Selective Stimulation of Cardiac Lymphangiogenesis Reduces Myocardial Edema and Fibrosis Leading to Improved Cardiac Function Following Myocardial Infarction

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Background—The lymphatic system regulates interstitial tissue fluid balance, and lymphatic malfunction causes edema. The heart has an extensive lymphatic network displaying a dynamic range of lymph flow in physiology. Myocardial edema occurs in many cardiovascular diseases, eg, myocardial infarction (MI) and chronic heart failure, suggesting that cardiac lymphatic transport may be insufficient in pathology. Here, we investigate in rats the impact of MI and subsequent chronic heart failure on the cardiac lymphatic network. Further, we evaluate for the first time the functional effects of selective therapeutic stimulation of cardiac lymphangiogenesis post-MI.

Methods and Results—We investigated cardiac lymphatic structure and function in rats with MI induced by either temporary occlusion (n=160) or permanent ligation (n=100) of the left coronary artery. Although MI induced robust, intramyocardial capillary lymphangiogenesis, adverse remodeling of epicardial precollector and collector lymphatics occurred, leading to reduced cardiac lymphatic transport capacity. Consequently, myocardial edema persisted for several months post-MI, extending from the infarct to noninfarcted myocardium. Intramyocardial-targeted delivery of the vascular endothelial growth factor receptor 3–selective designer protein VEGF-C_{125}, using albumin-alginate microparticles, accelerated cardiac lymphangiogenesis in a dose-dependent manner and limited precollector remodeling post-MI. As a result, myocardial fluid balance was improved, and cardiac inflammation, fibrosis, and dysfunction were attenuated.

Conclusions—We show that, despite the endogenous cardiac lymphangiogenic response post-MI, the remodeling and dysfunction of collecting ducts contribute to the development of chronic myocardial edema and inflammation-aggravating cardiac fibrosis and dysfunction. Moreover, our data reveal that therapeutic lymphangiogenesis may be a promising new approach for the treatment of cardiovascular diseases. (Circulation. 2016;133:1484-1497. DOI: 10.1161/CIRCULATIONAHA.115.020143.)

Key Words: edema ◼ edema, cardiac ◼ fibrosis ◼ heart failure ◼ lymphatic vessels ◼ therapeutics ◼ vascular endothelial growth factor C ◼ ventricular remodeling

The lymphatic system plays a crucial role in the maintenance of tissue fluid balance and immune surveillance in most vascularized tissues. The heart carries an elaborate lymphatic network, first described by Rudbeck.1-3 The heart depends heavily on its lymphatics for the return to the blood circulation of extravasated macromolecules and fluids.4 Accordingly, surgical obstruction of cardiac lymphatics rapidly leads to myocardial edema with well-described acute, and chronic, as well, deleterious effects on cardiac function.5-7 Clinically detectable myocardial edema is a frequent finding in the acute phase of myocardial ischemia,4 initially because of vascular hyperpermeability.8-11 Concurrently, there is rapid infiltration of neutrophils, macrophages, and other immune cells, which participate in the inflammatory tissue response to injury. These cells perform important beneficial roles such as the removal of dead cells and debris and the stimulation of reparative or regenerative processes, including angiogenesis.12,13 However, cardiac inflammation also has many

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deleterious effects, including induction of blood vascular rarefaction and dysfunction and stimulation of cardiac fibrosis, contributing to the development of chronic heart failure. Furthermore, many inflammatory mediators, and oxygen radicals generated during inflammation, as well, negatively affect lymphatic function, causing impairment of lymph flow and initiation of lymph edema and chronic inflammation. It is noteworthy that clinically detectable myocardial edema, extending beyond the infarct zone, may persist for up to 6 to 12 months post–myocardial infarction (MI) in humans, which is suggestive of lymphatic insufficiency.

Whether cardiac lymphatic dysfunction occurs after myocardial injury, and the impact this may have on myocardial fluid balance and cardiac inflammation, remains to be investigated. Moreover, although the advent of molecular lymphatic markers has fueled investigations into lymphatic anatomy, function, and growth in many organs, only a handful of articles have assessed lymphangiogenesis in the heart. It was recently shown that cardiac lymphangiogenesis and lymphatic remodeling occur in humans with cardiovascular diseases, such as infective endocarditis, acute or chronic myocardial ischemia, and terminal chronic heart failure. However, little information exists on the functional role of cardiac lymphangiogenesis in the native heart, and it is unknown whether insufficient lymphangiogenesis contributes to chronic myocardial edema, inflammation, and fibrosis in cardiovascular diseases.

Stimulation of lymphatic vessel growth (therapeutic lymphangiogenesis) has been proposed as a treatment to resolve peripheral edema of different etiologies, including secondary lymphedema. Promisingly, experimental research over the past decade has shown that stimulation of lymphangiogenesis may reduce edema and limit or resolve chronic inflammation. To the best of our knowledge, targeted stimulation of cardiac lymphangiogenesis has never before been attempted in the MI setting.

Here, we present a molecular study of the cardiac lymphatic tree describing the endogenous lymphangiogenic response to myocardial injury and its impact on cardiac lymphatic function, myocardial interstitial fluid balance, and immune cell infiltration. Furthermore, we investigate for the first time whether targeted intramyocardial lymphangiogenic therapy may exert beneficial effects on cardiac remodeling and function post-MI.

Methods

Animals and Reagents
Male Wistar rats (220 g) were obtained from Janvier. Animal housing and experiments were in accordance with National Institutes of Health guidelines, and the study ethically approved by the Normandy University regional review board according to French and EU legislation (project no. 01181.01). Recombinant rat vascular endothelial growth factor (VEGF)-C\textsubscript{C152S} was purchased from Reliatech. Sources of antibodies and other reagents are listed in the online-only Data Supplement Methods (Tables I through III in the online-only Data Supplement).

Surgical Model
Left ventricular (LV) MI was induced by left coronary artery anterior descending branch ligation (permanent model) or occlusion (45 minutes of ischemia; temporary model) as previously described. Albumin-alginate microparticles, loaded or not with VEGF-C\textsubscript{C152S}, were injected in the LV free wall after reperfusion in the temporary MI model.

Functional Evaluations

Echocardiography, MRI, and Hemodynamics
Echocardiography was performed in sedated rats as previously described. MRI analyses were performed in anesthetized rats, as described, and for T2-mapping of cardiac water content by using a 4.7\textsuperscript{T} horizontal bore scanner (Bruker). Invasive hemodynamic assessment of cardiac function was performed by LV catheterization as previously described. See online-only Data Supplement Methods.

Lymphangiography
Invasive cardiac lymphangiography was performed at 4, 8, or 12 weeks post-MI. Fluorescent quantum dots (Molecular Probes) were injected intramyocardially, and fluorescein isothiocyanate-dextran (Sigma-Aldrich) was injected intravenously, followed by macroconfocal imaging (TCS LSI, Leica) of the anterolateral epicardium. Images were assembled using NIH ImageJ software for 3D reconstruction of cardiac surface blood and lymphatic vascular networks. For details see online-only Data Supplement Methods.

Gravimetry
Cardiac water content was evaluated by wet weight–dry weight method after desiccation of tissues for 5 days at 65°C, similar to what has been described.

Immunohistochemistry and Histology

Immunohistochemistry
Hearts were arrested in diastole and either fixed in 3% paraformaldehyde or snap-frozen. Whole hearts or sequential cryosections (8 μm) were immunolabeled by using lymphatic markers (LYVE, Podoplanin, Prox-1, vascular endothelial growth factor receptor 3 (VEGFR3), FOXC2, and CCL21), blood vessel markers (CD31, SMA), immune cell markers (CD68, CD11b, CD11c), and wheat germ agglutinin for cardiomyocyte contours. 4′,6-Diamidino-2-phenylindole provided nuclear stain. Signals were visualized with either peroxidase-based colorimetry or immunofluorescence. Images were assembled using ImageJ software and 3D-modeled using Amira software (FEI). For details, see online-only Data Supplement Methods.

Histology
Cardiac cryosections (8 μm) were stained with Sirius Red. Infarct size was calculated as: (% Infarct area/total LV area. Cardiac interstitial collagen density (fibrosis) in noninfarct areas was evaluated as described. For details, see online-only Data Supplement Methods.

Western Blot
Cardiac LV samples were extracted, and denatured samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted to nitrocellulose membranes incubated with antibodies as detailed in online-only Data Supplement Methods.

Statistics
Data are presented as mean ± standard error of the mean. Differences between multiple groups were evaluated by parametric 1-way analysis of variance followed by Tukey post hoc test for samples with normal distribution and n>7 rats/group, or alternatively for samples with non-Gaussian distribution, or n≤8 rats/group, using nonparametric Kruskal-Wallis analysis followed by the Dunn post hoc test. Parametric Pearson or nonparametric Spearman rank order tests were used for evaluating correlations. Statistical analyses were performed with GraphPad Prism.
Results

Remodeling of Cardiac Lymphatics Post-MI

First, we performed immunohistochemical analyses to assess the molecular and structural profile of the cardiac lymphatic network in the LV of healthy, adult rats. The density of intramyocardial LYVE-1+ lymphatics was 10 times lower than the corresponding density of CD31+ blood vessels; whereas the blood vessel-to-cardiomyocyte ratio was ≈1:1, the lymphatic-to-cardiomyocyte ratio was 1:10 (Figure 1A through 1F, Figure 1A in the online-only Data Supplement). Cardiac LYVE-1+ lymphatic vessels expressed VEGFR3 (Figure 1B and 1C in the online-only Data Supplement) and Prox-1 online-only Data Supplement Figure IE). In the subepicardium, LYVE-1+/VEGFR3+ precollector-like vessels of at least 20 μm in diameter also expressed podoplanin (Figure 1C and 1D, Figure IC in the online-only Data Supplement). However, in midmyocardial areas lymphatic podoplanin expression was less frequent (Figure ID in the online-only Data Supplement). Indeed, the vast majority (>80%) of LYVE-1+ vessels were small (lumen diameter <5 μm) lymphatic capillaries that showed variable expression of VEGFR3, were negative for podoplanin, and devoid of smooth muscle cells (Figure IIA in the online-only Data Supplement), and did not express macrophage markers (Figure IIB through IID in the online-only Data Supplement). The intramyocardial lymphatics drain centrifugally, with each cardiac contraction, toward the superficial epicardial lymphatic network also underwent significant remodeling, again extending beyond the scar region (Figure 1K).

Molecular Regulation of Cardiac Lymphangiogenesis

Next, we assayed the levels of lymphangiogenic regulators VEGF-A, VEGF-C, and VEGFR3 in cardiac samples. In agreement with previous studies, we found that, whereas VEGF-A levels, peaking early after ischemia, were reduced to sham levels by 12 weeks post-MI, the cardiac levels of VEGF-C and VEGFR3 remained elevated, and even tended to increase, from 4 to 12 weeks (Figure 2A), in support of active cardiac lymphangiogenesis. The cardiac VEGF-C may in part be derived from recruited macrophages, where its expression is induced by both inflammatory mediators and osmotic pressure–regulated pathways.

Alteration of Cardiac Lymphatic Transport and Myocardial Fluid Balance Post-MI

In view of the structural alterations of the cardiac lymphatic network post-MI, we investigated cardiac lymphatic function. Cardiac lymphangiography, based on a macroconfocal ex vivo imaging approach developed in our laboratory, revealed attenuated lymphatic transport by 4 weeks, and partially restored transport capacity by 12 weeks post-MI (Figure 2B). This transient functional deficit mirrors the timeline of structural modifications, notably the decrease in the percentage of precollectors and total area of open lymphatics (Figure 1D and 1K), indicating that transport function and lymphatic precollector structure, rather than lymphatic capillary density, are linked.

Similar to previous studies, we found that cardiac water content, as a proxy for myocardial edema, was increased in the LV infarct zone for up to 16 weeks post-MI (Figure 2C). Moreover, we also detected significantly increased water content in the noninfarcted LV free wall for at least 4 weeks post-MI (Figure 2C). These findings of slowly dissipating myocardial edema, extending to noninfarcted areas and persisting long after the acute ischemia–induced vascular hyperpermeability has subsided, point to insufficient cardiac lymphatic transport as a limiting factor for reinstatement of cardiac fluid homeostasis post-MI.
Figure 1. Alterations of cardiac lymphatics following MI. LYVE-1+ lymphatic density (A), LYVE-1+/podoplanin+ precollector density (C), and percentage of podoplanin+ vessels (among LYVE-1+ lymphatics; D) in noninfarcted LV subepicardium following MI induced by permanent LCAd ligation (n=8–13 rats/group). Dotted line indicates healthy sham levels. CD31+ blood vessel density (E), CD31+ blood vessel-to-CM ratio (F), SMA+ arteriolar density (G), and percentage of arterioles (among CD31+ blood vessels; H) in rat myocardium (n=8–16 rats/group). Lymphatic vessel sizes were evaluated, and the frequency of open lymphatics (diameter>5 μm) among LYVE-1+ vessels was calculated (I). Average vessel diameter among open lymphatics (J), and total open lymphatic area per mm² (K). (n=8–10 rats/group). Example of the rat subepicardial lymphatic network (L): LYVE-1 (red); cardiomyocytes (green); nuclei (blue). Scale bar, 50 μm. Colorimetric whole-mount imaging of epicardial surface lymphatics in healthy sham rats (M) or at 4 weeks post-MI (N). Valved collectors indicated by white arrows in inset (M). Permanent LCAd ligation in blue (N). Scale bar, 2 mm. Values represent mean±SEM. *P<0.05; **P<0.01; ***P<0.001 vs sham. #P<0.05; ##P<0.01 as indicated.

LCAd indicates left coronary artery anterior descending branch; LV, left ventricle; MI, myocardial infarction; and SEM, standard error of the mean.
To assess whether lymphangiogenic therapy might improve cardiac lymphatic transport and reduce cardiac water imbalance post-MI, we developed a targeted approach using microparticle-based intramyocardial delivery of the VEGFR3-selective designer protein VEGF-CC152S. Our biodegradable, injectable albumin-alginate microparticles release bioactive growth factors, including VEGF-C152S over several weeks, as assessed in vitro (Figure VA through VC in the online-only Data Supplement), leading to increased therapeutic efficacy over naked protein delivery, and spatiotemporally controlled effects, as well, as demonstrated previously.27

One of the many advantages of our microparticle-based approach is that it is associated with minimal immunological interference, as opposed to adenoviral therapy, which in
Figure 3. Targeted delivery of VEGF-C152S, selectively stimulates cardiac lymphangiogenesis. Examples of rat subepicardial lymphatics (A): Top row, LYVE-1+ (red)/podoplanin-+ (green) precollectors; nuclei, blue. Bottom row, Cardiomyocytes (green); CD31- blood vessels (red); LYVE-1- (blue). LYVE-1- lymphatic density (B), LYVE-1- vessel-to-CM ratio (C), and relative density of podoplanin-+ precollectors (D) in the noninfarcted subepicardium at 3 weeks or 8 weeks post-MI (n=6–13 rats/group). CD31- blood vessel density (E) and CD31- blood vessel-to-CM ratio (F), and cardiomyocyte sizes (G) (n=7–11 rats/group). Frequency of open lymphatics (diameter>5 μm) among LYVE-1- vessels (H), lymphatic vessel diameter (I), and total area of open lymphatics (J) (n=5–11 rats/group). Dotted line indicates healthy sham levels (○). Controls (●) and VEGF-C152S-+treated rats (low dose, △; high dose, ▽). Values represent mean±SEM. $P=0.08; *P<0.05 vs sham; #P<0.05 as indicated. Scale bar, 50 μm. CM indicates cardiomyocyte; HD, high dose; LD, low dose; MI, myocardial infarction; SEM, standard error of the mean; and VEGF, vascular endothelial growth factor.
addition may lead to lymphatic uptake and transport of active virions. In contrast, our microparticles are small enough to be readily injectable, yet large enough to be entrapped between cardiomyocytes at the injection site, thus limiting spatially, in addition to temporally, the release of bioactive factors.

We opted for a temporary occlusion MI model in our subsequent studies because of its higher pertinence to the clinical situation, and to avoid confounding effects on cardiac lymphatic transport caused by inadvertent permanent ligation of one of the main cardiac collectors that run alongside the left coronary artery anterior descending branch (Figures 1M and 2B). Our experimental design included sham-operated animals, MI controls injected with empty microparticles, and 2 groups of VEGF-CC152S–treated MI rats: low-dose (1.5 μg/rat: VEGF-C_{152S}) and high-dose VEGF-C (5μg/rat VEGF-C_{152D}) (Figure VD and VE in the online-only Data Supplement). Despite similar infarct sizes at 3 weeks and 8 weeks post-MI, the cardiac hypertrophy, present in control MI rats by 8 weeks, was less pronounced in VEGF-CHD–treated rats (Figure VI).

Despite similar infarct sizes at 3 weeks and 8 weeks post-MI, although capillary-to-cardiomyocyte ratios were already diminished in the permanent ligation model, and the lymphatic capillary wall, the lymphangiogenic response in controls was slower than in VEGF-CLD–treated rats (Figures VI through VIC in the online-only Data Supplement), suggesting attenuated LV remodeling in the group receiving high-dose VEGF-C_{152D}.

Similar to our findings in the permanent ligation model, control MI rats displayed an endogenous capillary lymphangiogenic response together with lymphatic remodeling in the infarct (Figure VID through VIH in the online-only Data Supplement). However, neither lymphatic capillary density nor lymphatic sizes in the infarct area were further increased by VEGF-C_{152S} therapy, in comparison with controls (Figure VII, VIII, and VIH in the online-only Data Supplement). In the adjacent subepicardium of the noninfarcted LV free wall, the lymphangiogenic response in controls was slower than in the permanent ligation model, and the lymphatic capillary density was still not significantly increased by 8 weeks post-MI, although capillary-to-cardiomyocyte ratios were already increased by 3 weeks (Figure 3A through 3C). Furthermore, only VEGF-C_{152D} significantly increased lymphatic capillary density and lymphatic-to-cardiomyocyte ratios in the subepicardium by 3 weeks in comparison with controls (Figure 3A through 3C), and further tended to increase midmyocardial lymphatic density (Figure VII through VID in the online-only Data Supplement), indicating an accelerated cardiac lymphangiogenesis. By 8 weeks, the lymphatic capillary densities were similar among all 3 MI groups, demonstrating that VEGF-C_{152S} therapy increased the speed, but not the extent, of the cardiac lymphangiogenic response post-MI. However, in this temporary MI model, where the relative rarefaction of LYVE-1/podoplanin+ precollectors was less pronounced (Figure 3D), VEGF-C_{152S} therapy did not significantly alter precollector densities, indicating lymphatic capillary expansion as the main effect. The MI-induced myocardial blood vessel rarefaction was less pronounced in controls in this temporary occlusion MI model (Figure 3E).

In contrast, VEGF-C_{152D}–treated rats, the frequency of larger (>30 μm diameter, red) epicardial precollectors was increased in comparison with sham and controls. This prevention by VEGF-C_{152D} treatment of MI-induced epicardial lymphatic profile shifting, together with increased intramyocardial lymphatic density (Figures 3B, 3C, and 4), but also the enlargement of intramyocardial lymphatics, as observed in VEGF-C_{152D}–treated rats (Figure 3I and 3J), could potentially lead to improved cardiac lymphatic drainage in comparison with MI controls. However, cardiac lymphangiography revealed no major improvement in lymphatic transport capacity in the anterior LV surface (encompassing both infarct and peri-infarct regions) of VEGF-C_{152D}–treated versus controls at 8 weeks post-MI (Figure 5).

Effect of Therapeutic Lymphangiogenesis on Myocardial Edema, Inflammation, and Cardiac Fibrosis

Despite the lack of evidence of macroscopically improved cardiac lymphatic function in the VEGF-C_{152D}–treated group, we investigated whether our lymphangiogenic therapy influenced myocardial fluid balance post-MI. Similar to
our observations in the permanent ligation model, cardiac water content was significantly increased in controls for up to 8 weeks post-MI (Figure 6). Again, the myocardial edema extended from the infarct to noninfarcted areas. In VEGF-C_{LD}-treated rats, the MI-induced myocardial water imbalance, assessed by gravimetry, was reduced by 3 weeks in the noninfarcted LV free wall and septum, but not in the infarct or right ventricle (Figure 6A). In the VEGF-C_{HD} group, there was no difference from MI controls in cardiac water content at 3 weeks (data not shown), and, by 6 weeks, only a small, nonsignificant reduction was found (−10±3% of T2 signal, \( P=0.12 \)), as assayed by MRI T2 mapping (Figure 6B and 6C). However, by 8 weeks, there was a significant reduction of both right ventricular and total cardiac water content, as assessed by gravimetry, in VEGF-C_{LD} rats in comparison with controls (Figure 6E).
Apart from the role of lymphangiogenesis in restoring interstitial fluid balance, expansion and remodeling of cardiac lymphatics may influence the extent, duration, or quality of the inflammatory response to myocardial injury. Examining infarcted (Figure 7B) and noninfarcted (Figure 7A and 7C) LV at 3 weeks post-MI, when the acute inflammatory response has subsided, we found that VEGF-CC\textsubscript{152S} treatment led to a dose-dependent reduction in CD68\textsuperscript{+} macrophage levels in both the infarct (VEGF-CHD: 48±3% of control levels, \(P<0.05\)) and the noninfarcted LV (VEGF-C\textsubscript{HD}: 68±5% of controls, \(P<0.05\)). By 8 weeks, immune cell infiltration was similarly reduced in controls (data not shown), indicating that targeted delivery of VEGF-C\textsubscript{G152S} modulates the duration, but not the extent, of the inflammatory response in the chronic phase of MI. This may involve increased egress of immune cells, notably antigen-presenting cells such as macrophages and dendritic cells, by cardiac lymphatics. Indeed, CD68\textsuperscript{+} cells were occasionally observed in the lumen of intramyocardial lymphatics.
Figure 7. Therapeutic lymphangiogenesis limits macrophage levels and cardiac fibrosis. 

A. Examples of macrophage infiltration in noninfarcted LV at 3 weeks post-MI. Quantification of CD68⁺ macrophage density in infarct (B) and noninfarcted LV (C) (n=5–11 rats/group). CD68⁺ cells (green) were occasionally observed in the lumen of precollection lymphatics (red). 

D. Correlation between macrophages and intramyocardial lymphatic density. E. Quantification and example of CD11c⁺ dendritic cell density in the noninfarcted LV. F. Correlation between dendritic cells and CCL21-expressing lymphatics. Quantification (G) and example (H) of interstitial cardiac collagen levels in noninfarcted LV at 3 weeks or 8 weeks post-MI (n=5–10 rats/group). Values represent mean±SEM. *P<0.05; ***P<0.001 vs sham. #P<0.05; ###P<0.001 as indicated. Scale bar, 50 μm. DAPI indicates 4',6-diamidino-2-phenylindole; HD, high dose; LD, low dose; LV, left ventricle; MI, myocardial infarction; SEM, standard error of the mean; and VEGF, vascular endothelial growth factor.
precollectors, increasing the total area of open lymphatics (Figure 7C, Figure IIB through IID in the online-only Data Supplement), and CD68⁺ cell density correlated inversely with lymphatic density (Figure 7D), but not with CCL21 expression. Lymphatic-selective expression of the chemokine CCL21 has been shown to regulate immune cell clearance.⁴₃ We found that VEGF-C⁺ HD⁴₄ treatment led to a nonsignificant increase in CD11c⁺ dendritic cell density (Figure 7E) associated with a trend for lower CCL21 expression levels in subepicardial lymphatics in the noninfarct LV, but not in the infarct, in comparison with controls (Figure VIII in the online-only Data Supplement). Indeed, in agreement with a prominent role for this chemokine in directing dendritic, but not macrophage, cell egress, we found that cardiac CD11c⁺ density correlated inversely with lymphatic CCL21 expression (Figure 7F).

Both myocardial edema and inflammatory mediators released by immune cells, including macrophages, stimulate collagen production in fibroblasts leading to the initiation of cardiac fibrosis extending to noninfarcted areas.⁴₆,⁴⁷ Whereas interstitial collagen levels in controls were significantly increased by 8 weeks post-MI in comparison with sham (135±5% of sham levels, P<0.001), we remarkably found that cardiac fibrosis was completely prevented in VEGF-C⁺ C₁₅₂S⁻ (135±5% of sham levels, P<0.001), we remarkably found that cardiac fibrosis extending to noninfarcted areas.³⁶,³⁷ In contrast, in the bordering noninfarcted LV wall, where VEGF-C⁺ C₁₅₂S⁻ therapy led to partial rescue of epicardial/subepicardial precollectors from MI-induced remodeling, cardiac lymphatic transport was still found to be inefficient when acutely challenged in our lymphangiography approach. However, given that we observed reductions in both cardiac water content and macrophage density in noninfarcted myocardium, the structural and molecular effects of VEGF-C⁺ C₁₅₂S⁻ treatment seem to have sufficed for improving physiologically relevant lymphatic drainage of the heart.

The marginal decrease in cardiac water content observed with VEGF-C⁺ HD⁴₄ therapy, in comparison with controls (0.8% reduction at 3 weeks), may seem anodyne, but given the heart’s exquisite sensitivity to changes in interstitial fluid volume, linked to its immediate impact on interstitial fluid pressure in this organ,² even small changes may significantly impact cardiac function. Indeed, an increase in cardiac water content by as little as 2.5% leads to a 30% to 40% reduction in cardiac output.⁴²,⁴³ Furthermore, the remarkable improvement of cardiac function seen with our lymphangiogenic therapy is likely attributable to a combination of both direct (interstitial fluid pressure normalization) and indirect (cardiac fibrosis) effects of limiting myocardial edema. Different from lymphatic networks in other organs,⁴⁴,⁴⁵ notably the essential absence of smooth muscle cells on cardiac precollectors, and only sparse muscular cells on its collecting ducts,⁴⁶ the heart largely depends on extrinsic factors for regulation of its lymphatic drainage. Consequently, cardiac contractile (systolic) dysfunction is likely a major contributing factor to the insufficient cardiac lymphatic drainage observed post-MI.⁴⁶,⁴⁷

VEGF-C therapy has previously been investigated in clinical trials in patients with myocardial ischemia. However, these
trials were performed with the native protein almost 15 years ago, with the rationale at the time being that VEGF-C (also called VEGF-2) would stimulate cardiac angiogenesis. Of note, although VEGF-C in its spliced, mature form indeed may activate VEGFR2 in blood endothelial cells, leading to stimulation of angiogenesis, it is a far more powerful activator of VEGFR3 on lymphatic endothelial cells leading to the stimulation of lymphangiogenesis. Surprisingly, in retrospect, the potential effects of VEGF-C therapy on cardiac lymphatics and dependent parameters, including myocardial edema, inflammation, and fibrosis, were completely overlooked in these early studies. It thus remains unknown whether VEGF-C gene or protein therapy in humans stimulated cardiac lymphangiogenesis, and further if this mechanism may have contributed to any beneficial cardiac functional effects in patients. Our current findings strongly support renewed interest in VEGF-C as a potential therapeutic option for ischemic heart disease, notably in patients with signs of chronic myocardial edema and inflammation. Conversely, our study indirectly suggests that humans with genetic or acquired lymphangiogenic deficits may be at increased risk to develop chronic myocardial edema and severe cardiac dysfunction in the advent of increased blood vascular permeability, eg, during myocardial ischemia.

Most studies on therapeutic lymphangiogenesis have focused on gene or protein delivery of VEGF-C, which stimulates both sprouting lymphangiogenesis and lymphatic vessel enlargement (lymphatic hyperplasia). In addition, mature native VEGF-C, acting on VEGFR2, may also increase blood as well as lymphatic vascular permeability, causing transient lymphatic dysfunction. However, over the past decade, many additional growth factors have been found to stimulate lymphangiogenesis, including VEGF-A, angiopoietins, platelet-derived growth factors, insulin-like growth factors, fibroblast growth factor 2, and hepatocyte growth factor. It is still unknown...
what growth factor(s) therapy yields stable lymphatic vessels that durably increase lymphatic transport. Although in our study, VEGF-C\textsuperscript{CC152S} monotherapy was sufficient to improve lymphatic network structure and reduce edema, future studies should perhaps envisage the use of growth factor combinations.\textsuperscript{5,6} Indeed, developmental studies have shown that multiple growth factors are necessary for the correct differentiation and patterning of lymphatic endothelial cells into a functional lymphatic system. Interestingly, a recent study revealed synergistic stimulation of corneal lymphangiogenesis by using VEGF-C combined with fibroblast growth factor 2.\textsuperscript{5,6}

In conclusion, our data show that MI leads to significant remodeling of the cardiac lymphatic network with an endogenous lymphangiogenic response that is insufficient to prevent lymphatic transport dysfunction and initiation of chronic myocardial edema. Promisingly, targeted lymphangiogenic therapy improves both precollection remodeling and capillary lymphangiogenesis, leading to accelerated resolution of myocardial edema and inflammation, and prevention of cardiac fibrosis and dysfunction. Our study represents a striking example of a previously unexplored aspect of cardiac pathophysiology, which may significantly impact future therapeutic options for patients with MI or chronic heart failure.

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Disclosures

None.

References

Role of Cardiac Lymphangiogenesis

The pathophysiological role of the lymphatic system has become increasingly recognized over the past decade, extending from control over interstitial fluid balance to regulation of inflammation, but also impacting tumor metastasis in patients who have cancer. However, the lymphatic network of the heart has been largely neglected. Although therapeutic lymphangiogenesis is currently being proposed as a novel treatment against edema or chronic inflammation in peripheral organs, it has remained unknown whether lymphangiogenesis in the heart may impact cardiac function, notably in cardiovascular diseases such as myocardial infarction and chronic heart failure.

In this study, we show that myocardial infarction induces severe cardiac lymphatic network remodeling and lymphatic transport dysfunction, which together with insufficient lymphangiogenesis contribute to the initiation of chronic myocardial edema and inflammation. Furthermore, we assessed the impact of selective targeted lymphangiogenic therapy in the heart. Promisingly, we demonstrate for the first time that cardiac therapeutic lymphangiogenesis is beneficial post-myocardial infarction because it leads to expedited lymphatic clearance of both excess tissue fluids and infiltrating macrophages. As a result, cardiac fibrosis, remodeling, and dysfunction are reduced. Our findings provide new mechanistic insight into the pathophysiology of cardiac remodeling and dysfunction, and open up novel therapeutic approaches to treat cardiovascular diseases including, but not limited to, myocardial infarction and chronic heart failure. Notably, our data may bring renewed interest in vascular endothelial growth factor C therapy for ischemic heart disease, because they provide a new rationale for the clinical trials that were already performed with this angiogenic/lymphangiogenic growth factor 15 years ago.
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SUPPLEMENTAL MATERIAL
Supplemental methods

Preparation, characterization, and loading of albumin-alginate microparticles

Albumin-alginate microspheres were prepared using a transacylation reaction in emulsion. This method avoids the use of toxic cross-linking reagents or volatile solvents, while allowing the formation of microparticles made of a stable and biocompatible network of covalently linked protein and polysaccharide\(^1\). Briefly, 6 mL of an aqueous phase consisting of 2 % propylene glycol alginate (FMC BioPolymer) and 20 % human serum albumin (LFB) in water was emulsified in 40 mL of isopropyl myristate (SDF) at a stirring speed of 3000 rpm. After 5 min stirring, 2 mL of a 2 % NaOH solution in 95 % (v/v) ethanol (Charbonneaux-Brabant) was added and agitation was continued for 15 min to allow the transacylation reaction to occur. Then, 2 mL of an 8.5 % (v/v) acetic acid solution in ethanol was added for neutralization of the emulsion. After 15 more min, agitation was stopped and the microspheres were then washed, congealed and lyophilized.

The particles were sized by laser diffraction granulometry (Coulter) and scanning electron microscopy (LEO 982 microscope) was performed on lyophilized samples after coating with a thin layer of Au/Pd.

Microparticles were passively loaded with growth factors by imbibition, as previously described\(^2\). Briefly, rat VEGF-C\(_{152S}\) was added at the dose of 1-10 microgram per mg desiccated microparticles. After swelling of the microparticles in the VEGF-C-containing solution for 60 min at +4°C, they were resuspended in an artificial interstitial fluid mimetic release buffer as described to a final concentration of 4 mg microparticles/mL in sterile eppendorf tubes. Microparticle-suspensions were incubated at 37°C at 3 rpm and aliquots were taken every to every other day to determine the daily release levels of VEGF-C using ELISA (Reliatech).

To verify that the microparticle-released VEGF-C\(_{152S}\) retained full bioactivity, we assayed its effects on stimulation of VEGFR3-expressing PAE cell proliferation *in vitro*. Briefly, PAE-VEGFR3 cells were
plated in 12-well plates and incubated in DMEM medium supplemented with 1% fetal calf serum for cell cycle arrest 24h prior to stimulation of cell proliferation with either recombinant “fresh” VEGF-C_{C152S} (Reliatech), or the same protein released from our microparticles at the concentrations of 50 and 100 ng/mL. After 48h in culture, the released VEGF-C_{C152S} displayed the same stimulation of cell proliferation as the “fresh” growth factor (WST-1 colorimetric assay), confirming full retention of bioactivity of the microparticle-released recombinant growth factor.

For lymphangiogenic therapy, loading was performed in the same manner at the dose of 5 or 10 microgram rat VEGF-C_{C152S} per mg microparticles, and the solution was resuspended at the concentration of 7.5 mg/mL before intramyocardial injections. The total dose per rat heart was 1.5 (low dose) or 5 microgram (high dose) of VEGF-C_{C152S} distributed equally in four spots in the anterolateral LV free wall.

**Surgical model**

Left ventricular (LV) MI was induced by LCAd ligation (permanent model) or occlusion (45 min of ischemia; temporary model) in ketamine-xylazine-anaesthetized, mechanically-ventilated rats. Albumin-alginate microparticles, loaded or not with VEGF-C_{C152S}, were injected in the LV freewall after reperfusion in the temporary MI model. Total dose of VEGF-C_{C152S} was 1.5μg/heart in the low-dose group “VEGF-C_{LD}” and 5μg/heart in the high-dose group “VEGF-C_{HD}”.

**Functional evaluations**

**Echocardiography**

Non-invasive echocardiography was performed in sedated (Sodium Methohexital: 50mg/kg IP) rats at 3 and 6 weeks post-MI using a Vivid 7 ultrasound echograph equipped with a M12L linear probe operating at 14MHz and outfitted with Echopac PC software (GE medical).
**Hemodynamics**

Invasive hemodynamic assessment of cardiac function was performed by LV catheterism at 8 weeks post-MI using a Millar probe (model SPR-838) connected to a pressure-conductance unit (MPCU-200, Millar).

**MRI**

MRI analyses were performed in anaesthetized (Sodium Methohexital: 50 mg/kg IP) rats at 6 weeks post-MI (temporary model) for cardiac perfusion analysis, as previously described\(^2\), and for T2-mapping of cardiac water content using a 4.7 T horizontal bore scanner (Bruker). Briefly, after adiabatic double inversion pulses to cancel the blood signal in the cavities and the blood flow T2 contribution, a single slice 12 spin echo readout was acquired in short axis views located at the base of the papillary muscles. The acquisition was synchronized with cardiac rhythm to acquire the signal only during diastole. The T2 map was generated after exponential regression using paravision 5.1 software.

**Lymphangiography**

Invasive cardiac lymphangiography was performed in anaesthetized (ketamine-xylazine) rats at 4, 8 or 12 weeks following MI (permanent and temporary MI models). Briefly, 10 μL fluorescent quantum dots (20 pmol QDot\(_{655}\), Molecular Probes) was injected intramyocardially in the apex of the heart. After 20 minutes, 0.8 mL of a 30 mg/mL solution of FITC-dextran (\(M_w\: 500\) kDa, Sigma-Aldrich) was injected intravenously, followed by animal sacrifice and extraction of the heart subsequently arrested in diastole and mounted for macroconfocal imaging (Leica). Anterolateral cardiac views were acquired by assembling partially overlapping fields. Cardiac areas lacking quantum dot signals were omitted from imaging to expedite the process (about 8-10 microscopic fields required for full anterior view of the heart with 10 min. acquisition time per field) generating occasionally only partial views of the heart. 3D images were assembled using Image J software (NIH) for reconstruction of perfused cardiac blood vasculature and quantum dot-filled epicardial draining lymphatics.
**Structural and molecular evaluations**

**Western Blot**

Cardiac tissue samples (LV infarct, freewall and septum pooled) were extracted using a standard Tris SDS lysis buffer including protease inhibitors. Bradford method was used to determine total protein concentrations. Samples were denatured for 10 min at 95°C, and 150 or 300 μg protein per sample were separated by 4-12% bis-Tris SDS-PAGE, followed by transfer to either 0.22 μm (VEGF-A, VEGF-C) or 0.45 μm (VEGFR3) HyBond ECL membranes (Amersham Biosciences, Uppsala, Sweden) during 90 min at 100 V (Mini Trans-Blot Cell; Bio-Rad Laboratories). Membranes were incubated over night at +4°C with primary antibodies diluted in PBS/milk (table 1). After repeated washing, horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, goat anti-rat, or goat anti-mouse, Jackson ImmunoResearch Laboratories) were used. Target proteins were visualized using an ECLplus chemiluminescence kit (Lumi-Light, Roche Molecular Biochemicals) and bands were analyzed using Image J software. For VEGF-A blots, several bands were visible (notably in sham rats) ranging from 18-28 kD in size. These correspond to rat VEGF-A 120, 164 and 188 a.a. isoforms. All three bands were included in the densitometric analyses to quantify total VEGF-A. Absolute densitometric levels were normalized to beta-actin, probed after brief incubation of membranes in stripping buffer (GeneBio Application), and reported values represent averages of duplicate samples run on separate gels (n=3-10 rats/group) expressed as fold of sham levels.

**Table 1. Antibodies used for Western Blot:**

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<tr>
<th>antigen</th>
<th>code</th>
<th>supplier</th>
<th>species reactivity</th>
<th>host</th>
<th>dilution</th>
<th>target size</th>
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<td>VEGF-C</td>
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<td>rabbit</td>
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<td>VEGFR3</td>
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<td>Abcam</td>
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<td>rat</td>
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<td>VEGF-A</td>
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<td>Santa Cruz</td>
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<td>rabbit</td>
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<td>beta-actin</td>
<td>sc-8432</td>
<td>Santa Cruz</td>
<td>human, rat</td>
<td>mouse</td>
<td>1/500</td>
<td>43 kDa</td>
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Immunohistochemistry

Cardiac sections were cut on a cryostat (8 μm or 30 μm thickness) and collected on SuperFrost plus glass slides. After fixation in acetone for 10 min, non-specific binding sites were blocked using Dako Diluent, followed by Biotin-Avidin Blocking kit (Thermo Scientific) when streptavidin (SA)-conjugates were used to detect biotinylated secondary antibodies. Primary antibodies, diluted in blocking buffer, were incubated on the sections at r.t. for 1h (table 2), followed by repeated washing in PBS and incubation with secondary antibodies for 30 minutes to 1h (table 3). Double or triple stainings were performed sequentially, and negative controls included omission of primary antibodies. Slides were mounted in Vectashield containing DAPI, and images were acquired using x5, x10, x20, or x40 objectives on a Zeiss epifluorescence microscope (AxioImager J1) equipped with an apotome and Axiovision Mosaic X software (Zeiss). Images were analyzed by an operator blinded to the treatment groups using ImageJ software (NIH).

Table 2. Primary antibodies used for immunohistochemistry:

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<th>species reactivity</th>
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<th>dilution</th>
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<td>CCL21</td>
<td>AF457</td>
<td>RnD system</td>
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<td>MCA1441</td>
<td>AbD Serotec</td>
<td>rat</td>
<td>mouse</td>
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<td>CD31/PECAM</td>
<td>555026</td>
<td>BD</td>
<td>rat</td>
<td>mouse</td>
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<td>Biorad</td>
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<td>mouse</td>
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<td>FOXC2</td>
<td>Ab55004</td>
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<td>rabbit</td>
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<td>VEGFR3</td>
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Table 3. Secondary antibodies used for immunohistochemistry:

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<td>FP-CA5570</td>
<td>Interchim fluoroprobes</td>
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<td>Donkey anti-mouse FITC</td>
<td>715-095-151</td>
<td>Jackson Immunoresearch</td>
<td>1/400</td>
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<td>Donkey anti-rabbit Cy3</td>
<td>711-165-152</td>
<td>Jackson Immunoresearch</td>
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Donkey anti-rabbit Cy5 711-605-152 Jackson Immunoresearch 1/400
Donkey anti-goat FITC A50-201D2 Bethyl Laboratories 1/400
Donkey anti-goat Cy3 A50-201D3 Bethyl Laboratories 1/400
Biotinylated donkey anti-goat RPN10251 GE Healthcare 1/300

**Image analysis of cardiac sections**

Blood vessels were defined as strongly CD31-positive structures. These clearly differed from lymphatic vessels that either lacked or displayed very weak CD31 signal. Conversely, in adult rat hearts, both sham and post-MI, blood vessel endothelial cell were consistently found not to express lymphatic markers: LYVE-1, Podoplanin, Prox-1, or VEGFR3. Blood to cardiomyocyte ratios were evaluated in sections double stained for CD31 and WGA imaged at x40. Individual endothelial cells were not counted, rather vessels structures were assessed. If two vessels were adjacent and partly overlapping, they were counted as two vessels. For one vessel that divides into two branches, two vessels were counted.

Arterioles were defined as smooth muscle actin-positive, CD31-positive vessels with a diameter <150 micrometers. Photos were captured at x10.

Lymphatic vessels were defined as strongly LYVE-1-positive structures. These clearly differed from CD68-expressing macrophages that either lacked or displayed very weak LYVE-1 signal. However, in the infarct zone (1-3 months post-MI), large, rounded CD68+ macrophages were found weakly positive for LYVE-1 and/or VEGFR3. These macrophages, also differing in size and morphology from the elongated cell body and nucleus typical of lymphatic endothelial cells, were however readily excluded from lymphatic counts in the infarct. Additional analyses with the marker Prox-1, particularly useful in the infarct (where no or only few Prox-1+ cardiomyocytes reside), revealed that these CD68+/LYVE-1+/VEGFR3+ infarct macrophages, in contrast to true CD68+/LYVE-1+/VEGFR3+ lymphatics, lack Prox-1 signal (*data not shown*). Photos were captured at x5, x10, x20 or x40.

Pre-collectors were identified as LYVE-1-positive structures co-expressing podoplanin. Intramyocardial “open” vessels (diameter >5 µm) expressed LYVE-1, VEGFR3, Podoplanin., and often CCL21, and were
preferentially located in the subepicardium, rather than in the mid-myocardium. To assess the size and frequency of open lymphatic vessels, an average of 2.2±0.2 mm² of the LV freewall, or 0.4±0.1 mm² of the infarct zone, was imaged and analyzed for each rat. The absolute density (open/mm²) was measured and used to calculate relative open density (open/mm² normalized to LYVE-1+ lymphatics/mm²; “open lymphatics % of total”). Lumen areas were measured and used to calculate mean vessel diameter. The parameter “area of open lymphatics, %” was calculated as the total sum of all measured open vessel areas divided by the total LV area analyzed for each rat.

The sizes of intramyocardial capillaries ranged from <5-15 µm diameter, precollectors 20-50 µm diameter, and collectors 50-200 µm diameter, as assessed in cardiac cryosections. In whole mount stainings of the epicardial surface layer lymphatics, vessels were larger (as not compressed in-between cardiomyocytes): blunt-ended capillaries ranged from 10-80 µm in diameter, pre-collector segments that drain these initial lymphatics ranged from 50-100 µm, and larger, valved collectors of 100-200 µm in diameter were observed.

**Whole mount**

Hearts were arrested in diastole and fixed in 3% paraformaldehyde for 6h at +4°C. Following dehydration in graded methanol baths, and post-fixation in Dent’s fixative, samples were either directly rehydrated (for fluorescence imaging) or first bleached in 3% H₂O₂ to block endogenous peroxidases (for colorimetric samples). After extensive blocking of non-specific binding sites and tissue permeation with Triton-X100, the cardiac surface lymphatics were revealed using LYVE-1 (rabbit polyclonal, Reliatech, 1/500) and/or podoplanin (mouse monoclonal, Reliatech, 1/250)-reactive antibodies visualized using either peroxidase-coupled secondary reagents (antirabbit N-Histofin, Cosmo Bio) followed by DAB colorimetric development and direct imaging under a dissecting light microscope, or using fluorescence-coupled secondary antibodies (Cy3-conjugated donkey antirabbit, 1/400, and Cy5-conjugated goat anti mouse, 1/400) followed by macroconfocal imaging (Leica). Z-stacked images were acquired from partially overlapping fields in the anterior surface of the heart. About 10-12 microscopic fields were required for a
full anterior view of the heart. With as long as 20-30 minutes acquisition time per field, only partial views of the heart were technically feasible for macroconfocal whole mount fluorescence imaging of cardiac surface lymphatics in rats. Images were assembled and 3D projected using Image J software (NIH). 3D-modeling (skeletonization) and vessel profile analyses were performed using Amira software (FEI).

Histology

Cardiac cryosections (8μm) of rat hearts were processed for Sirius Red staining as described[^32]. Infarct sizes were evaluated in 4-5 sections spanning the LV, and calculated as: (% Infarct area/ Total LV area. Cardiac interstitial collagen density (“fibrosis”) was evaluated in Sirius Red-stained sections imaged on a light microscope (Zeiss) equipped with a x20 objective. Image analysis was performed in ImageJ using an in-house developed macro-program.
Supplemental Figures
Suppl. Figure 1  Molecular profile and structure of intramyocardial lymphatics

Immunohistochemical analyses of intramyocardial lymphatics in rats revealed that only lymphatics, but not blood vessels (CD31+, red), expressed LYVE-1 (blue, a). Lymphatics ranged from small, "closed" capillaries (upper row, a), to large, "open" lymphatics (vessel lumen >5 μm, bottom row, a). The lymphatic-to-cardiomyocyte ratio, visualized by co-staining for wheat geam agglutinin (WGA, green), was about 10 times lower than the blood-to-cardiomyocyte ratio (a). VEGFR3 (red) was strongly expressed in a subset of LYVE+ vessels (b, c), but was not present in blood vessels (CD31, blue, b). In the subepicardium, many VEGFR3+ LYVE+ vessels expressed Podoplanin (green, c) and these frequently displayed "open" lumen, and were designated as precollectors. In contrast, in mid-myocardial regions of the LV lymphatic podoplanin expression and "open" lumen was less frequent, and here the lymphatic network was essentially composed of small capillaries (d). LYVE-1 (green) lymphatics, as well as surrounding cardiomyocytes, expressed Prox-1 (red, e). Scale bar = 20 μm. Photos taken using x20 or x40 objectives.
Suppl. Figure 2  Molecular profile of intramyocardial lymphatics vs. macrophages

Immunohistochemical analyses of intramyocardial lymphatics in rats revealed that only arterioles, but not lymphatics (LYVE-1+, red), were coated with smooth muscle cells (SMA, green, a). Cardiac CD11b+ macrophages (green) did not express LYVE-1 (red), but were sometimes found in the close vicinity of lymphatic vessels (b). Further, LYVE-1 CD68+ macrophages (green) were seen entering strongly LYVE-1 expressing (red) lymphatics (white asterix, c). Some CD68+ macrophages (white) were weakly positive for LYVE-1 (red, white arrow), but generally did not express VEGFR-3 (green, d, e). In the infarct scar, at 1 month post-MI, many large, rounded CD68+ macrophages (white) were found to be weakly positive for LYVE-1 (red) and/or VEGFR3 (green) (white arrowhead, e). Scale bars = 50 μm in a; 20 μm in b-e. Photos taken using x10, x20, or x40 objectives.
Suppl. Figure 3  
**Structure of epicardial surface collector lymphatics**  
Immunohistochemical whole mount analysis of rat LV surface lymphatics revealed that epicardial collecting vessels of 100-200 μm in diameter contain bicuspid valves, outlined by Prox-1 (*green*), LYVE-1 (*red*), and FOXC2 (*blue*) staining. Scale bar = 50 μm. Maximal intensity 3D projection of a 280 μm z stack image taken using a Leica macroconfocal.
Suppl. Figure 4 Rarefaction of subepicardial precollectors and absence of lymphangiogenic remodeling in mid-myocardium after MI
Schematic illustration of infarct ("I") vs subepicardial ("S") vs mid-myocardial ("M") regions in the non-infarcted LV free wall (a). LYVE-1+ lymphatics (red), cardiomyocytes (green). Examples of LYVE-1+ (red) and podoplanin+ (green) double-labeled lymphatic pre-collectors in the subepicardium of healthy (sham) rats, and at 4 or 12 weeks post-MI (b). Nuclei (blue). Mid-myocardial LYVE-1+ lymphatic density
(c), podoplanin+ LYVE-1+ pre-collector density (d), and percentage podoplanin+ (among LYVE-1+ lymphatics; e). Values represent mean ± SEM (n= 8-9 rats/group). Scale bars = 2 mm (a), or 50 μm (b).
Suppl. Figure 5  **Targeted delivery of VEGF-C<sub>C152S</sub> by albumin-alginate microparticles**

Albumin-alginate microparticles displayed an average diameter of 90±50 µm, as determined by granulometry (a), and a porous surface and interior (b), as revealed by scanning electron microscopy. Scale bar = 5 µm. The daily release rate (c) of VEGF-C<sub>C152S</sub> was proportional to the payload (amount growth factor per mg of microparticles): at 5 µg VEGF-C<sub>C152S</sub> per mg microparticles (the payload used in our in vivo study for low dose treatment) the daily release levels by 14 days of incubation was around 200 ng VEGF-C<sub>C152S</sub> per mg microparticles. In vivo, we applied 0.3 mg microparticles/heart for a total dose of 1.5 µg VEGF-C<sub>C152S</sub> per rat (“VEGF-C<sub>LD</sub>”), or 0.5 mg microparticles/heart for a total dose of 5 µg VEGF-C<sub>C152S</sub> per rat (“VEGF-C<sub>HD</sub>”). Experimental setup used in our therapeutic lymphangiogenesis study (d-e): Control and “VEGF-C” male Wistar rats were exposed to 45 min of ischemia (induced by temporary LCA occlusion), followed by 10 minutes of reperfusion, after which microparticles loaded or not with VEGF-C<sub>C152S</sub> were injected intramyocardially in 4 spots of the LV free wall. Sham operated rats were exposed to thoracotomy without tying of the snare. Structural and functional evaluations were carried out as indicated.
Suppl. Figure 6  Infarct size and infarct lymphatic remodeling during VEGF-C<sub>C152S</sub> therapy in temporary occlusion model of MI

Infarct sizes were determined in serial histological sections stained with sirius red (a-b). Infarct size, assayed at 3 and 8 weeks post-MI (n= 5-11 rats/group). Dotted line indicates average infarct size. Body weight gain was not different between groups throughout the study. Cardiac dry weight to body weight ratio (c) (n= 5-13 rats/group). Example of infarct zone podoplanin<sup>+</sup> (green) LYVE-1<sup>+</sup> (red) lymphatics at 1 month post-MI (d). Quantification of LYVE-1<sup>+</sup> lymphatic density (e), relative frequency of “open” lymphatics (diameter >5 µm) (f), “open” lymphatic mean vessel diameter (g), and total area of open lymphatics/mm² (h). (n=6-13 rats/group). Control rats (filled circles) and VEGF-C<sub>C152S</sub>-treated rats (low dose, open triangles; high dose, filled triangles) at 3 or 8 weeks post-MI. Healthy sham rats (open circle).
One-way ANOVA (f, g, h) or Kruskal Wallis (a, c, e): * $p<0.05$; *** $p<0.001$ vs sham. # $p<0.05$ as indicated. Scale bar = 500 μm.
Suppl. Figure 7  Lymphangiogenesis in mid-myocardium following VEGF-C<sub>C152S</sub> therapy
Examples of LYVE-1<sup>+</sup> (red) capillaries and few podoplanin<sup>+</sup> (green) lymphatics in the mid-myocardium of the non-infarcted LV (a). Scale bar = 50 μm. Mid-myocardial LYVE-1<sup>+</sup> lymphatic density (b), podoplanin<sup>+</sup> LYVE-1<sup>+</sup> “pre-collector” density (c), and percentage podoplanin<sup>+</sup> lymphatics (among LYVE-1<sup>+</sup> vessels; d). Dotted line indicates healthy sham levels. Control rats (filled circles) and VEGF-C<sub>C152S</sub>-treated rats (low dose, open triangles; high dose, filled triangles) at 3 or 8 weeks post-MI. Healthy sham rats (open circle). (n=5-13 rats/group) Values represent mean ± SEM. Kruskal Wallis (b-d): * p<0.05; ** p<0.01 vs sham. Example of LYVE-1<sup>+</sup> podoplanin<sup>+</sup> lymphatics (ranging from blunt-ended ‘terminal’ capillaries to larger pre-collectors and collector lymphatics) in the anterior cardiac surface of a healthy, adult rat as visualized by macroconfocal whole mount fluorescence imaging (e). White arrows point to weakly LYVE-1<sup>+</sup> podoplanin<sup>+</sup> lymphatic collectors endowed with valves. White arrow head point to unidentified podoplanin<sup>+</sup> structures that lack LYVE-1 expression. Scale bar = 500 μm.
Suppl. Figure 8  Cardiac lymphatic CCL21 expression following VEGF-C\textsubscript{C152S} therapy

Examples of CCL21 expression (green) in LYVE-1\textsuperscript{+} (red) lymphatics in the subepicardium of the non-infarcted LV (a). Note the grainy appearance of the CCL21 signal, representing lymphatic secretion (yellow) and extracellular matrix deposition (green) of the chemokine. Scale bar = 20 μm. Photos taken at x20. Quantification of lymphatic CCL21 expression (presented as % of CCL21 positive LYVE-1\textsuperscript{+} vessels) among all subepicardial lymphatics in the non-infarct LV freewall (b), or in the infarct scar lymphatics (c). Dotted line indicates healthy sham levels. Control rats (filled circles) and VEGF-C\textsubscript{C152S}-treated rats (low dose, open triangles; high dose, filled triangles) at 3 weeks post-MI. Healthy sham rats (open circle). (n=4-10 rats/group). Values represent mean ± SEM. Kruskal Wallis (b, c): $p=0.07$ vs sham.
Supplemental References
