

FIGURE 2

The reduced expression of LEC-S1pr1 deteriorates cardiac function after acute myocardial infarction. (A–D) Representative images of echocardiography of post-MI hearts in WT and *Lyve1-Cre-S1pr1^{flox/wt}* mice (A) with quantification of left ventricle ejection fraction (LVEF%), left ventricle fractional shortening (LVFS%), and LV mass (mg) (B,D) (*n* = 3). (E) The ratio of heart weight to bodyweight of the indicated groups (HW/BW, mg/g) (*n* = 3). Data are mean ± S.E.M. n.s., no statistical significance.

LECs in 7 days post-MI hearts (Supplementary Figure 3). These data demonstrated that the reduced LEC-S1pr1 expression worsened post-MI pathological cardiac remodeling without any

significant influences on the lymphatic vessel formation in post-MI hearts.

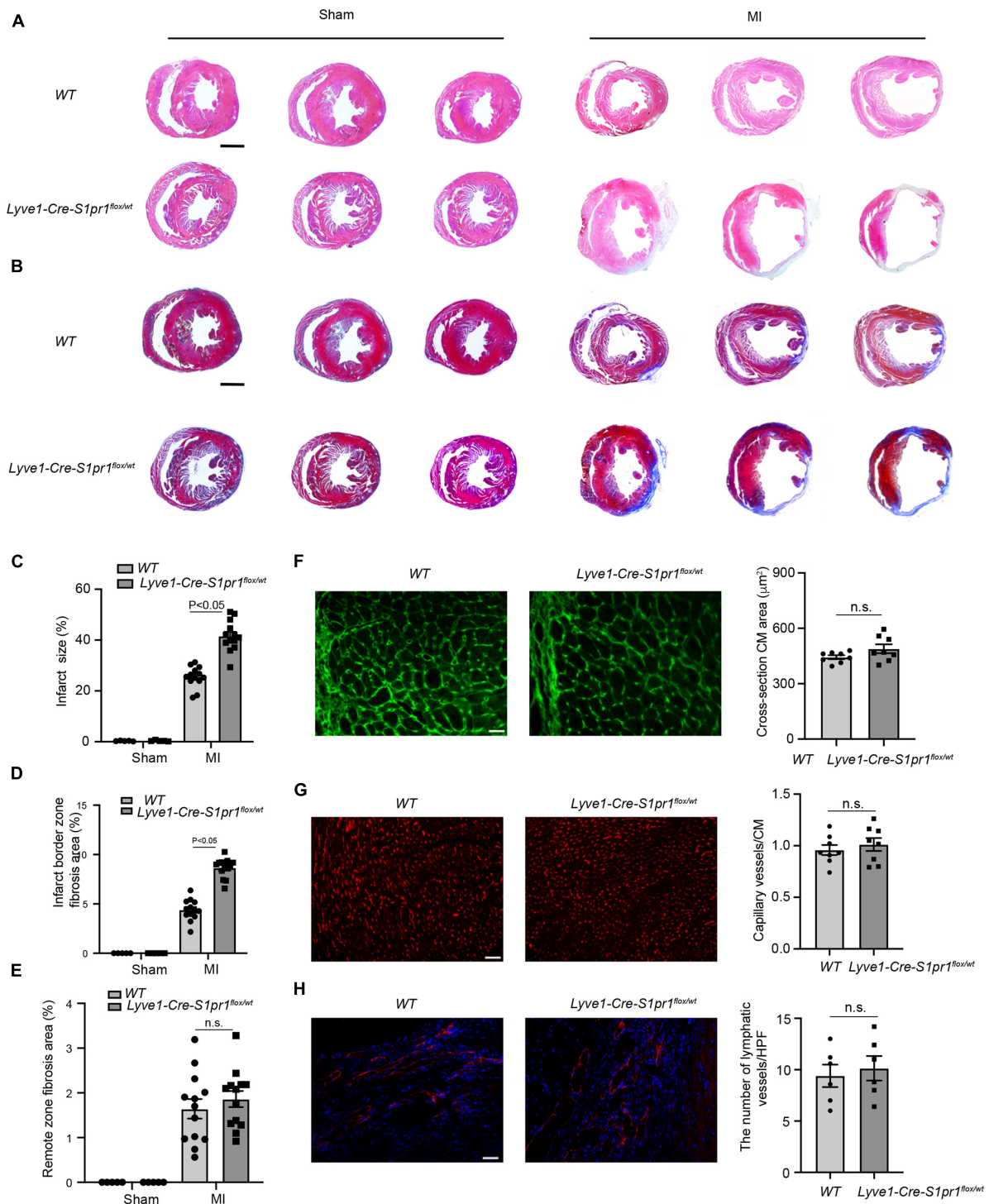


FIGURE 3

The reduced expression of LEC-S1pr1 aggravates post-MI pathological cardiac remodeling. (A) Representative images of H&E staining of serial sections from hearts in WT and Lyve1-Cre-S1pr1^{flox/wt} mice at 28 days after MI (n = 13). (B–E) Representative images of Masson's Trichrome staining of serial sections from hearts in WT and Lyve1-Cre-S1pr1^{flox/wt} mice at 28 days after MI (B), with quantification of the percentage of infarct size and cardiac fibrosis in left ventricle myocardium (C–E) (n = 13). (F) Representative images of WGA staining of hearts in WT and Lyve1-Cre-S1pr1^{flox/wt} mice at 28 days after MI, with quantification of the cross-section cardiomyocyte area in hearts (n = 8). (G) Representative images of isolectin-B4 staining of hearts in WT and Lyve1-Cre-S1pr1^{flox/wt} mice at 28 days after MI, with quantification of capillary density in post-MI myocardium (n = 8). (H) Representative images of Lyve-1 (LEC marker) staining of hearts in WT and Lyve1-Cre-S1pr1^{flox/wt} mice at 28 days after MI, with quantification of capillary lymphatic density in post-MI myocardium (n = 6). Data are mean ± S.E.M. Scale Bars: (A,B), 2 mm; (F–H), 50 µm; n.s., no statistical significance.

Sphingosine 1-phosphate receptor 1 regulates lymphatic endothelial cell migration, proliferation, and angiogenic activity

To better understand the effects of S1pr1 in LECs, cell migration and cell proliferation were performed in human lymphatic endothelial cells (HLECs). Lentivirus carrying S1PR1 (S1PR1 OE) or S1PR1 shRNA was generated to achieve elevated or reduced expression of S1PR1 in HLECs, respectively. Overexpression of S1PR1 in HLECs markedly increased cell migration and cell proliferation as shown by the transwell chemotactic assay and scratch wound healing assay and MTT assay, respectively, and the reduced S1PR1 expression significantly inhibited LEC migration and proliferation (Figures 4A–D), demonstrating that S1pr1 enhances lymphatic endothelial cell migration and proliferation. We next analyzed the effect of S1PR1 on *in vitro* angiogenic activity of HLECs using tube formation assay. The higher angiogenic tube formation was observed in HLEC-expressing S1PR1 shRNA (Figure 4E), suggesting that S1PR1 might restrict lymphatic endothelial cell tube formation. This result is consistent with Geng X et al.'s report in which LEC-S1pr1 has been shown to prevent sprouting from quiescent lymphatic vessels (19). Our *in vitro* data suggested that the inhibitory effect of S1pr1 on LEC tube formation and the enhancing effect of S1pr1 on LEC migration/proliferation might counteract each other's effect on lymphatic vascular angiogenesis, which might explain that no difference in lymphangiogenesis in post-MI myocardium between *WT* and *Lyve1-Cre-S1pr1^{flox/wt}* mice. This suggests that the effect of LEC-S1pr1 on post-MI cardiac remodeling was mediated by non-lymphangiogenic mechanisms.

The reduced expression of LEC-S1pr1 retards macrophage clearance in injured hearts

It has been shown that lymphatic vessels regulated the clearance of the infiltrated leukocytes in post-MI myocardium, and therefore influenced cardiac acute inflammatory responses and heart injury after MI (13). We first compared the number of infiltrating leukocytes in post-MI hearts between *Lyve1-Cre-S1pr1^{flox/wt}* mice and *WT* littermates. Although no difference was observed in the number of neutrophils (Figure 5A) in infarct zone of myocardium at 1 day after MI in *Lyve1-Cre-S1pr1^{flox/wt}* mice compared with *WT* littermates, a significant increase was detected in the number of macrophages in the infarct zone of hearts at 7 days after MI in *Lyve1-Cre-S1pr1^{flox/wt}* mice (Figure 5B). To further investigate whether *Lyve1-Cre-S1pr1^{flox/wt}* mice influenced cardiac resident

macrophage after myocardial infarction, we performed co-immunostaining of CCR2 and F4/80 in post-MI hearts. Our results showed that there was no difference in the number of cardiac resident macrophages (CCR2⁺F4/80⁺) in the peri-infarct zone between *Lyve1-Cre-S1pr1^{flox/wt}* mice and *WT* littermates (Supplementary Figure 4A), suggesting that cardiac resident macrophages may not be influenced by *Lyve1-Cre-S1pr1^{flox/wt}* mice. In the infarct zone, there were minimal resident macrophages in infarct zone 3 days post-MI in both *Lyve1-Cre-S1pr1^{flox/wt}* mice and *WT* littermates (Supplementary Figure 4B). These results were well consistent with previous investigations which reported that resident cardiac macrophages were lost within infarct myocardium and that resident cardiac macrophages accounted only 2–5% of the total cardiac macrophages within the infarct zone during the first a few weeks post-infarct (23). To test whether LEC-S1pr1 influences the infiltration of macrophages and/or the clearance of macrophages in the infarct zone of post-MI hearts, we first examined whether circulating monocyte subpopulation was altered in *Lyve1-Cre-S1pr1^{flox/wt}* mice. Our cytometric analysis showed that *Lyve1-Cre-S1pr1^{flox/wt}* mice displayed similar monocyte subpopulations including CD11b⁺Ly6c^{high} and CD11b⁺Ly6c^{ow} as *WT* mice (Supplementary Figure 5) indicating that circulating monocyte/macrophage profile might not be influenced by heterozygous deletion of S1pr1 in *Lyve1-Cre*-expressing cells. We next enumerated infiltrated macrophages in myocardium at 3 days after MI, since those infiltrated macrophages reach a peak in the infarct zone of injured myocardium at 3 days after MI and that macrophage clearance is not evident until 7 days after MI following the establishment of an extensive lymphatic network draining the infarcted area (13). Our data showed that the number of macrophages in the infarct zone of *Lyve1-Cre-S1pr1^{flox/wt}* mice was as similar as *WT* mice at 3 days after MI, compared with *WT* mice (Figure 5C). These results suggested that a reduced expression of LEC-S1pr1 didn't alter the infiltration of macrophages in an early inflammatory phase of MI. We next investigated whether LEC-S1pr1 might affect macrophage clearance in the later phase of MI. It has been shown that MLN as the secondary lymphatic organ serving the heart and that infiltrating macrophages in myocardium were transported via afferent cardiac lymphatics into draining mediastinal lymph nodes (MLNs). To investigate cardiac immune cell trafficking to MLNs, we employed adoptive cell transfer using ubiquitous EGFP expressing transgenic mice. Specifically, splenic EGFP⁺ monocytes were isolated and transferred to a recipient *Lyve1-Cre-S1pr1^{flox/wt}* mice or *WT* littermates, via intramyocardial delivery, at the time of coronary artery ligation (Figure 5D). Less EGFP⁺ cells were detected in draining MLNs of *Lyve1-Cre-S1pr1^{flox/wt}* mice compared with *WT* mice, while more EGFP⁺ cells in hearts of *Lyve1-Cre-S1pr1^{flox/wt}* mice on day 7 following MI (Figures 5E,F). These data demonstrated that the reduced

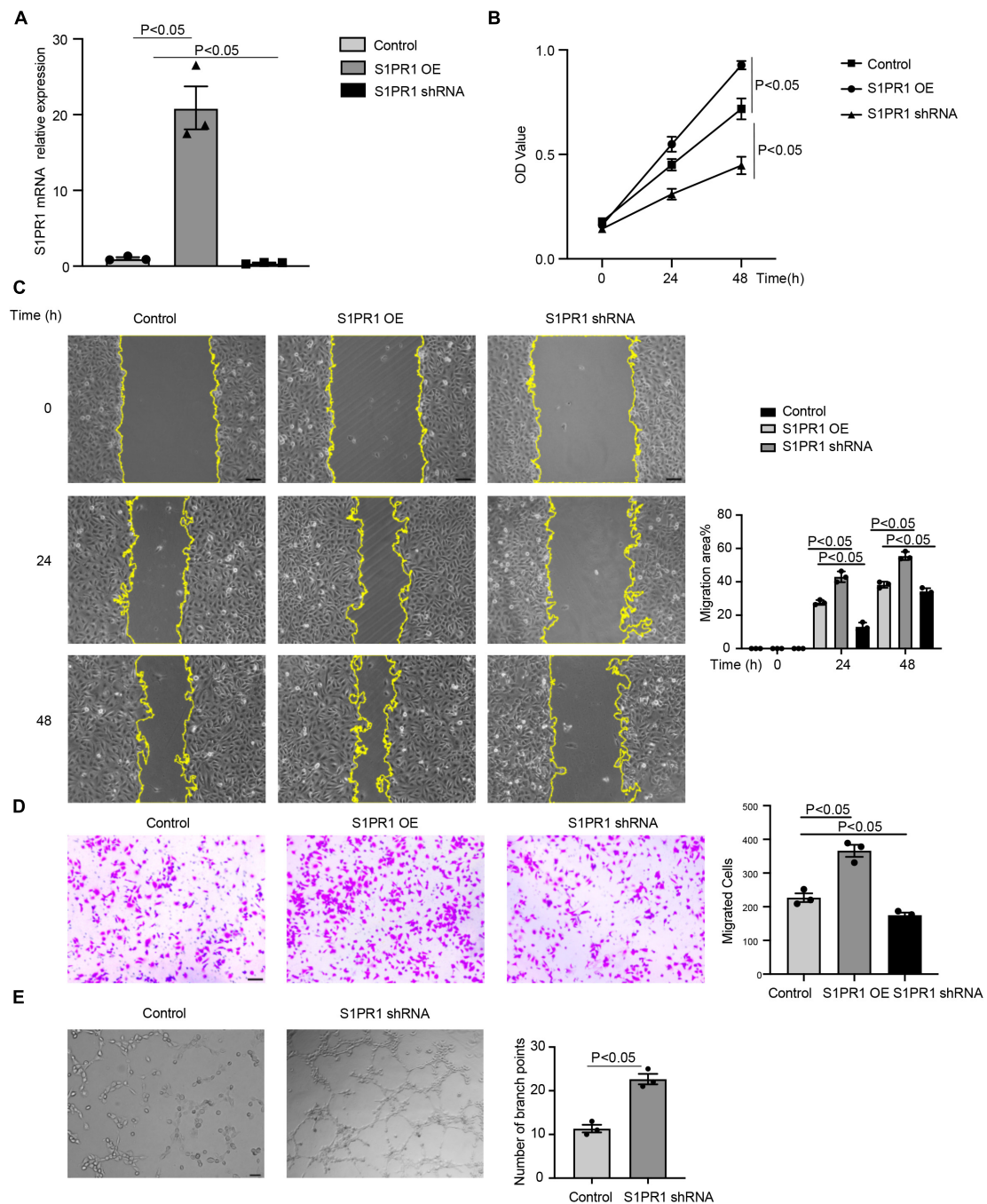


FIGURE 4

S1pr1 regulates lymphatic endothelial cell migration, proliferation, and angiogenic activity. (A) RT-qPCR analysis of S1PR1 mRNA levels in LECs of the indicated group ($n = 3$). (B) MTT assay showed the proliferation of cells of the indicated group ($n = 3$). (C) Scratch wound healing assay showed the cell migration, with quantification of cell migration area (%) of the indicated group ($n = 3$). (D) Boyden chamber assay showed the cell migration of cells of the indicated group, with quantification of migrated cells of the indicated group ($n = 3$). (E) Tube formation assay showed S1PR1 shRNA promoted LEC tube formation, with quantification of the number of branch points ($n = 3$). Control, scramble RNA lentivirus. OE, S1PR1-overexpressing lentivirus. shRNA, S1PR1 shRNA lentivirus. Data are mean \pm S.E.M. Scale Bars: (C–E), 100 μ m.

expression of LEC-S1pr1 didn't influence the infiltration of macrophages in an early inflammatory phase of MI, but significantly affected macrophage clearance in the later phase

of MI via afferent cardiac lymphatics, and thus aggravated post-MI cardiac acute inflammatory responses and worsened pathological cardiac remodeling.

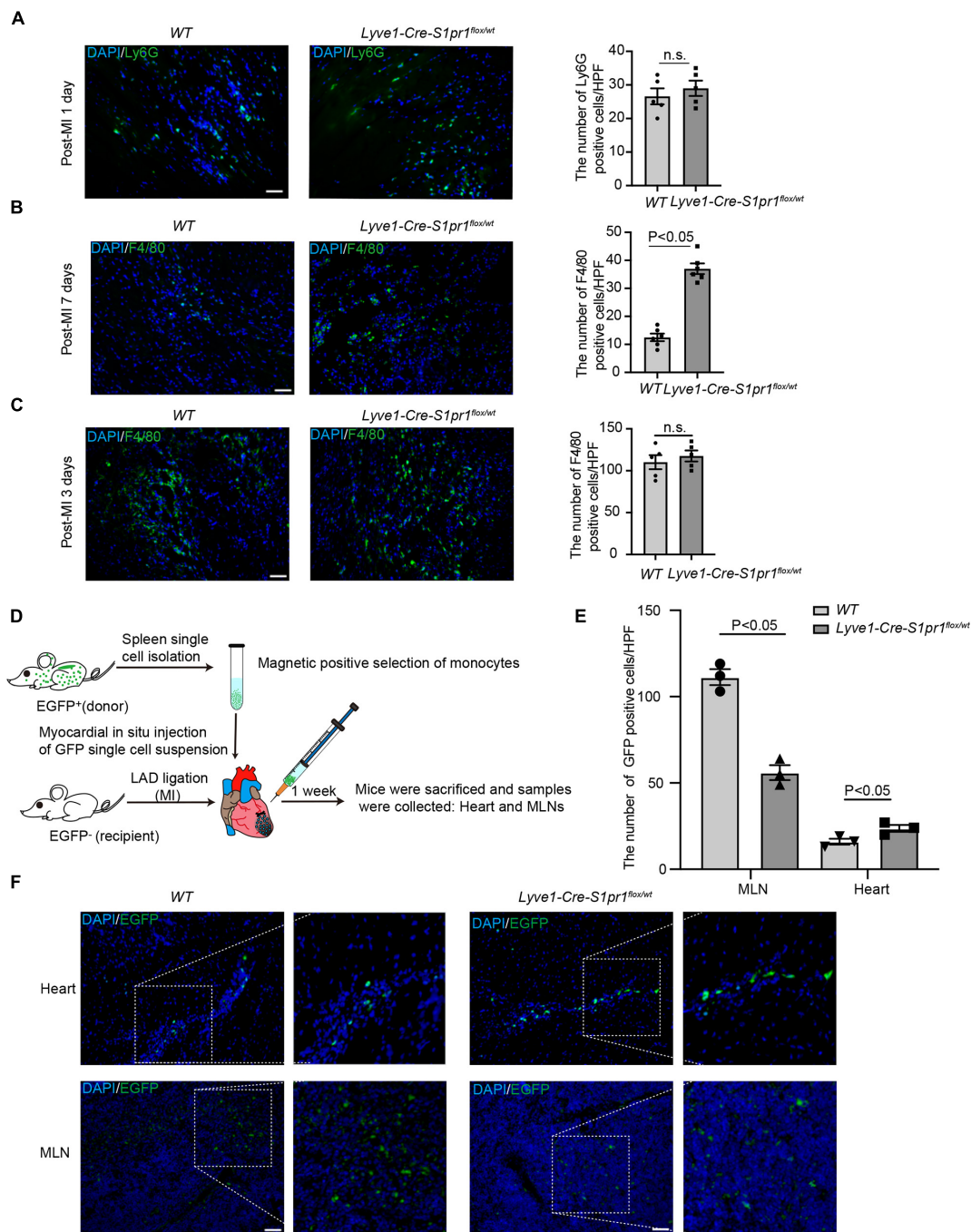


FIGURE 5

The reduced expression of LEC-S1pr1 retards macrophage clearance in injured hearts. (A) Representative images of immunostaining of Ly6G (neutrophil marker) from hearts in WT and *Lyve1-Cre-S1pr1^{fllox/wt}* at 1 day after MI, with quantification of the number of neutrophils in infarct zone of myocardium of the indicated groups ($n = 5$). (B) Representative images of immunostaining of F4/80 (macrophage marker) within infarct zone of hearts in WT and *Lyve1-Cre-S1pr1^{fllox/wt}* at 7 days after MI, with quantification of the number of macrophages in myocardium of the indicated groups ($n = 6$). (C) Representative images of immunostaining of F4/80 (macrophage marker) from hearts in WT and *Lyve1-Cre-S1pr1^{fllox/wt}* at 3 days after MI, with quantification of the number of macrophages in infarct zone of myocardium of the indicated groups ($n = 5$). (D) Schematic diagram of the adoptive cell transfer approach. EGFP transgenic mice were used as splenic EGFP⁺ monocyte donors, and recipient WT or *Lyve1-Cre-S1pr1^{fllox/wt}* adult mice received donor EGFP⁺ monocytes via intramyocardial delivery at the time of LAD ligation and EGFP donor monocyte trafficking was monitored in post-MI hearts and their draining MLNs ($n = 3$). (E, F) Representative fluorescence images of hearts and draining MLNs in WT and *Lyve1-Cre-S1pr1^{fllox/wt}* at 7 days after MI, with quantification of the number of EGFP⁺ macrophages in the myocardium of the indicated groups. Less donor GFP⁺ macrophages were detected in draining MLNs of *Lyve1-Cre-S1pr1^{fllox/wt}* mice compared with WT mice, while more donor EGFP⁺ macrophages in hearts of *Lyve1-Cre-S1pr1^{fllox/wt}* mice ($n = 3$). Scale bars for (A–C, F), 50 μ m. Data are mean \pm S.E.M. n.s., no statistical significance.

LEC-S1pr1 regulates macrophage trafficking via ERK/CCL2 signaling pathway

To further identify the molecular mechanism by which LEC-S1pr1 influences macrophage clearance, we tested the effect of HLEC-conditioned medium on the migration of macrophages *in vitro*. Our data showed that the conditioned medium from S1PR1-overexpressing HLEC culture significantly enhanced macrophage transmigration *in vitro* (Figure 6A), suggesting that LECs might secrete chemokines via S1P/S1pr1 signaling to recruit macrophages and boost their migration in a paracrine manner. We next investigated whether LEC-S1pr1 regulated key chemokines which control macrophage chemotactic migration. Among various chemokines, CCL2 was the major chemokine expressed in HLECs, and significantly higher expression levels of CCL2 were observed in S1PR1-overexpressing HLECs, while lower expression in S1PR1-silencing HLECs (Figure 6B). As expected, we detected lower expression levels of Ccl2 in LECs from hearts of *Lyve1-Cre-S1pr1^{fllox/wt}* mice at 7 days following MI, in comparison with *WT* littermates (Figure 6C); however, the main lymphatic chemokine, Ccl21, was not altered in LECs of *Lyve1-Cre-S1pr1^{fllox/wt}* mice (Figure 6C). Our results further showed that inhibition of CCL2 expression by siRNA blocked the enhancing effect of HLEC-S1PR1 overexpression on macrophage transmigration (Figure 6D). To further investigate which signaling pathway was involved in the regulation of LEC-S1PR1 on CCL2 expression, we investigated the influence of LEC-S1PR1 on the ERK signaling pathway which has been widely reported to be activated by S1PR1 in various cells (3). Our western-blot analysis showed that the active levels of ERK were significantly higher in S1PR1-overexpressing HLECs, while lower in S1PR1-silencing HLECs, suggesting S1PR1 up-regulated ERK activity in LECs (Figure 6E). To further investigate whether the effects of LEC-S1pr1 on CCL2 expression are dependent on ERK signaling pathway, we treated LECs with ERK inhibitor, U0126, in S1PR1-overexpressing LECs *in vitro*. The inhibition of the ERK signaling pathway reversed the up-regulated expression of CCL2 in S1PR1-overexpressing HLECs (Figure 6F). These results suggest that LEC-S1pr1 might regulate CCL2 expression via ERK signaling pathway. Taken together, our results demonstrate that LEC-S1pr1 might regulate macrophage recruitment and migration via the ERK-CCL2 pathway in a paracrine manner (Figure 7).

Discussion

There are two vascular circulatory systems in mammals: one is the blood vasculature; the other is the lymphatic vasculature. It has been well documented that S1pr1 was essential for maintaining vascular stability under both physiological and pathophysiological conditions (24). However, the effect of S1pr1

on lymphatic vasculature has been just recently reported by Geng X et al. (19). Geng X et al. showed that loss of S1pr1 in LECs induced lymphatic vascular hyper-sprouting and hyperbranching in a Vegfr3-dependent manner *in vivo* and that S1pr1 controlled lymphatic vessel maturation via RhoA signaling pathway by promoting membrane localization of the tight junction molecule claudin-5 (19). S1pr1 dampens LSS/VEGF-C signaling, suggesting that S1pr1 plays an important role in lymphatic vascular hemostasis during embryo development (19). However, whether LEC-S1pr1 is involved in the regulation of pathological ventricular remodeling is unknown. In this report, our data showed that the expression of S1pr1 in LECs was significantly reduced in hearts after myocardial infarction. Our further *in vivo* experiments showed that a reduced expression of S1pr1 in LECs deteriorated pathological ventricular remodeling and worsened cardiac dysfunction after myocardial infarction, suggesting that LEC-S1pr1 exerts a key role in improving post-MI cardiac remodeling and functions.

The lymphatic vasculature is a unidirectional conduit with a range of functions, including maintenance of interstitial fluid balance and immune cell trafficking (18). After heart injury, leukocyte transport determines the extent of cardiac injury and influences the following myocardial healing (13). Vieira JM et al. reported that cardiac lymphatic vessels promoted leukocyte clearance from injured hearts, resulting in dampening cardiac injury and improving cardiac healing (13). They suggested that VEGFC enhanced post-MI lymphangiogenesis and thus promoted leukocyte exit from injured hearts (13). However, one recent report reported that lymphangiogenesis may not contribute to the beneficial effect of VEGFC on cardiac repair after MI (25). The molecular mechanism by which cardiac lymphatic vasculature regulates the egress of immune cells from hearts and its role in cardiac repair after heart injury are not yet fully understood. Herein, we identified that LEC-S1pr1 was critical for the clearance of infiltrating macrophages in the myocardium, since a reduction of LEC-S1pr1 significantly reduced macrophage exists from the post-MI hearts. Our further data suggested this effect of LEC-S1pr1 on macrophage clearance from ischemic hearts might not be due to post-MI lymphangiogenesis, as demonstrated by a similar lymphatic vessel density in myocardium between *WT* and *S1pr1* transgenic mice.

It is known that the passage of immune cells from tissue interstitium toward afferent lymphatic vessels depends on chemotaxis (18). Previous investigations showed that lymphatic endothelium expressed and secreted various chemokines, including CCL2, CCL12, CCL5, CCL20, CXCL2, and CX3CL1 (fractalkine), which direct selective egress from tissues of leukocytes (26). We next asked whether LEC-S1pr1 regulated the expression of chemokines, which directs leukocytes toward LECs and enhanced their egress from injured hearts. Our data showed that LEC-S1pr1 influenced the expression of

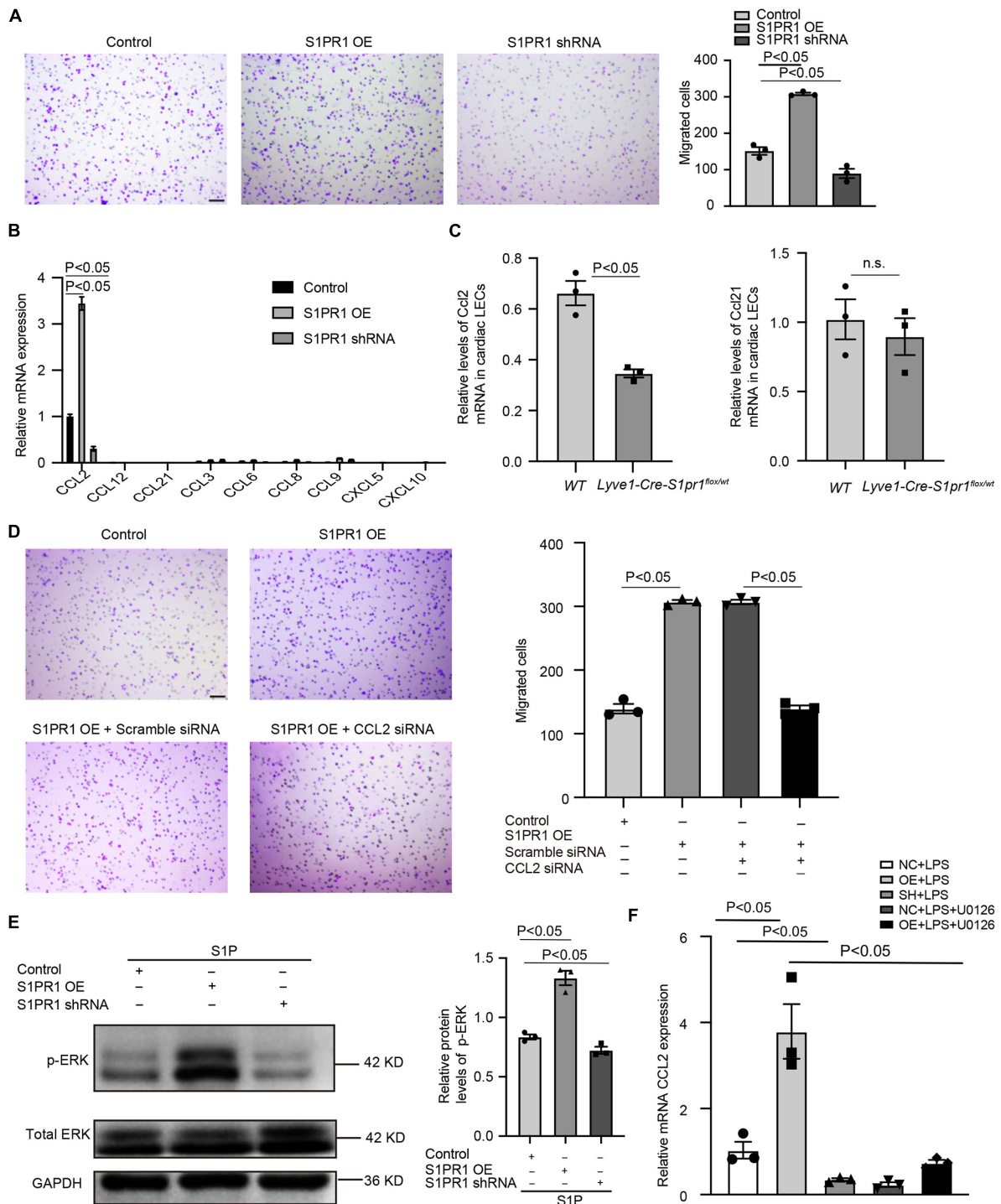
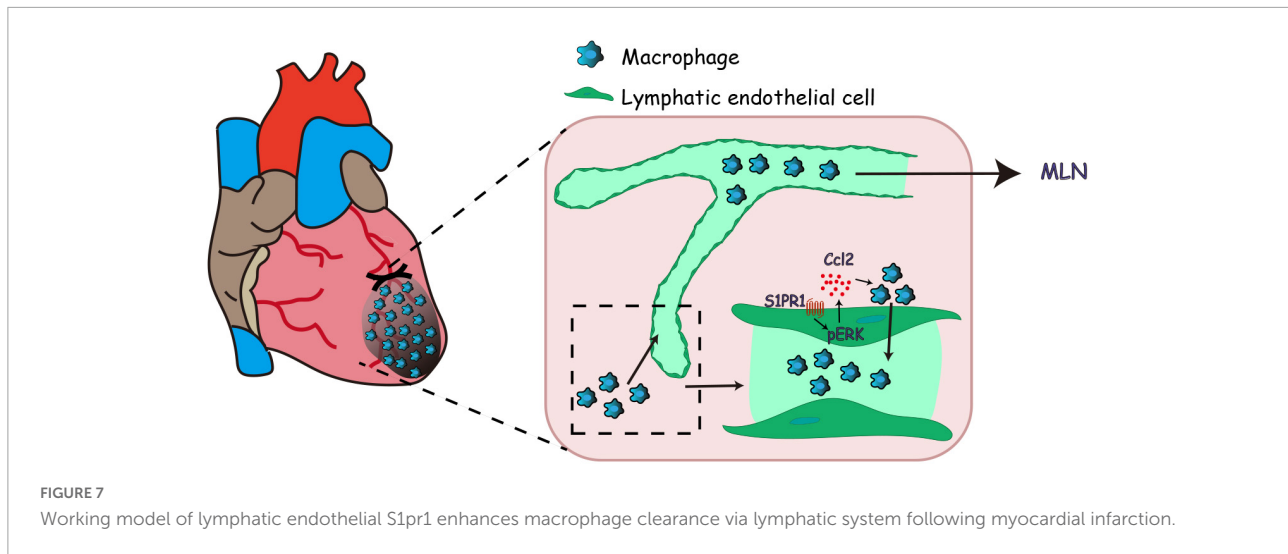


FIGURE 6 LEC-S1pr1 regulates macrophage trafficking via ERK/CCL2 signaling pathway. **(A)** The representative images of transwell cell migration assay of THP-1 cell lines, which were co-cultured with condition medium obtained from LECs of the indicated groups, with their quantification of migrated cells ($n = 3$). **(B)** RT-qPCR analysis showed the relative mRNA expression levels of different chemokines in the indicated groups ($n = 3$). **(C)** RT-qPCR analysis showed the relative Ccl2 and Ccl21 mRNA expression levels in LECs from hearts of the indicated groups at 7 days after MI ($n = 3$). **(D)** The representative images of Boyden chamber assay of THP-1 cell lines, which were co-cultured with the conditioned medium obtained from LECs of the indicated groups, with their quantification of migrated THP-1 ($n = 3$). **(E)** Western blotting analysis of ERK activation status in LECs treated with S1P in the indicated groups with their quantification of the ratio of p-ERK/total ERK ($n = 3$). **(F)** RT-qPCR showed the CCL2 mRNA expression of the indicated groups ($n = 3$). U0126, ERK inhibitor. Scale bars for **(A,C)** 100 μ m. Data are mean \pm S.E.M. n.s., no statistical significance.



multiple chemokines, including CCL2, CCL3, and CCL6. Among these cytokines, CCL2 is the major chemokine which was expressed and significantly regulated by S1pr1 signaling in LECs. It has been well documented that CCL2, monocyte chemoattractant protein-1 (MCP-1), played an essential role in myocardial pathology and was sharply upregulated in post-MI myocardium (27). *In vivo* animal studies have shown that CCL2 regulated monocytes/macrophages recruitment, activation, and polarization in the injured heart after MI, and thus influenced pathological ventricular remodeling and cardiac repair (27). Previous studies revealed that LECs expressed CCL2, suggesting that LEC-CCL2 is involved in the regulation of leukocyte chemotactic migration (28, 29). In consistent with previous reports (28, 29), we identified that CCL2 was expressed in LECs, and that LEC-expressing CCL2 was regulated by S1pr1. Previous studies showed that ERK signaling pathway was involved in the regulation of the expression of multiple genes, including CCL2 (17, 30, 31). In consistence with these reports (17, 30, 31), our data showed that S1pr1 enhanced the expression of CCL2 in LECs via the ERK signaling pathway.

Taken together, previous investigations indicated that S1pr1 tightly controls lymphatic vascular homeostasis during embryogenesis; however, the LEC-specific effects of S1pr1 on post-MI pathological ventricular remodeling are unknown. Using LEC-specific S1pr1 transgenic mice, we presented that the reduced expression of S1pr1 in LECs worsened post-MI ventricular remodeling and cardiac dysfunctions, providing strong *in vivo* evidence to support an essential role of LEC-S1pr1 in the regulation of cardiac repair and functions after MI. Mechanically, S1pr1 signaling activates ERK signaling pathway in LECs, and boosts CCL2 expressions, resulting in the recruitment of infiltrating macrophages in myocardium toward lymphatic endothelium and an enhancement in macrophage clearance from ischemic myocardium via afferent cardiac lymphatics, and consequently dampens post-MI inflammation

and improves cardiac functions (Figure 7). Our study reveals a novel role of LEC-S1pr1 in the regulation of post-MI cardiac remodeling and functions, providing a potential therapy by modulation of LEC-S1pr1 to resolve cardiac inflammation and to improve cardiac functions after myocardial infarction.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject, PRJNA818802.

Ethics statement

The animal study was reviewed and approved by Tongji University Institutional Animal Care and Use Committee.

Author contributions

SD and JW contributed to the conception and design of the study, and wrote the manuscript. QL performed experiments and acquire data. CZ, KZ, and YD quantify experimental data. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcvm.2022.872102/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Flow cytometric analysis showed the purity of isolated cardiac LECs without monocyte/macrophage contamination. (A) The representative

images of flow cytometric analysis of cardiac cells from post-MI hearts of WT mice before LECs separation. (B) The representative images of flow cytometric analysis of cardiac cells from post-MI hearts of WT mice after LECs separation. (C) The representative images of flow cytometric analysis of peripheral blood cells of WT mice by staining CD45 or CD11b or Ly6C antibody and its corresponding IgG control validated the optimization of flow cytometric analysis in our study.

SUPPLEMENTARY FIGURE 2

Relative mRNA expression levels in cardiac LECs of mice. (A) Relative mRNA expression levels of S1pr2 in cardiac LECs of mice at 3 days after sham or LAD operation ($n = 6$). (B) Relative mRNA expression levels of S1pr3 in cardiac LECs of mice at 3 days after sham or LAD operation ($n = 6$). (C) Relative mRNA expression levels of S1pr4 in cardiac LECs of mice at 3 days after sham or LAD operation ($n = 6$). Data are mean \pm S.E.M. n.s., no statistical significance.

SUPPLEMENTARY FIGURE 3

The reduced expression of LEC-S1pr1 did not influence the proliferation of lymphatic endothelial cells after myocardial infarction. Representative images of immunostaining of Ki67 (cell proliferation marker) and Lyve-1 (LEC marker) from hearts in WT and *Lyve1-Cre-S1pr1^{flox/wt}* at 7 days after MI, with quantification of the number of proliferating lymphatic endothelial cells in myocardium of the indicated groups ($n = 5$). Scale bars, 50 μ m. Data are mean \pm S.E.M. n.s., no statistical significance.

SUPPLEMENTARY FIGURE 4

The reduced expression of LEC-S1pr1 did not influence the resident macrophages in peri-infarct zone after myocardial infarction. (A) Representative images of immunostaining of CCR2 and F/480 from hearts in WT and *Lyve1-Cre-S1pr1^{flox/wt}* at 3 days after MI, with quantification of the number of CCR2⁻F4/80⁺ cells (cardiac resident macrophages) in the peri-infarct zone of indicated groups ($n = 5$). (B) Representative images of immunostaining of CCR2 and F/480 from hearts in WT and *Lyve1-Cre-S1pr1^{flox/wt}* at 3 days after MI, with quantification of the number of CCR2⁺F4/80⁺ cells (the recruited macrophages from circulation) in the infarct zone of indicated groups ($n = 5$). Scale bars for (A–B), 50 μ m. Data are mean \pm S.E.M. n.s., no statistical significance.

SUPPLEMENTARY FIGURE 5

The monocyte subpopulation profile in peripheral blood. The representative cytometric images of circulating CD11b⁺Ly6C^{high/low} monocyte subpopulations with their quantification in WT and *Lyve1-Cre-S1pr1^{flox/wt}* mice.

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