $^{+/+}$ mice underwent optogenetic VNS ([Figure 5D](#)). Mice injected with rAAV-ChAT-GFP were used as controls. VNS significantly elevated ACh protein concentration in both genotypes ([Figure 5E](#)). In the control mice, genetic $\alpha7nAChR$ deletion aggravated cardiac remodeling, dysfunction, and $Ccr12^{+}$ macrophages. The application of VNS significantly

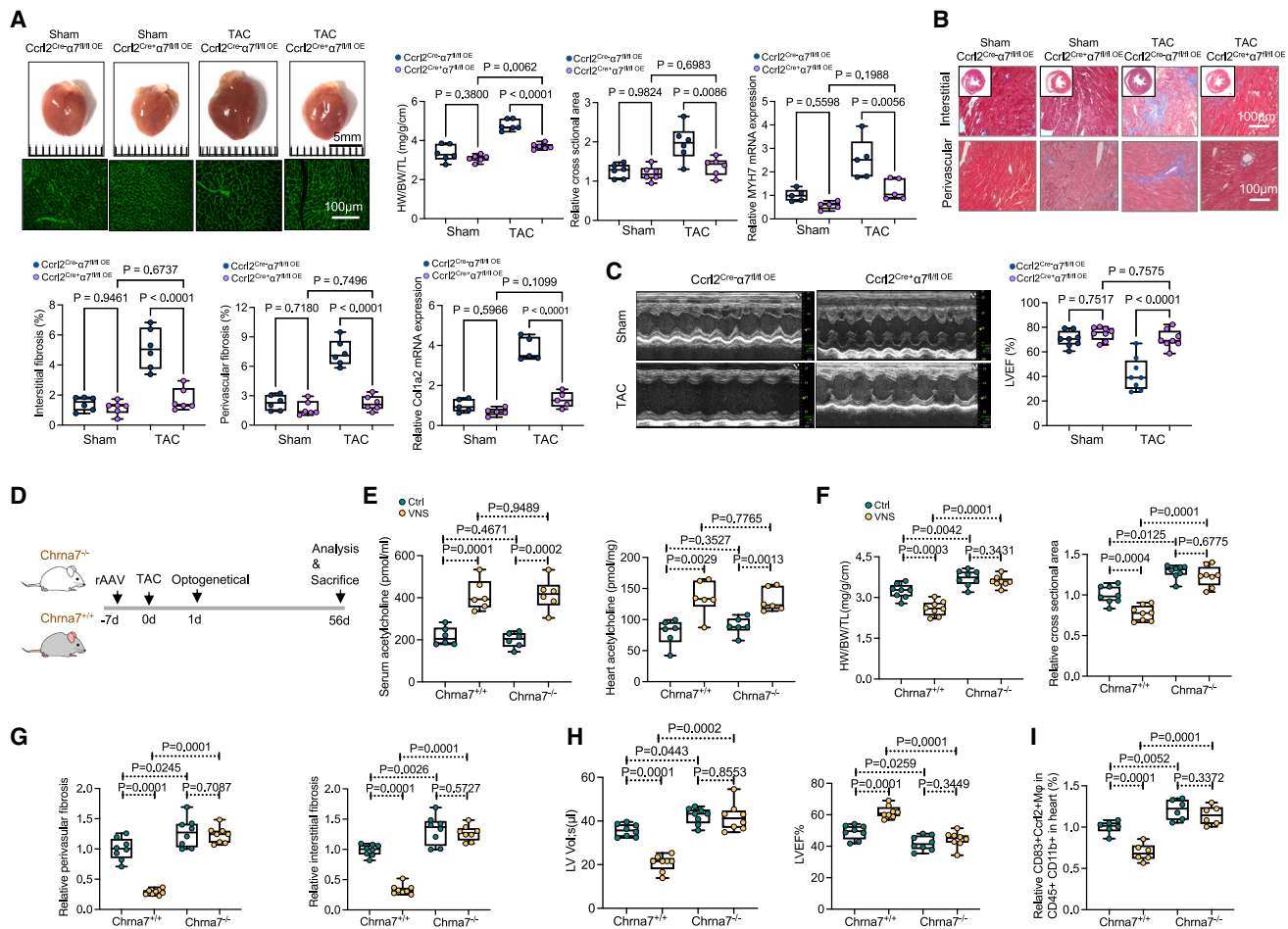


Figure 5. α7nAChR accounts for the cardioprotective effect of VNS

(A) Representative images of gross heart morphology and WGA staining. Quantification of HW/BW/TL, cross-sectional area of CMs, and *Myh7* mRNA expression in the heart from *Ccr2^{Cre-α7^{fl/fl} OE}* and *Ccr2^{Cre-α7^{fl/fl} OE}* mice after sham operation or TAC. *n* = 6 per group in biologically independent experiments.

(B) Representative images of Masson trichrome staining and quantification of perivascular and interstitial fibrosis and *Col1a2* mRNA expression in the hearts. *n* = 6 per group in biologically independent experiments.

(C) Representative M-mode echocardiograms and echocardiogram data to show LVEF. *n* = 8 per group in biologically independent experiments.

(D) *Chrna7^{+/+}* and *Chrna7^{-/-}* mice were injected with rAAV-ChAT-GFP (Ctrl) or rAAV-ChAT-hChR2(H134R)-GFP (VNS) followed by TAC.

(E) Serum and cardiac ACh concentration in *Chrna7^{+/+}* and *Chrna7^{-/-}* mice that received VNS or Ctrl stimuli after 8 weeks of TAC. *n* = 6 per group in biologically independent experiments.

(F–I) HW/BW/TL ratio, CM cross-sectional area, quantitation of fibrosis, LV vol/s, LVEF, and CD83⁺Ccr2⁺ Mφ in *Chrna7^{+/+}* and *Chrna7^{-/-}* mice that received VNS or Ctrl stimuli after 8 weeks of TAC. *n* = 6–8/group in biologically independent experiments.

Statistical significance was determined using two-way ANOVA followed by Tukey's multiple-comparisons test (A–I). All data are presented as mean ± SEM. This figure is also related to Figure S7.

reduced all indices of cardiac pathology in *Chrna7^{+/+}* mice. However, VNS-mediated protection was abolished in *Chrna7^{-/-}* mice after TAC (Figures 5F–5I). The collective data indicate that α7nAChR is essential for the cardioprotection conferred by VNS.

CCR2⁺ macrophage signature is associated with heart dysfunction in HF patients

The activation of vagus-α7nAChR diminished HF by restricting Ccr2⁺ macrophages in our mouse model. We verified these observations in patients with HF. First, immunostaining revealed the presence of CD68⁺Ccr2⁺ macrophages within the LV of HF patients (Figure 6A). α7nAChR colocalized with Ccr2⁺ macrophages (Figure 6B). Ccr2⁺-cell-enriched areas exhibited fibro-

sis, hypertrophy, and apoptosis, unlike non-enriched areas. The pathological changes were positively correlated with the number of Ccr2⁺ cells (Figures 6C and 6D). We analyzed the association between vagal activity and TNF-α-responsive genes. These patients were divided into low and normal vagus nerve activity (LVNA and NVNA, respectively) groups according to 24-h Holter monitoring (Figures S7H and S7I; Table S4). Patients with LVNA showed significantly lower heart rate variability indices and reduced serum ACh concentration compared with those with NVNA (Figure 6E). Plasma concentrations of TNF-α-responsive proteins and chemerin (a chemokine for Ccr2) increased from healthy to NVNA to LVNA patients, especially chemerin (Figure 6F). In patients with HF, circulating ACh

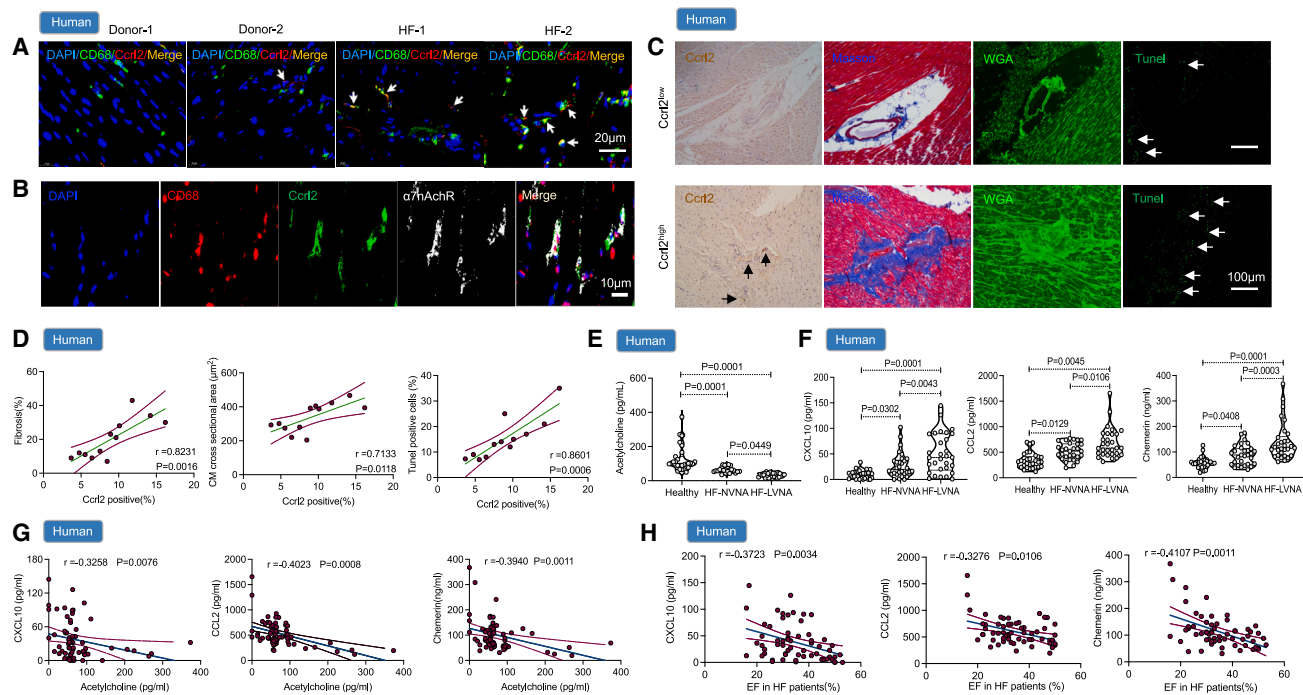


Figure 6. Ccr2⁺ macrophages and TNF- α -responsive signature are associated with heart dysfunction in patients with HF

(A) Representative images of Ccr2⁺CD68⁺ Mφ in left ventricles from healthy donors and HF patients.

(B) Representative images of Ccr2⁺α7nAChR⁺ in left ventricles of HF patients.

(C) Representative images of Ccr2, Masson trichrome, WGA, and TUNEL staining in serial heart sections from HF patients.

(D) Spearman's correlation between Ccr2⁺ cells and fibrosis, cardiac cross-sectional area, and TUNEL⁺ cells. Dashed lines represent 95% confidence intervals.

(E and F) Serum ACh, CXCL10, CCL2, and chemerin concentration in HCs and HF patients with NVNA or LVNA. $n = 30$ per group in biologically independent experiments.

(G and H) Spearman's correlation between serum CXCL10/CCL2/chemerin concentration and serum ACh, EF in HF patients. $n = 60$; dashed lines represent 95% confidence intervals.

Statistical significance was determined using the Kruskal-Wallis test with Dunn's multiple-comparison test (E and F). All data are presented as mean $\pm$ SEM.

concentration showed significant negative correlations with both LVEF and the circulating levels of CXCL10, CCL2, and chemerin (Figures 6G and 6H). Clinical analysis supported the association of decreased ACh-α7nAChR signals and increased Ccr2⁺ macrophage signature with HF.

Pharmacological activation of ACh-α7nAChR axis mitigates HF

We attempted to activate the vagal-α7nAChR axis via pharmacological tools and investigated which type of therapy—enhancing ACh contents or activating α7nAChR—was more effective for treating HF. The selective α7nAChR agonist EVP-6124 was used, as it is well tolerated and has successfully progressed to phase 3 clinical trials.¹⁸ In addition, the acetylcholinesterase inhibitor donepezil was used, as it improves heart dysfunction in rats with acute myocardial infarction.¹⁹ To determine whether drug treatment could mitigate the progression of HF after cardiac remodeling, WT mice that underwent TAC for 4 weeks were divided into three randomized treatment groups and administered saline, donepezil (5 mg/kg/day), or EVP-6124 (2 mg/kg/day) (Figure 7A). No deaths occurred during the 4-week treatment period. The fraction of Ccr2⁺ macrophages decreased by 1.6- and 1.3-fold in EVP-6124- and donepezil-treated mice,

respectively, compared with that in saline-treated mice (Figure 7B). Both donepezil and EVP-6124 treatment attenuated the pathological phenotype of cardiac adverse remodeling and expression of TNF- α -responsive genes. These changes were more apparent in the EVP-6124 treatment (Figures 7C–7E). LVEF was similar among the three groups at 4 weeks following TAC. The LVEF, after 8 weeks of EVP-6124 treatment, was 60% higher than that saline treatment and 14% higher than that after donepezil treatment (Figure 7F).

EVP-6124 is more effective than donepezil in cardiac remodeling and HF. We determined the effects of the three treatments at the molecular level using bulk RNA-seq. Global Pearson correlation analysis identified 1,051 DEGs that were correlated with treatment, including 691 upregulated and 810 downregulated genes, with tendencies from saline and donepezil to EVP-6124 (Figure 7G). Single-sample gene set enrichment analysis of DEGs identified significant DEG sets among the three groups. A series of pathways related to inflammation and cardiac remodeling were significantly downregulated, whereas oxidative phosphorylation was significantly upregulated, in the EVP-6124 group compared with the saline group (Figure 7H). These results implicate selective α7nAChR agonists as a therapeutic strategy to improve heart function and reduce adverse remodeling.

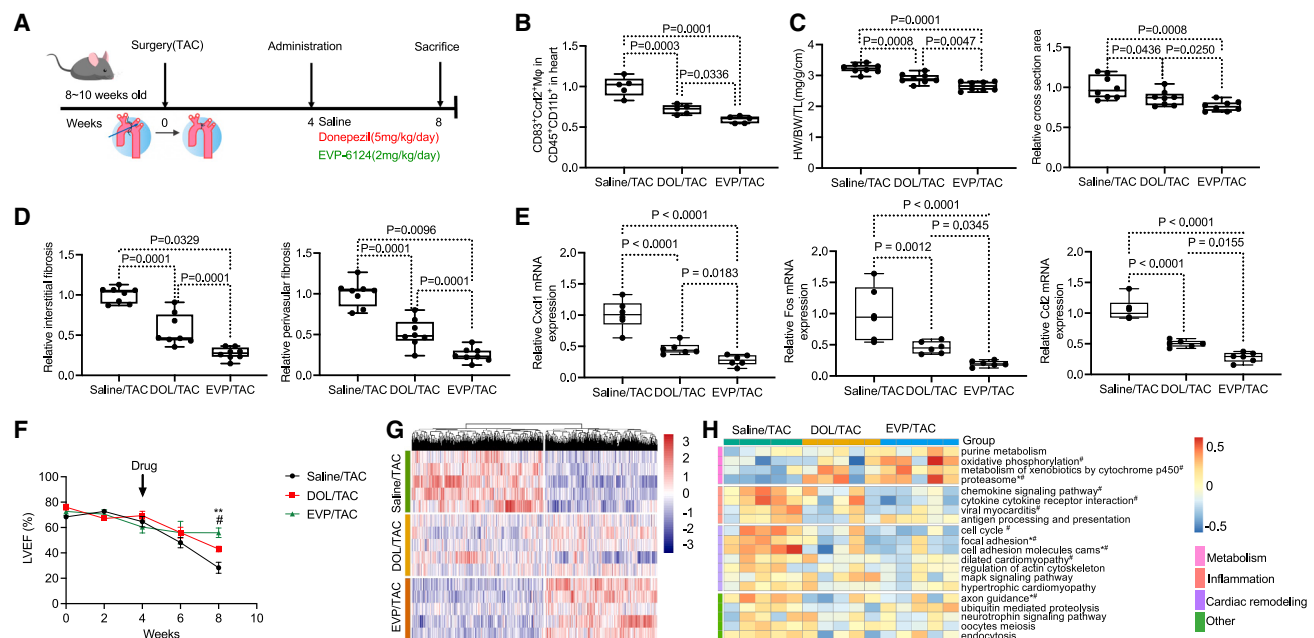


Figure 7. Therapeutic role of pharmacological enhancement of ACh- α 7nAChR signaling against HF

(A) Outline of pharmacological activation of ACh- α 7nAChR in mice subjected to TAC surgery.

(B–D) After 4 weeks of treatment, CD83⁺Ccr2⁺ macrophages; HW/BW ratio, CM cross-sectional area; perivascular and interatrial fibrosis areas in mice administrated saline, donepezil (DOL), or EVP-6124 (EVP). $n = 8$ per group in biologically independent experiments.

(E) Relative mRNA expression of TNF- α -responsive genes (*Cxcl1*, *Fos*, and *Ccl2*) in hearts from mice administrated saline, DOL, or EVP. $n = 6$ per group in biologically independent experiments.

(F) Longitudinal echocardiogram data showing the effects of TAC, over time, on LVEF in saline, DOL, or EVP treated mice. $n = 8$ per group. #Significantly between DOL and saline treatment ($p < 0.05$), *significantly between EVP and saline treatment ($p < 0.05$).

(G) Bulk RNA-seq was performed on hearts treated with saline, DOL, or EVP after TAC ($n = 5$ per group), followed by a Pearson correlation analysis between gene expression profiles with treatment groups. Heatmap of 1,501 genes with changes in expression significantly correlated with treatment ($p < 0.05$).

(H) Pathway profiles based on genes with upregulated and downregulated expression. Rows represent pathways. Each cell represents an enrichment score of pathway activity calculated using single-sample gene set enrichment analysis. *Significantly enriched pathway between DOL and saline treatment, #significantly enriched pathway between EVP and saline treatment ($p < 0.05$).

Statistical significance was determined using one-way ANOVA followed by Bonferroni's post hoc comparison test (B–F). All data are presented as mean $\pm$ SEM.

DISCUSSION

This study clarified the physiological roles of the neuroimmune axis in the heart in response to stressors and demonstrated that optogenetic stimulation of the vagal neurons of the DVMN suppressed cardiac remodeling and dysfunction induced by pressure overload. VNS suppressed the Ccr2⁺ macrophage subset, exhibiting a distinct TNF- α -responsive signature. Ccr2⁺ macrophage depletion prevented HF. The findings provide genetic and pharmacological evidence that the protection elicited by VNS depended on macrophage-specific α 7nAChR signaling.

The pre-ganglionic vagal neurons of the DVMN control ventricular contractility.²⁰ VNS-based anti-HF therapy can be used specifically to activate the vagal neurons of DVMN innervating the ventricles. The cardiac application of optogenetics has focused largely on the heart rhythmicity, defibrillation, and termination of arrhythmia.^{21–24} Optogenetic VNS suppresses cardiac remodeling and HF. Because autonomic neurons directly innervate the myocardium through fibers branching from the superior cervical ganglia,²⁵ neural modulation of the immune response involved in HF may occur locally in the heart. However, the interheart neuro-

immune axis has not yet been identified. Our findings demonstrate that the failing heart contains four distinct macrophage subsets. A previous study demonstrated the cardioprotective role of cardiac-resident macrophages in myocardial infarction.²⁶ Trem2⁺ macrophages have specialized functions in lipid metabolism and lesion calcification in atherosclerosis.²⁷ The abundance of CCR2⁺ macrophages/monocytes in infarcted hearts exhibiting heightened inflammatory activity has been described.²⁸ Ccr2⁺ macrophages are an undescribed population. Human HF is characterized by the presence of Ccr2⁺ macrophages. To clearly understand the macrophage classification, we analyzed the similarity between our findings and reported cardiac macrophages.²⁹ The analysis of marker genes and feature genes scores suggested that Ccr2⁺ macrophages were similar to CCR2⁺CD52^{hi} macrophages compared with Timd4⁺Lyve1⁺ macrophages, but only a portion of Ccr2⁺ macrophages express CCR2 in the heart after TAC (Figures S7J–S7M). Despite similarities, Ccr2⁺ macrophages are distinct from CCR2⁺ macrophage/monocyte subset.

Our study contributes to the literature by demonstrating the cellular and molecular mechanisms through which the vagal nerve controls immune cells in the heart. We identified Ccr2⁺

macrophages as an important link between vagal nerves and HF. Ccr2⁺ macrophages were located closer to ChAT⁺ neurons. Functionally, we identified a TNF- α -responsive role for Ccr2⁺ macrophages. The increase in Ccr2⁺ macrophages and the TNF- α responsiveness induced by TAC were attenuated in mice that received VNS. Ccr2⁺ macrophages infiltrate the heart during the early stages of HF. Chemokines and important regulators of cytokine expression were highly expressed in Ccr2⁺ macrophages. Inflammation was primarily driven by Ccr2⁺ macrophages before HF. Importantly, we revealed the pathological role of Ccr2⁺ macrophages in the regulation of cardiac inflammation, hypertrophy, and fibrosis. Clinically, cardiac Ccr2⁺ macrophages correlated with pathological changes, and the plasma concentration of chemerin correlated with LVEF in patients with HF. A cohort study implicated serum chemerin as a prognostic indicator of adverse cardiac events in HF patients.³⁰ The present study showed that VNS specifically inhibits Ccr2⁺ macrophages, impeding the pathological process of HF. We did not exclude the contribution of Ccr2⁺CD68⁺ cells to HF, as a few expressions of Ccr2 were observed on CD68⁺ cells in heart tissue from HF patients. scRNA-seq analysis also shows a low expression of Ccr2 in monocytes and granulocytes. The function of Ccr2⁺ non-macrophages requires future exploration.

In addition to its cardiac role, the suppression of hematopoiesis might play an equally significant role in VNS-mediated cardiac protection. Our data demonstrated that VNS inhibited hematopoiesis and circulating Ly6C^{hi} monocyte production during HF. Plenty of evidence showed the importance of hematopoiesis in HF. HF patients have an increased frequency of CD34⁺/CD38⁺ hematopoietic stem/progenitor cells in BM and monocytes compared with controls.³¹ Angiotensin-converting enzyme inhibitors, as a cornerstone therapy for HF, inhibited the mobilization of granulocyte-monocyte progenitors and reduced cardiac inflammation.³² Neuronal and non-neuronal BM cholinergic signals increased during stress such as chemotherapy or irradiation, and cholinergic signal activation of α 7nAChR in BM mesenchymal stromal cells led to hematopoietic stem/progenitor cells (HSPC) quiescence.¹⁴ Thus, VNS exerts an immunoregulatory effect in both BM and locally in the heart during HF.

This study provides evidence that VNS inhibits Ccr2⁺ macrophages through α 7nAChR-NRF2 signaling. Genetic analysis revealed that 14 eQTL were associated with the increased expression of CHRNA7, located in a major linkage hotspot, and were negatively associated with HF. *In vivo*, α 7nAChR was indispensable for the beneficial effects of VNS on HF. The neuroimmune interaction between cholinergic fibers and α 7nAChR-expressed macrophages has been established.³³ However, macrophage heterogeneity has long been recognized, and cellular specificity and molecular mechanisms of α 7nAChR on macrophage subtypes are not clear. α 7nAChR was highly expressed in Ccr2⁺ macrophages and Ccr2 macrophage-specific α 7nAChR OE protected against HF. Our research revealed that α 7nAChR activation inhibited TNF- α responsiveness by enhancing NRF2 protein expression. NRF2 is critical for the maintenance of redox, metabolism, and in the regulation of inflammation.³⁴ TNF- α -induced biphasic regulation of the NRF2 pathway with a low concentration of TNF- α (<10 ng/mL) mediates the activation of NRF2,³⁵ whereas a high concentration of TNF- α (>10 ng/mL)

suppresses the NRF2.³⁶ We demonstrated that NRF2 directly inhibited the expression of TNF- α -responsive genes in Ccr2⁺ macrophages. Consistent with our findings, NRF2 repression was identified as a regulator of proinflammatory astrocytes in autoimmune encephalomyelitis.³⁷ TNF- α responsiveness in Ccr2⁺ macrophages may be due to the inhibition of NRF2. The activity of NRF2 is regulated by the repressor protein Kelch-like ECH-associated protein 1 (KEAP1), which links the Cul3-based ubiquitin ligase complex with NRF2, leading to NRF2 ubiquitination and proteasomal degradation. NF- κ B p65 is a KEAP1-interacting protein that decreases NRF2 transactivation.³⁸ Akt signaling increases the nuclear import of NRF2 by inhibiting glycogen synthase kinase-3 beta (GSK-3 β) and reducing the nuclear localization of the tyrosine kinase Fyn.³⁹ α 7nAChR decreases NF- κ B p65 protein expression,⁴⁰ but increases the activation of Akt signaling.⁴¹ Our results support a model wherein NRF2 deactivation potentiates the TNF- α responsiveness of Ccr2⁺ macrophages. VNS upregulates NRF2 protein expression via α 7nAChR signaling, leading to the repression of Ccr2⁺ macrophages.

In this study, the optogenetic or pharmacological modulation of the vagus nerve was considered therapeutic. Optogenetic techniques are currently limited to animal experiments. Advances in rAAV gene transfer to the nervous system and implantable wireless optogenetic devices for peripheral nerves may promote future clinical applications of this technology.^{42,43} The potential impact on HF of drugs related to the ACh- α 7nAChR signaling should be investigated. Donepezil improves heart dysfunction following acute myocardial infarction (AMI),¹⁹ consistent with our results. The therapeutic action of EVP-6124 on cardiac remodeling and HF was superior to that of donepezil, as ACh binds to both nAChRs and mAChRs. However, the activation of mAChR can produce both inhibitory and stimulatory effects on the heart.⁴⁴ Peripheral blockade of α 7nAChR was reported to significantly attenuate the cardioprotective effects of donepezil on chronic HF rats.⁴⁵ Our findings provide evidence that encourages a pilot study to investigate the therapeutic effects of selective α 7nAChR agonists on HF patients.

We attempted to explain why ACh concentration decreased in the TAC model. An enhanced sympathetic nervous system may counteract the parasympathetic nervous system via multiple pathways. Sympathetic transmitters act locally on cholinergic neurons to reduce the release of ACh.⁴⁶ Sympathetic axons release adrenergic transmitters that bind to α 2a adrenergic receptors and cause presynaptic inhibition of the release of ACh.⁴⁷ Moreover, galanin (a non-adrenergic transmitter) is released during cardiac sympathetic hyperactivity and directly inhibits the release of ACh.⁴⁸ Furthermore, sympathetic nerve terminals preferentially target cholinergic excitatory cells in the myenteric plexus.⁴⁹ Monosynaptic input from adrenergic neurons to iii parasympathetic preganglionic neurons was demonstrated at the ultrastructural level.⁵⁰ Angiotensin II is locally generated in the heart and brain, in addition to its systemic production during HF. The upregulated angiotensin II interacts with the arterial baroreflex at several sites within the blood-brain barrier to inhibit vagal discharge and increase central sympathetic outflow.⁵¹

This study identifies a neuroimmunological axis for the prevention of HF by inhibiting Ccr2⁺ macrophages and proposes α 7nAChR as an immunomodulatory therapeutic target for HF.

Limitations of the study

Although we observed reduced vagal tone and increased sympathetic activation in HF, the precise neuroanatomical and functional crosstalk between autonomic pathways—particularly the roles of central nuclei such as the DMV, NTS, and RVLM—remain undefined. It is unclear whether VNS directly suppresses sympathetic overactivity or operates independently, warranting further investigation. Additionally, although VNS attenuated expansion of pro-inflammatory Ccr2⁺ macrophages and their BM precursors, the molecular mechanisms underlying VNS-mediated suppression of myelopoiesis and macrophage differentiation remain uncharacterized. Finally, although optogenetic VNS enabled precise neuromodulation in mice, clinical translation faces challenges, including the invasiveness of central nerve targeting and technical barriers to chronic stimulation. Non-invasive or clinically adaptable VNS strategies remain critical unmet needs, despite our clinical correlations linking vagal dysfunction to HF severity.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yulin Li (lyllyl_1111@163.com).

Materials availability

The materials are available upon request from the corresponding authors.

Data and code availability

- RNA-seq data have been deposited at GEO (GSE295812, GSE295813, GSE296034, and GSE296255) and are publicly available as of the date of publication. Accession numbers are listed in the [key resources table](#). This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Yulin Li conceptualized and supervised the study. G.L., C.Z., J.Y., Y.S., and X. Z. performed *in vivo* and *in vitro* experiments. Yang Li and S.Z. performed bioinformatic analysis. K.M., J.W., and P.L. collected and analyzed the clinical data. Yulin Li, Z.L., and X.-L.M. provided input on the study design. L.W. provided optogenetics technical support. Yulin Li and G.L. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
NRF2	Abcam	Cat# ab62352RRID:AB_944418
CEBPβ	Abcam	Cat# ab32358 RRID:AB_726796
c-Fos	Abcam	Cat# ab214672 RRID:AB_3939046
c-Rel	Abcam	Cat# ab309131 RRID:NA
Tyrosine hydroxylase	Abcam	Cat# ab113 RRID:AB_297905
Choline acetyltransferase	Abcam	Cat# ab34419 RRID:AB_726869
Dopamine beta hydroxylase	Abcam	Cat# ab19353 RRID:AB_731851
CD68	Abcam	Cat# ab955 RRID:AB_307338
CD74	Abcam	Cat# ab289885 RRID:NA
α7nAChR	Abcam	Cat# ab216485 RRID:NA
β-actin	ZSGB-bio	Cat# TA-09 RRID:AB_2636897
GAPDH	ZSGB-bio	Cat# TA-08 RRID:AB_2747414
GFP	Abcam	Cat# ab13970 RRID:AB_300798
Lyve1	Invitrogen	Cat# MA5-32512 RRID:AB_2809789
Ccr12	Invitrogen	Cat# MA5-24209 RRID:AB_2610321
Trem2	Invitrogen	Cat# PA5-119690 RRID:AB_2913263
Tropomyosin	Santa	Cat# SC-74480 RRID:AB_2272017
Ki-67	Santa	Cat# SC-7846 RRID:AB_2142374
α-SMA	Sigma	Cat# A5228 RRID:AB_262054
CCR2	Servicebio	Cat# GB11326-100 RRID:NA
PGP9.5	Abcam	Cat# ab227157 RRID:NA
Neun	GeneTex	Cat# GTX00837 RRID:AB_2937041
PE-CF594 Rat Anti-Mouse CD45	BD	Cat# 562420 RRID:AB_11154401
BV510 Rat Anti-Mouse CD11b	BD	Cat# 562950 RRID:AB_2717913
APC Rat Anti-Mouse TREM2	R&D	Cat# FAB17291A RRID:AB_884527
PE Rat Anti-Mouse LYVE1	eBioscience	Cat# 13-0443-82 RRID:AB_1724157
FITC Rat Anti-Mouse CD163	eBioscience	Cat# 11-1631-82 RRID:AB_2815035
BV786 Rat Anti-Mouse CCRL2	BD	Cat# 743688 RRID:AB_2741675
BV421 Rat Anti-Mouse CD9	BD	Cat# 752985 RRID:AB_2917940
BB700 Rat Anti-Mouse CD83	BD	Cat# 742263 RRID:AB_2871445
BV711 Rat Anti-Mouse CD74	BD	Cat# 740748 RRID:AB_2740416
Super Bright 645 Rat Anti-Mouse MHC Class II (I-A/I-E)	eBioscience	Cat# 64-5321-82 RRID:AB_2662402
APC Rat Anti-Mouse Mouse CCR2	R&D	Cat# FAB5538A RRID:AB_10645617
PE/Cyanine7 Rat Anti-Mouse CD64	BioLegend	Cat# 164413 RRID:AB_3083142
BV605 Rat Anti-Mouse Ly-6C	BD	Cat# 563011 RRID:AB_2737949
APC-Cy7 Rat Anti-Mouse Ly-6A/E	BD	Cat# 560654 RRID:AB_1727552
AF700 Rat Anti-Mouse CD34	BD	Cat# 560518 RRID:AB_1727471
PerCP-Cy5.5 Rat Anti-Mouse CD16/CD32	BD	Cat# 560540 RRID:AB_1645259
PE-Cy7 Rat anti-Mouse CD117	BD	Cat# 558163 RRID:AB_647250
V450 Rat Anti-Mouse Lineage Cocktail	BD	Cat# 561301 RRID:AB_10611731
Goat Anti-Rabbit IgG H&L Secondary Antibody, AF800	Thermo Fisher	Cat# A32735 RRID:AB_2633284
Goat Anti-Mouse IgG H&L Secondary Antibody, AF680	Thermo Fisher	Cat# A32788 RRID:AB_276283

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
FITC Conjugated Goat anti-Rabbit IgG Antibody	ServiceBio	Cat# GB22303 RRID:AB_2904189
Alexa Fluor® 488 Conjugated Goat anti-Mouse IgG Antibody	ServiceBio	Cat# GB25301 RRID:AB_2904018
Cyanine 3 Conjugated Donkey Anti-Goat IgG Antibody	ServiceBio	Cat# GB21404 RRID:AB_2868507
Cyanine 3 Conjugated Goat Anti-Rabbit IgG Antibody	ServiceBio	Cat# GB21303 RRID:AB_2861435

Bacterial and virus strains

rAAV-ChAT-HChR2[H134R]-GFP	BrainVTA	PT-2313
rAAV-ChAT-GFP	BrainVTA	PT-1722
rAAV-Efla-DIO-EYFP	BrainVTA	PT-0012
rAAV-EF1 α -DIO-hChR2(H134R)-EYFP-WPRE-hGH polyA	BrainVTA	PT-0001
AAV2/1-hSyn-FLEX-EGFP-WPRE-pA	Taitool Bioscience	S0373-1
Lv-Nfe2l2	GENECHEM Co. Ltd	SV0285243
Lv-Con	GENECHEM Co. Ltd	SV0280001

Chemicals, peptides, and recombinant proteins

WGA (FITC conjugate)	Sigma-Aldrich	Cat#L4895
Masson's trichrome	Solarbio	Cat#G1346
OCT	SAKURA	Cat#4583
Diphtheria toxin	Sigma-Aldrich	Cat#D0564
PUN-282987	Sigma-Aldrich	Cat#CAS123464-89-1
Methyllycaconitine citrate	Sigma-Aldrich	Cat# CAS112825-05-5
Recombinant mouse TNF- α	R&D	Cat#410-MT- 010
Recombinant mouse M-CSF	PeproTech	Cat#315-02
Si-m-Nfe2l2	RiBOBIO	Cat#siBDM1999A
Donepezil	Abmole	Cat#M8888
Encencline	Abmole	Cat#M7227

Critical commercial assays

Anti-mouse Acetylcholine ELISA kit	Bio-vision	Cat#E3353
Anti-human Acetylcholine ELISA kit	Bio-vision	Cat#E3352
Anti-human CXCL10 ELISA kit	Bio-vision	Cat#E4826
Anti-human CCL2 ELISA kit	Bio-vision	Cat#E4833
Anti-human Chemerin ELISA kit	Bio-vision	Cat#E5040
Anti-human TNF α ELISA kit	Bio-vision	Cat#DRT100
Neonatal Heart Dissociation Kit	MACS	Cat#130-098-373
iScript cDNA Synthesis kit	Bio-Rad	Cat#1708891
TUNEL	Promega	Cat#G3250

Experimental models: Organisms/strains

Mouse: Gt[ROSA]26Sortm4 [ACTB-tdTomato,-EGFP] ^{Luo/J}	The Jackson Laboratory	JAX: 007576
Mouse:LysMCre+ mice	The Jackson Laboratory	JAX: 004781
Mouse: Chrna7 ^{-/-}	The Jackson Laboratory	JAX: 003232
Neonatal C57bl6/J mice	Beijing Viewsolid Biotechnology Co., Ltd.	VSM10001
Ccr12 Cre+	Cyagen Biosciences, Inc	N/A
Rosa26-iDTR	Cyagen Biosciences, Inc	N/A
$\alpha 7$ nAChR ^{fl/fl} overexpression ($\alpha 7^{fl/fl}$ OE)	Cyagen Biosciences, Inc	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Si-m-Nfe2l2 (targeting sequence: 5'-tCTCCGCCTCTAAGTTCTt-3')	RiBOBIO	Cat#siBDM1999A
Primers used in quantitative real-time PCR analysis, see Table S5	This paper	N/A
Software and algorithms		
GraphPad Prism 9	Graphpad	https://www.graphpad.com/
R version 4.0.3	R Foundation for Statistical Computing	https://www.r-project.org/
ssGSEA	Barbie et al. ⁵²	https://bioconductor.org/packages/GSVA/
EBSeq R package	Leng et al. ⁵³	https://doi.org/10.1093/bioinformatics/btt087
SCENIC	Aibar et al. ⁵⁴	https://doi.org/10.1038/nmeth.4463
CytoTRACE v0.3.3	Aibar et al. ⁵⁴	https://cytotrace.stanford.edu
Monocle 2 v2.32.0	Gulati et al. ⁵⁵	http://cole-trapnellab.github.io/monocle-release
GENIE3	Open source software	https://bioconductor.org/packages/GENIE3/
Deposited data		
Microarray	This paper	Accession number: GEO: GSE295812
Microarray	This paper	Accession number: GEO: GSE295813
RNA sequencing	This paper	Accession number: GEO: GSE296034
RNA sequencing	This paper	Accession number: GEO: GSE296255

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study cohort

This was a cross-sectional study of participating patients with HF having 24 h Holter monitoring data and healthy controls (HCs) between March and December 2020 at the Beijing Anzhen Hospital. The study protocol was approved by the local Institutional Review Board of the Institute of Biophysics and Anzhen Hospital under the number GZR-3-011. All patients fulfilled the criteria for HF in accordance with the European Society of Cardiology Guidelines 2021.⁵⁶ They presented with classical symptoms (e.g., breathlessness, ankle swelling, and fatigue) and/or signs (e.g., elevated jugular venous pressure, pulmonary crackles, and peripheral edema) of HF, N-terminal pro-B-type natriuretic peptide (NT-proBNP) ≥ 125 pg/mL or BNP ≥ 35 pg/mL. Patients underwent thorough clinical examination, laboratory evaluation, and 24 h Holter monitoring. Participants with liver disease, lung disease, or lack of baseline data, samples, or informed consent were excluded. The heart rate variability (HRV) tests were performed under standardized conditions that included overnight fasting, avoidance of caffeine and tobacco products for 8 h before the test and withholding of prescriptions and over-the-counter medicines (except for basal insulin) until testing was completed. Patients with non-sinus rhythms, atrial fibrillation (persistent or intermittent), or pacemakers were excluded.⁵⁷

First, 40 patients with HF and 40 age- and sex-matched HCs were chosen for testing serum ACh. Second, thirty patients with normal vagus nerve activity (HF-NVNA) or low vagus nerve activity (HF-LVNA) were identified based on heart rate variability (HRV) tests. Age- and sex-matched propensity scores for 30 patients with normal vagal activity and 30 HCs were chosen. Four time-domain HRV parameters were calculated on a 24 h recording after excluding non-sinus cardiac cycles: 1) the standard deviation of all normal the time elapsed between two successive R-waves of the QRS signal (RR) intervals [SDNN in ms], and 2) the standard deviation of the averaged normal RR intervals for all 5 min segments (SDANN in ms), both of which reflect long-term HRV and hence sympathetic activity or sympathovagal balance⁵⁸; 3) the root mean square of the difference between the coupling intervals of adjacent R-R intervals (RMSSD in ms), and 4) the percentage of adjacent R-R intervals that varied by more than 50 ms (pNN50 in %), both of which reflect short-term beat-to-beat HRV and hence vagal activity.⁵⁹ Each HRV indices below the lower limit of reference value are recorded 1 point. The reference value of HRV indices is based on previously published research by Bauer et al.¹ Low vagal activity was defined as the score was > 2 . Normal vagal activity was defined as the score was ≤ 2 . Clinical characteristics of all participants are presented in [Tables S6](#) and [S7](#).

Mice

Mice expressing Cre recombinase under the control of the ChAT promoter (ChAT^{Cre+} mice) were kindly provided by the Luo laboratory⁶⁰ (National Institute of Biological Sciences, Beijing, China); mT/mG reporter mice (Gt[ROSA]26Sortm4 [ACTB-tdTomato, -EGFP]Luo/J; strain 007576; Jackson Labs) were kindly provided by Wei Kong (Peking University Health Science Center, Beijing, China); LysM^{Cre+} mice (strain 004781) and ChRNA7^{-/-} mice (stock #003232) were purchased from Jackson Laboratory. Transgenic mice expressing Cre under the control of the Ccr12 promoter (abbreviated as Ccr12^{Cre+}), Rosa26-iDTR mice and floxed mice of $\alpha 7$ nAChR overexpression (abbreviated as $\alpha 7^{fl/fl}$ OE) mice were generated by Cyagen Biosciences, Inc. (Suzhou, China). Ccr12 macrophages specific- $\alpha 7$ nAChR overexpression mice were generated using Cre-LoxP technology. $\alpha 7^{fl/fl}$ OE mice were established as follows: the Rosa26 locus is targeted with a DNA construct encoding CHRNA7, preceded by a STOP cassette flanked by loxP sites, cloned downstream of the Rosa26 promoter. Expression of $\alpha 7$ nAChR from the targeted Rosa26 locus occurs following the excision of the STOP cassette by Cre recombination of loxP sequences. To generate transgenic mice with overexpression of $\alpha 7$ nAChR in Ccr12⁺ cells (abbreviated as Ccr12-Cre⁺ $\alpha 7^{fl/fl}$ OE), $\alpha 7^{fl/fl}$ OE mice were bred with Ccr12^{Cre+} mice in a C57BL/6 genetic background.

Ccr12^{Cre+}iDTR mice were achieved by crossing Ccr12^{Cre+} mice with homozygous ROSA26-iDTR mice. For diphtheria toxin (DT, Sigma, D0564-1MG) administration, DT was dissolved in phosphate-buffered saline, and mice were injected intraperitoneally at 10 ng/g body weight. LysM^{Cre+} strain was crossed with mT/mG reporter mice to map the distribution of LysM-Cre-positive macrophages. We generated a conditional overexpression mouse model ($\alpha 7^{fl/fl}$ OE) in which a loxP-STOP-loxP-mus mus $\alpha 7$ nAChR construct expressing the exogenous mus mus $\alpha 7$ nAChR gene through the CAGGS promoter was inserted at the Rosa26 locus. Ccr12^{Cre+} $\alpha 7^{fl/fl}$ OE mice were achieved by crossing $\alpha 7^{fl/fl}$ OE with Ccr12^{Cre+} mice. Genotyping primers were shown in Table S5. The mice were maintained on a C57BL/6 background, fed standard rodent feed (12% calories from proteins, 12% from fats, and 66% from carbohydrates), and housed in a pathogen-free barrier facility with a 12 h light/dark cycle. Male mice, aged 10–12 weeks, were used in the experiments. Neonatal mice were purchased from ViewSolid (Beijing ViewSolid Biotechnology Co., Ltd., Beijing, China). All animal handling complied with the animal welfare regulations of the Capital Medical University. The animal study protocol was approved by the Animal Subjects Committee of the Capital Medical University.

METHOD DETAILS

Human sample collection

Left ventricular tissue was obtained from patients with HF undergoing heart transplantation or from normal heart donors. Blood was collected from HCs and patients with HF within 24 h of admission. Blood was collected in tubes and centrifuged (4,000 × g, 4 °C) for 15 min. All plasma samples underwent one freeze-thaw cycle, and 200 μ L aliquots were delivered into 0.5 mL Lobind tubes and stored at -80 °C. This study was conducted in accordance with the principles set out in the Declaration of Helsinki, revised in 2000 (Scotland 2000).

In vivo animal models

To establish mouse models of HF, mice were subjected to surgical transverse aortic constriction (TAC) for 8 weeks, as described previously.⁶¹ Briefly, 10–12 weeks old male mice were anesthetized by 1% Pentobarbital (Sigma, 1507002), the mouse was placed in the supine position, the skin was opened, and a 1.5–2 cm incision at the midline of the neck and chest was made. Next, the thymus covering the aortic arch was separated from it. 27-gauge curved needle was placed under the arch, the diameter of the aortic arch was narrowed to 0.5 mm. The sham operation only separated the aorta without ligation. Pulsed wave Doppler imaging was used for the non-invasive assessment of the pressure gradient across the aortic constriction.

Pharmacological treatment

Pressure-overloaded HF was induced by TAC in C57BL/6 mice, aged 8–10 weeks. After 4 weeks of TAC, the surviving mice were randomly divided into three groups (each of 15 mice), and the test drugs were administered orally in drinking water. The $\alpha 7$ nAChR agonist group was administered encenicline (EVP-6124) at a dosage of 2 mg/kg/day (Cat# M7227, AbMole, Texas, USA), and the acetylcholinesterase inhibitor group was administered donepezil at a dosage of 5 mg/kg/day (Cat# M8888, AbMole, Texas, USA). As EVP-6124 was tested at 1 mg/kg, which was found to be the most effective dose to enhance memory consolidation in rats.⁶² We converted the rat dose to mouse dose according to the following equation⁶³: Mouse equivalent dose = Rat dose (1 mg/kg) × Km ratio (6/2), the mouse equivalent dose is 2 mg/kg. The dosage of donepezil treatment was selected as previous report.⁶⁴ Control mice received an equal volume of physiological saline.

Cardiac function analysis by echocardiograph

At 8 weeks after sham operation or TAC, mice were anesthetized with isoflurane (2.5% induction and 1.5% maintenance) and placed on a heating pad providing constant surface heat between 35 °C and 38 °C. Cardiac function was evaluated using the Vevo 770 high-resolution microimaging system (FUJIFILM VisualSonics, Toronto, Canada). Echocardiography was performed by an experienced echocardiographer and completed within 10 min of anesthesia administration. The heart was viewed from the parasternal short-axis in 2D mode. Stored data were analyzed offline in a blinded fashion using the VevoLab software system (FUJIFILM VisualSonics). All measurements were performed over eight consecutive cardiac cycles. The left ventricular end-systolic volume (LV vol;s) and ejection fraction (EF%) were calculated.

Optogenetic stimulation of vagus neurons

Viral vectors

For optogenetic stimuli experiments, two different recombinant adeno-associated viral vectors (rAAV) were used. The first expressed the light-sensitive cation channel channelrhodopsin-2 (ChR2) under the choline acetyltransferase (ChAT) promoter (rAAV-ChAT-HChR2[H134R]-GFP). rAAV-Efla-DIO-hChR2(H134R)-EYFP expressed an enhanced version of ChR2 fused to the EYFP. In this vector, the ChR2-GFP sequences were flanked by double lox sites. rAAV-ChAT-GFP and rAAV-Efla-DIO-EYFP served as controls. All rAAVs were purchased from BrainVTA (Wuhan, China).

Injection of viral vectors into the dorsal vagal motor nucleus

Mice were anesthetized with pentobarbital (80 mg/kg, intraperitoneal [IP] injection) and placed on a brain stereotaxic device. The injection site dorsal vagal motor nucleus (DVMN) refers to the description in *Mouse Brain in Stereotaxic Coordinates*.⁶¹ Using a micro-syringe pump (nanoliter 200 Injector, WPI), rAAV vectors (2×10^{12} particles/mL) were slowly injected (30 nL/min) into the DVMN area (coordinates = anterior-posterior -6.6–-6.7 mm; Medial-Lateral 0.12 mm; Dorsal Ventral 4.5 mm) for a total volume of 150 nL.

An Cre-dependent AAV driving the expression of enhanced green fluorescent protein (EGFP) for the anterograde tracing of ChAT + neurons. In ChAT-Cre mice, 2×10^{10} particles of AAV2/1-hSyn-FLEX-EGFP-WPRE-pA was injected into the bilateral DMVN. The mice recovered in their home cage. After 2 weeks to allow for AAV expression, the mice were used for immunofluorescent examination. AAV were purchased from Taitool Bioscience (Shanghai, China).

Placement and evaluation of optical fibers

An optical fiber (Diameter: 0.125 mm, Length: 4 mm, numerical aperture: 0.39) (Inper, Hangzhou, China) was inserted above the rAAV injection site and secured to the skull with cyanoacrylate adhesive. Prior to implantation, the optical fibers were glued to a zirconia ferrule. Mice were treated with ibuprofen (5 mg/kg/day subcutaneously) for 3 days and allowed to recover for 1 week. After fibers implantation, magnetic resonance imaging (MRI) was performed using a 2.35T Bruker Avance DBX-100 system (Bruker Corporation, Billerica, MA, USA).⁶⁵ Excitation and signal reception were achieved using a Helmholtz coil (100 mm) and a surface coil (16 mm), respectively. As shown in [Figure S1J](#), sets of 3D gradient-echo images (spoiled FLASH, Repetition Time /Echo Time 50/1 ms, 20° flip angle) in the transverse plane (window level: 44, window width: 80, thickness: 1.0 mm) were obtained to assess the distance between the position of the DMVN and the implanted fiber.

Optogenetic activation of vagus neurons

The mice were implanted with an optical fiber. Photostimulation of vagus nerves (wild type [WT], or *Chrna7*^{-/-} mice with injections of rAAV-ChAT-HChR2[H134R]-GFP, ChAT^{Cre+} mice with injections of rAAV-ChAT-GFP or control stimulation (same strains with injections of rAAV-ChAT-GFP or AAV-Efla-DIO-EYFP, respectively) was performed while the mice were confined to a plethysmography chamber. The mice were placed in the chamber for 2 h twice for 1 week for habituation to the environment. After TAC, mice received 30 minutes of light stimulation at 8pm (zeitgeber time 12) every 24 hours, for up to 8 weeks. Briefly, the mice were short-term anesthetized, and the connection between the implanted fiber optics and the laser was established (Aurora 300, Newdoon Inc., Hangzhou, China). Blue light pulses (10Hz, 445nm) triggered precisely timed depolarization and action potential firing in DVMN neurons expressing ChR2.

Recording heart rate and the efferent activity of the vagus nerve

Heart rate and efferent activity of the vagus nerve were recorded as previously described.⁶⁶ Mice were anesthetized with isoflurane (2.5% induction and 1.5% maintenance). Heart rate was monitored using a cardio tachometer (model 1321; San-Ei, Tokyo, Japan) triggered by an electrocardiogram (ECG) signal recorded via subcutaneous electrodes implanted into the chest skin. For the measurement of the cervical vagus nerve, a right cervical vagus bundle was dissected carefully from the surrounding tissues under stereoscopic microscopy. The efferent activity was measured using a bipolar electrode made of Teflon-coated wire (Cooner Wire, Chatsworth, CA, USA) connected to the LabChart system (AD Instruments) at 10 Hz blue light and sampled at a rate of 5 kHz using a Power1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge, United Kingdom).

Heart rate variability analysis

To assess the impact of VNS on heart rate variability (HRV) in conscious mice, we surgically implanted ECG devices in the mouse cranium. In brief, this implantable device is composed of an 8-pin 'female' connector and three highly flexible insulated electrical wires. Three single lead electrodes are utilized for peripheral ECG measurements. Before implantation, the 8-pin 'female' connector was covered with latex to prevent direct contact with the skin and subcutaneous tissue during the procedure. adult male mice were anesthetized with 2% isoflurane. The right front chest of the mice was surgically sutured with one electrode, while the other two electrodes were sutured to the muscular areas on each side of the thigh. To alleviate potential post-operative discomfort, Motrin (ibuprofen 7 mL/L) was administered via drinking water for a duration of 4 days following implantation. After a recovery period of 10 days, the mice underwent optogenetics and VNS procedures. The ECG signals were acquired using a receiver (Amplifier 1700, A-M systems, Carlsborg, WA). Subsequently, the signals underwent bandpass filtering (1 Hz–1 KHz) and were digitized at a sampling rate of 2 KHz using an A/D converter (PCI-6024E, National Instruments, Austin, TX). For HRV analysis, ECG recordings were exported to the Kubios HRV software (University of Eastern Finland).⁶⁷

Preparation of single-cell suspensions

Mice were received rAAV-ChAT-hChR2[H134R]-GFP (vagal nerve stimulation [VNS] group) or rAAV-ChAT-GFP (control [Ctrl] group), followed by TAC. Left ventricle tissues of three hearts per group mixed and diced into 1 mm³ fragments and transferred to enzyme digestion solution (Neonatal Heart Dissociation Kit, MACS, Bergisch Gladbach, Germany). Tissue pieces were incubated at 37 °C and 5% CO₂ for 10–15 min, after shaking on a gentleMACS™ Dissociator for 37 s for two cycles. The digested mixture was filtered using an Easy Strain 100 μm cell strainer into a 50 mL Falcon tube. Complete medium (Dulbecco's Modified Eagle Medium [DMEM] with 10% fetal bovine serum [FBS]) was poured through the filter into the Falcon tube up to 40 mL, then centrifuged at 200 × g for 10 min at 4 °C. The supernatant was decanted, and 1 mL of PBS (phosphate buffered saline) with 0.04% bovine serum albumin (BSA) was added to gently resuspend the centrifugate. When the cell viability exceeded 85% as assessed with trypan blue assay (Thermo Scientific, Massachusetts, USA), single cells were prepared for sequencing.

Single-cell RNA-sequencing

Libraries from isolated single cells were generated using a chromium controller (10x Genomics, California, USA) with the following modifications. Libraries were tagged using the Nextera XT Library Prep kit (Illumina, California, USA) with custom barcode adapters (sequences available upon request). Libraries from cells with unique barcodes were combined and sequenced with paired-end, 38-base reads using a NextSeq 500 sequencer (Illumina). A single-cell suspension of the pool was generated in 0.04% PBS-BSA, and approximately 20,000 single cells were loaded to the chromium controller, with a targeted recovery of 10,000 cells. Single cells and single gel beads containing barcoded oligonucleotides were encapsulated into nanoliter-sized droplets and subjected to reverse transcription. Droplets were broken, and the barcoded cDNAs were purified using Dynabeads and amplified by PCR. Thirty gene expression and TotalSeq antibody-derived barcode cDNAs were size-selected and separated with SPRIselect Reagent (Beckman Coulter, California, USA) and then fragmented, end-repaired, and ligated with index adapters. Quality control of the resulting libraries was performed using the Bioanalyzer High Sensitivity DNA Analysis (Agilent 2100 High Sensitivity DNA Assay Kit, Agilent Technologies, California, USA). The constructed gene expression library was sequenced using the Illumina HiSeq X Ten platform (Annoroad Gene Tech. Co., Ltd., Beijing, China).

scRNA-seq analysis

For the analysis of immune cells only, immune cells were computationally isolated from the cell subtypes based on the expression of CD45. The variable genes were obtained for two samples using FindVariableFeatures. For clustering, CD45⁺ immune cell integration was performed following the Seurat vignette to integrate all genes common among samples, using the functions FindIntegrationAnchors and IntegrateData. These 'integrated' batch-corrected values were then set as the default assay, and gene expression values were scaled before running principal component analysis (PCA). Uniform manifold approximation and projection (UMAP), based on PCA cell embeddings, was generated from the integrated counts batch normalized by Seurat. The first 10 principal components (PCs) were then visualized using RunUMAP, and clustering at a chosen resolution of 0.5 was conducted using FindNeighbors.

To identify cluster markers and variable genes between conditions, the Seurat function FindAllMarkers was used with the 'test.use' function. As recommended in the best practice of Seurat (<https://www.github.com/theislab/single-cell-tutorial>), non-batch normalized counts were used for differentially expressed comparison. Genes were identified as marker genes when they had a log-transformed fold change (FC) > 0.5, P < 0.05, and expression in at least 60% of cells. Default Seurat thresholds of log-transformed FC > 0.25 (absolute value), adjusted P-value (Bonferroni correction) < 0.05 and expression in greater than 10% of cells were required to consider a gene differentially expressed genes (DEGs). The pheatmap package was used to create heatmaps. To assess the statistical significance of TNFα-responsive genes, a TNFα-responsive score was calculated for each cell based on a given list of TNFα-responsive genes.⁶⁸ For each cluster in the VNS-TAC group, the distribution of the calculated scores was compared with that of cells collected from Ctrl-TAC, using Wilcoxon's signed-rank test. To assess the transcriptional regulatory characteristics of macrophage subsets, a gene regulatory network analysis was performed using SCENIC.⁵⁴ Instead of interrogating predefined regulons, individual regulons were constructed from macrophage subset data. The first transcription factor (TF)-gene co-expression modules were defined in a data-driven manner using GENIE3 (<https://bioconductor.org/packages/GENIE3/>). Subsequently, these modules were refined using RcisTarget by only retaining the genes that contained the respective TF binding motif. Once the regulons were constructed, AUCell was used to score individual cells by assessing each TF separately for the enrichment of target genes in the top quantile of the cell signature.

Trajectory analysis with CytoTRACE and Monocle2

Differentiation state and pseudotime trajectories of macrophages subpopulations were comprehensively inferred using CytoTRACE v0.3.3 (<https://cytotrace.stanford.edu>, accessed on 17 August 2024) and Monocle 2 v2.32.0 (<http://cole-trapnellab.github.io/monocle-release>, accessed on 17 August 2024). CytoTRACE can re-establish the relative differentiation status of cellular subpopulations based on gene expression profiles¹¹ and assess the immature of different cellular subpopulations. Monocle 2 identified cellular alterations during cellular differentiation as a means of inferring the developmental trajectory of the cellular subpopulation.⁵⁵

Microarray of sorted or co-cultured cells

Lyve1⁺, CD74⁺, Ccr12⁺, and Trem2⁺ macrophages were sorted from the six mixed hearts of WT mice at 1 week after TAC using Cell Sorter (FACSaria III; Becton Dickinson, New Jersey, USA). Total cell RNA was extracted with TRIzol. The microarray assay was performed as described previously.⁶⁹ Briefly, total RNA was reverse transcribed, amplified with Whole Transcript Pico Reagent Kit (Affymetrix, Thermo Fisher Scientific, California, USA), labeled, and hybridized with Affymetrix Clariom S Array (mouse) according to manufacturer instructions. Hybridized GeneChips were scanned using the Affymetrix GeneChip Command Console installed in the GeneChip Scanner 3000 7G. Robust Multichip Average (RMA) algorithms were employed for background correction, normalization, and summary of the AffyBatch probe-level data to obtain gene expression data. Microarray data sets were analyzed using the Agilent Genespring GS 11 software. The values were presented as log₂ RMA signal intensities. DEGs between Ccr12⁺ and CD74⁺, Lyve1⁺, or Trem2⁺ were filtered based on an FC ≥ 2.

Ccr12⁺ and Ccr12[−] macrophages were sorted from the heart tissue and then co-cultured with cardiac fibroblasts (CFs) or cardiomyocytes (CMs). After co-culture, CFs and CMs were prepared for microarray analysis. DEGs between CF co-culture with Ccr12⁺Mφ (CF-Ccr12⁺Mφ) and CF co-culture with Ccr12[−]Mφ (CF-Ccr12[−]Mφ) or CM co-culture with Ccr12⁺Mφ (CM-Ccr12⁺Mφ) and CM co-culture with Ccr12[−]Mφ (CM-Ccr12[−]Mφ) were filtered with an FC ≥ 2.

Bulk RNA sequencing

Heart tissue from mice (including Ctrl-TAC, VNS-TAC, Saline-TAC, donepezil-TAC, and EVP-6124-TAC) was collected for RNA extraction at 8 weeks post-TAC. Library construction was performed using the Illumina TruSeq RNA Library Prep Kit according to manufacturer protocol. After the final library quality control, the library preparations were sequenced on an Illumina Novaseq platform, and 150 bp paired-end reads were generated. After adaptor removal and quality control filtering, the clean reads were mapped to the mouse genome (Mm9) using Hisat2 v2.0.5 and featureCounts v1.5.0-p3.

Bulk RNA sequencing analysis

Raw RNA-sequencing results were quantified and summarized to genes based on a mapping file retrieved from the Ensembl Biomart website,⁷⁰ by filtering only for protein-coding transcripts and genes. The negative binomial test was used in the EBSeq R package to filter DEGs.⁵³ The empirical Bayesian method was used to obtain *P*-values, and the Benjamini-Hochberg procedure was used for multiple test corrections. Significant DEGs of each pairwise comparison were selected (adjusted *P* < 0.05, FC ≥ 1.5).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to determine the significant functions and pathways of DEGs. Only pathway categories with a false discovery rate (FDR)-corrected *P*-value of less than 0.05, were considered significantly enriched. Trend genes were defined as increasing or decreasing trends in gene expression in the three groups (saline-TAC, donepezil-TAC, EVP-6124-TAC) (*P* < 0.05). Single-sample gene set enrichment analysis (ssGSEA) was conducted to uncover the differentially enriched signaling pathways between saline-TAC and donepezil-TAC or EVP-6124-TAC. The enrichment score of each sample from different gene sets were calculated with the ssGSEA method and the GSVA package (<https://bioconductor.org/packages/GSVA/>). Significantly differentially enriched gene sets between the two groups were identified using the limma package.

Immunofluorescence staining and analysis

Mice were euthanized by anesthetic overdose and perfused with ice-cold PBS containing 1 U/mL heparin. Mouse brain tissues were fixed in 4% paraformaldehyde (PFA) 12 h at 4 °C, rinsed in PBS, and incubated overnight at 4 °C in 30% sucrose before freezing in optimal cutting temperature (OCT) compound. Serial 10 μm coronal floating sections were blocked with 3% BSA and 0.25% Triton X-100 in PBS, then stained with primary antibodies overnight at 4 °C, followed by staining with the corresponding secondary antibodies for 1 h at 21–22 °C. Stained hearts and brains were fixed by formalin perfusion, embedded in paraffin, and sliced into 5-μm sections. The sections were then incubated overnight with primary antibodies at 4 °C, followed by treatment with a secondary antibody. Sections were mounted with 4',6-diamidino-2-phenylindole (DAPI). CFs and CMs were prepared for staining in Penetrable cell culture cell (Netwell™, Corning, New York, USA). Cells were incubated with primary antibodies for 12 h at 4 °C and treated with a secondary antibody. Cell slides were mounted with DAPI. Images were captured using a Nikon Eclipse TE2000-S microscope (Nikon, Tokyo, Japan) and a Leica ST5 laser scanning confocal microscope. The list of primary antibodies and secondary antibodies are shown in [key resources table](#).

To quantify the average distance from Lyve1⁺Mφ, Trem2⁺Mφ, Ccr12⁺Mφ, and CD9⁺Mφ to ChAT across each image, the PANNORAMIC panoramic slice scanner was utilized to position the tissue sections onto the machine, and subsequently, the sections were incrementally advanced beneath the lens of the scanner. To visualize the proximity of macrophages to ChAT-expressing nerves, images from TAC-8 weeks hearts were collected using a ×10 objective with 0.7 zoom through an approximately 40 μm Z stack. To quantify the proximity of these cells, the images were analysed using Halo v3.0311.314. The images were first flattened to a maximum Z projection and subjected to deconvolution and background subtraction. In Halo, Lyve1⁺Mφ, Trem2⁺Mφ, Ccr12⁺Mφ, and CD9⁺Mφ and ChAT⁺ cells were separately defined as surfaces. Then, the “average distance” function in Imaris was applied to quantify the average distance from macrophages to ChAT⁺ cells across each image. This process was conducted in six total regions of interest across the hearts from post-TAC mice.

Other pathological staining

After eight weeks of sham operation or TAC, the mice were anesthetized with pentobarbital sodium (80mg/kg, i.p.) and perfused transcardially with 25 ml of saline. The brain tissues were fixed and embedded. Coronal sections from cryofixed tissue were collected and then stained with anti-c-Fos. Hearts were fixed in 10% formalin, processed, and paraffinized. For wheat germ agglutinin (WGA) staining, the sections were fixed by heat-induced epitope retrieval using a Microwave oven for 10 min at 100 °C, rinsed in PBS, and incubated with WGA conjugated with Alexa Fluor 488 (Sigma) (10 µg/mL) for 60 min. For fibrosis determination, heart sections were stained with Masson's (Solarbio) trichrome stain according to the manufacturer's protocols. For Ccr12 immunohistochemical staining, sections were incubated with primary antibody at 4 °C overnight. The signals were detected using a biotinylated corresponding secondary antibody in combination with Diaminobenzidine (DAB) Substrate (Thermo Fisher Scientific). For apoptosis determination, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Solarbio) staining was performed according to manufacturer protocol. High-resolution images of the entire and partial heart areas were captured using a Nikon Eclipse 80i upright microscope (Nikon). To assess the percentage of cardiac fibrosis, hypertrophy, Ccr12 levels, and apoptosis, areas were measured in 4–5 visual fields for similar positions in each section.

Isolation of bone-marrow-derived macrophages

Bone marrow-derived macrophages were extracted and cultured as previously described.⁷¹ Briefly, macrophages were isolated from the femurs and tibias of adult mice. Macrophages were then cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (supplemented with 10% FBS and 1% penicillin and streptomycin) supplemented with murine macrophage colony-stimulating factor (M-CSF) (50 ng/mL) at 37 °C for 5 days.

Co-culture experiments

CMs and CFs were isolated from neonatal mice. Briefly, the left ventricles were excised, washed three times, cut into small pieces within PBS, and digested with the Neonatal Heart Dissociation Kit according to manufacturer instructions. The mixture was then filtered and centrifuged at 200 × g. After culturing the cells for 2 h at 37 °C and 5 % CO₂, the unadherent cells in supernatant were removed to new culture dish, and were cultured as primary CMs with cardiac myocyte medium with 10% FBS and 100 mM BrdU (Bromodeoxyuridine). Adherent cells were further cultured as primary CFs in DMEM with 10% FBS. After 48 h, the medium was replaced with a serum-free maintenance medium for the co-culture experiments.

For the co-culture experiment, CMs or CFs (5 × 10⁵) were seeded in lower chambers, sorted macrophages (1 × 10⁵) were seeded in the upper chambers. The upper chamber was plugged into the lower chamber for 48 h as the following groups: CF-Ccr12⁺Mφ, CF-Ccr12⁺Mφ, CM-Ccr12⁺Mφ, CM-Ccr12⁺Mφ. After experiments, the lower chamber was washed with PBS for microarray analysis or IF staining.

Lentiviral vectors or siRNA transfection and pharmacological stimulation

The lentiviral vector encoding the mouse *Nfe2l2* gene (Lv-Nfe2l2) and its negative control (Lv-con) were constructed, packaged, purified, and titrated at GENECHM Co. Ltd (Shanghai, China). For transfections, Lv-Nfe2l2 or Lv-con was transfected into macrophages at a final concentration of 60 PFU/cell for 72 h. The overexpression efficiency of LV-Nfe2l2 was evaluated by western blotting.

Knockdown of nuclear factor erythroid 2-related factor 2 (Nfe2l2, also known as NRF2) was performed by small interfering RNA (siRNA) transfection. Macrophages were seeded at a density of 1.0 million/well in a six-well dish for 24 h to approximately 80% confluence, then washed with PBS. A serum-free medium was then added. Transient transfection of siRNA (100 nM) was performed using the Lipofectamine[™] RNAiMAX reagent according to manufacturer instructions. Macrophages were incubated with siRNA negative control, siRNA Nfe2l2 (targeting sequence: 5'-tCTCCGCCTCTAAGTTCt-3') (RiboBio). After 24 h, macrophages were treated with TNF-α (50 ng/mL) and PUN-282987 (50 µM) or methyllycaconitine (MLA) (50 µM) (Sigma-Aldrich) for 24 h. The efficiency of siRNA knockdown was evaluated by immunoblotting (Figure S6C).

Immunoblotting

Western blotting was performed as described previously.⁷² Briefly, total proteins from heart tissues or macrophages were homogenized, and lysed with Radio-immunoprecipitation Assay (RIPA) Cell Lysis Buffer (10X) (Thermo Scientific) supplemented with protease inhibitor (Thermo Scientific). Protein extracts (40–80 µg) were electrophoresed at 80 V on 10% or 12% SDS-PAGE, and then transferred to a nitrocellulose filter membrane (PALL BioTrace, Port Washington, NY, USA). The membranes were blocked and incubated overnight with anti-NRF2, anti-CEBPβ, anti-c-Fos, anti-c-Rel, GAPDH (TA-08), or β-Actin at 4 °C overnight, and then incubated with infrared dye 800-conjugated secondary antibodies (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) for 1 h at 21–22 °C. A Bio-Rad Imager (Bio-Rad Laboratories, California, USA) was used to detect protein expression. Bands were quantified using Image Lab Software (Bio-Rad) and normalized to GAPDH or β-Actin.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using TRIzol reagent and Direct-zol RNA MicroPrep columns (Zymo Research). RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad), and quantitative real-time PCR analysis was performed using SYBR master mix

(Thermo Fisher Scientific) and analyzed using a CFX-96 (Bio-Rad) system according to the manufacturer's instructions. Fold change in mRNA expression were calculated using the comparative cycle method ($2^{-\Delta C_t}$) normalized to the housekeeping gene *Gapdh*.⁵⁴ The list of primer sequences for mRNA is presented (Table S5).

Flow cytometry and fluorescence-activated cell sorting

After TAC surgery, cardiac cells were prepared as previously described.¹³ The prepared cell suspensions were stained in PBS containing 2% FBS with the antibodies at 4°C for 30 min. Antibodies are shown in table template. Flow cytometry was conducted using a BD LSRFortessa cytometer (Becton Dickinson) according to manufacturer protocol. Macrophages were sorted from cardiac cells suspension by BD FACS Aria III cytometer (Becton Dickinson) into four subpopulations: Lyve1⁺, CD74⁺, Ccr12⁺, and Trem2⁺ macrophages. The sorted macrophages were used for RNA extraction, microarray analysis, or co-culture with other cells.

Enzyme-linked immunosorbent assay

Heart tissue and serum acetylcholine (ACh) concentrations were measured using enzyme-linked immunosorbent assay (ELISA) with specific anti-mouse (Bio-vision) or anti-human (Bio-vision) ELISA kits. Serum cytokine concentrations were determined using ELISA with specific anti-human ELISA kits (R&D), the list of ELISA kits is shown in [key resources table](#). Assays were performed according to manufacturer protocols and read at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

Genetic analysis

Acetylcholine receptors family includes CHRM1-5, CHRNA1-7, CHRNA9, CHRNA10, and CHRNA2-4. We checked the expression level of each family member in inflammatory related tissues including spleen and whole blood in GTEx (V8) (<https://www.gtexportal.org/>), including genotype data from 714 donors and 11,688 RNA-seq samples. Expression quantitative trait locus (eQTL) ($P < 0.001$) of target genes in spleen and/or whole blood were selected, and their associations with HF were evaluated in the published statistical statistics of HF GWAS (HERMES: <http://kp4cd.org/datasets/mi>) including over 140,000 HF cases. The genes with eQTLs associated with HF ($P < 0.05$) were considered to play a role in inflammatory-mediated HF process. LocusZoom plots of the eQTL and GWAS signals at the target loci for target genes were constructed in R using the ggplot2 package (version 3.3.2).

QUANTIFICATION AND STATISTICAL ANALYSIS

For analysis of mouse and cell experiments, data are presented as mean $\pm$ SEM. Statistical outliers were identified using the ROUT method (threshold of $Q = 0.01$). Shapiro-Wilk test (threshold of $p=0.05$) was used to evaluate the normality of data distribution. For normally distributed data and two groups, unpaired Student's t-test was used. For >2 groups, one-way ANOVA with Tukey's multiple comparisons test was performed to determine differences. For experiments set up with >1 variable, 2-way ANOVA with Tukey's multiple comparisons test was used. For not normally distributed data or sample size <6 , Kruskal-Wallis test with Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli multiple comparisons test was performed. Multiplicity corrected p values are reported where multiple comparisons are tested.

For human studies, continuous data were described as mean $\pm$ SD if normally distributed, or as median (interquartile range) if non-normally distributed. Categorical variables were described as counts (percentages). Student's t-test and Mann-Whitney U test was used to compare the distribution of normally and non-normally distributed continuous variables, respectively, and the χ^2 test was used for categorical variables. Fisher's exact test was used whenever the count for a particular test was less than 5. Spearman or Pearson correlation was used to examine the association between two groups of variables. In Pearson correlation analysis between gene expression profiles and treatment in mice, administration of drugs was considered as an ordinal variable (Rankings: saline as 1, donepezil as 2, EVP-1641 as 3).

Statistical analyses were conducted using R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 9. Statistical significance was set at $P < 0.05$.