Targeting Lymphatic Vessel Activation and CCL21 Production by Vascular Endothelial Growth Factor Receptor-3 Inhibition Has Novel Immunomodulatory and Antiarteriosclerotic Effects in Cardiac Allografts

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Background—Lymphatic network and chemokine-mediated signals are essential for leukocyte traffic during the proximal steps of alloimmune response. We aimed to determine the role of lymphatic vessels and their principal growth signaling pathway, vascular endothelial growth factor (VEGF)-C/D/VEGFR-3, during acute and chronic rejection in cardiac allografts.

Methods and Results—Analysis of heterotopically transplanted rat cardiac allografts showed that chronic rejection increased VEGF-C+ inflammatory cell and hyaluronan receptor-1 (LYVE-1)+ lymphatic vessel density. Allograft lymphatic vessels were VEGFR-3+, contained antigen-presenting cells, and produced dendritic cell chemokine CCL21. Experiments with VEGFR-3/LacZ mice or mice with green fluorescent protein–positive bone marrow cells as cardiac allograft recipients showed that allograft lymphatic vessels originated almost exclusively from donor cells. Intraportal adenoviral VEGFR-3-Ig (Ad.VEGFR-3-Ig/VEGF-C/D-Trap) perfusion was used to inhibit VEGF-C/D/VEGFR-3 signaling. Recipient treatment with Ad.VEGFR-3-Ig prolonged rat cardiac allograft survival. Ad.VEGFR-3-Ig did not affect allograft lymphangiogenesis but was linked to reduced CCL21 production and CD8+ effector cell entry in the allograft. Concomitantly, Ad.VEGFR-3-Ig reduced OX62+ dendritic cell recruitment and increased transcription factor Foxp3 expression in the spleen. In separate experiments, treatment with a neutralizing monoclonal VEGFR-3 antibody reduced arteriosclerosis, the number of activated lymphatic vessels expressing VEGFR-3 and CCL21, and graft-infiltrating CD4+ T cells in chronically rejecting mouse cardiac allografts.

Conclusions—These results show that VEGFR-3 participates in immune cell traffic from peripheral tissues to secondary lymphoid organs by regulating allograft lymphatic vessel CCL21 production and suggest VEGFR-3 inhibition as a novel lymphatic vessel–targeted immunomodulatory therapy for cardiac allograft rejection and arteriosclerosis. (Circulation. 2010;121:1413-1422.)

Key Words: angiogenesis ■ arteriosclerosis ■ endothelium ■ immune system ■ transplantation

A fter transplantation, interaction of innate and adaptive immunity leads to alloimmune response that may be detrimental to cardiac allografts and heart transplant recipients.1–3 The encounter of antigen-presenting cells (APCs) and T lymphocytes in secondary lymphoid organs is essential for the initiation of an alloimmune response1–3; therefore, intervening with leukocyte traffic could modulate alloimmunity. Although the currently used immunosuppressive drugs effectively inhibit proliferation of alloreactive T cells, they have several metabolic, infectious, renal, and malignant side effects4 and have not prevented the development of cardiac allograft arteriosclerosis, the main limitation of long-term survival of heart transplant patients.5

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The lymphatic network forms a link between the innate and adaptive immunity. Lymphatic vessels are thin walled and contain valves that provide easy access for inflammatory cells and their unilateral movement from peripheral tissues to secondary lymphoid organs.6 In addition to these structural
properties, lymphatic endothelial cells (ECs) also have distinct molecular properties that enhance the exit of APCs from peripheral tissues. In contrast to vascular ECs, lymphatic ECs produce CCL21, a chemokine that attracts activated CCR7+ dendritic cells (DCs).8 In experimental transplantation models, CCL21/CCR7 signaling has been shown to regulate DC traffic to secondary lymphoid organs and the development of alloimmune response.9,10

Vascular endothelial growth factor (VEGF) -C and -D and their receptor (VEGFR-3) have been identified as key molecular regulators of lymphatic vessel growth and maintenance.11,12 Excessive lymphangiogenesis is often seen during chronic inflammation,13-16 and macrophages are a rich source of VEGF-C.17 Lymphangiogenesis also occurs in kidney transplants,14 but so far, the functional effects of lymphatic vessels and their growth factors in solid organ transplantation have remained unknown.

In this study, we investigated the role of lymphatic vessels and VEGF-C/D/VEGFR-3 signaling in cardiac allografts. We found chronic cardiac allograft inflammation to be a potent stimulus for allograft VEGF-C expression and lymphangiogenesis. In addition, we found that VEGF-C/VEGFR-3 signaling regulates allograft CCL21 production, DC traffic to spleen, and initiation of alloimmune responses. Our results thus suggest VEGFR-3 inhibition as a novel lymphatic vessel–targeted immunomodulatory therapy after solid organ transplantation.

Methods

Please see the online-only Data Supplement for an expanded Materials and Methods.

Evaluation of Lymphangiogenesis in Acutely and Chronically Rejecting Cardiac Allografts

LYVE-1+ and VEGFR-3+ lymphatic vessel density was determined from normal Dark Agouti (DA) rat hearts, heterotopically transplanted acutely (no immunosuppression, 5 days) and chronically (cyclosporine A immunosuppression, 8 weeks) rejecting allografts (DA->Wistar Furth), and their respective syngeneic controls (DA->DA). Double immunofluorescence stainings with antibodies against lymphatic EC transcription factor Prox-1 and rat vascular EC antigen-1 were performed to differentiate vascular and lymphatic ECs. VEGF-C and -D levels were determined by immunohistochemistry and quantitative reverse-transcriptase polymerase chain reaction (RT-PCR).

Origin of Allograft Lymphatic ECs

Balb/c hearts were transplanted to 2 different marker gene mouse strains (C57/BL6/J mice with green fluorescence protein–positive [GFP+] bone marrow [BM] cells and VEGFR-3/LacZ mice with C57/BL6/J background) with tacrolimus immunosuppression to investigate whether BM-derived or recipient-derived VEGFR-3+ cells contribute to lymphangiogenesis of chronically rejecting cardiac allografts at 8 weeks.

Effect of Soluble VEGFR-3-Ig on Rat Cardiac Allograft Survival

Rat cardiac allograft recipients were perfused intraportally with adenovirus (Ad) vector encoding GFP or Ad.VEGFR-3-Ig (VEGF-C/D-Trap) at the time of transplantation and received low-dose cyclosporine A immunosuppression. For mechanistic analysis, recipient allografts and spleens were harvested at 5 days. In graft survival studies, cardiac allograft function was analyzed by daily palpation, and the grafts were removed when the heart rate was <30 bpm or 8 weeks after transplantation.

Effect of VEGFR-3 Monoclonal Antibody on Mouse Chronic Rejection

BALB/c hearts were transplanted to C57BL/6J recipients and treated with rat immunoglobulin G (IgG) or rat anti-mouse VEGFR-3–neutralizing antibody (mF4–31C1; ImClone, New York, NY) 800 μg IP every third day for 4 weeks and with tacrolimus immunosuppression.18 Allografts were harvested at 8 weeks for immunohistochemical and histological analyses.

Histology

Allograft arteriosclerosis was determined from paraformaldehyde-fixed paraffin sections stained with hematoxylin and eosin and Resorcin fuchsin for internal elastic lamina using computer-assisted image processing (Axiovision 4.4; Carl Zeiss, Oberkochen, Germany).

Immunohistochemistry

Cryostat sections were stained with the peroxidase ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, Calif), and the reaction was revealed by 3-amino-9-ethylcarbazole (Vector Laboratories). Immunofluorescence double stainings were performed with Alexa Fluor 488 (green) and Alexa Fluor 568 (red; Promega, Madison, Wis) secondary antibodies. Please see the online-only Data Supplement for detailed antibody information.

Real-Time PCR

Real-time RT-PCR reactions were carried out in a Rotor-Gene 6000 (Qiagen, Venlo, the Netherlands), and the results are given in relation to 18S ribosomal RNA (rRNA) molecule numbers. Please see the online-only Data Supplement for detailed primer information.

Flow Cytometry

Spleen cells were stained with FITC- and phycocyanin-conjugated antibodies and analyzed with a FACScan (Becton Dickinson, San Jose, Calif) flow cytometer. Please see the online-only Data Supplement for detailed antibody information.

Statistics

All data are given as mean±SEM and analyzed by nonparametric Mann–Whitney U test or by log-rank test with SPSS for Windows version 17.0 (SPSS Inc, Chicago, Ill). Values of P<0.05 were regarded as statistically significant. The n values for each experiment are given in the figure legends.

Results

Chronic Rejection Induces Myocardial Lymphangiogenesis in Rat Cardiac Allografts

Lymphatic vessel densities were compared between normal rat hearts, acutely and chronically rejecting cardiac allografts, and their syngeneic controls, with lymphatic endothelium-specific hyaluronan receptor-1 (LYVE-1) used as a marker for lymphatic vessels19 (Figure 1). We found small and large LYVE-1+ vessel structures in the myocardium of nontransplanted (Figure 1A) and transplanted (Figure 1B and C) hearts. These vessels opened to larger epicardial collecting LYVE-1+ lymphatic vessels (Figure 1C, arrow). In normal hearts and syngeneic controls, the density of LYVE-1+ lymphatic vessels was 2 times higher in the epicardium (Figure 1E) than in the myocardium (Figure 1D). Acute
rejection decreased the epicardial lymphatic vessel density \((P=0.001; \text{Figure 1E})\), possibly because of lymphatic vessel destruction during intense inflammation. Chronic rejection doubled the myocardial LYVE-1\(^+\) lymphatic vessel density \((P=0.01; \text{Figure 1D})\), suggesting active lymphangiogenesis during chronic allograft inflammation.

Lymphatic EC transcription factor Prox-1 expression was detected in the nucleus of the LYVE-1\(^+\) cells (Figure 1F, arrows); the lymphatic phenotype was further supported by the observations that LYVE-1 and rat vascular EC antigen-1 were not expressed in the same vessels (Figure 1G). CD4\(^+\) (data not shown) and CD8\(^+\) T cells (Figure 1H) were detected mainly outside the LYVE-1\(^+\) lymphatic vessels. ED1\(^+\) macrophages (Figure 1I) and OX-62\(^+\) DCs (Figure 1J) were found both outside and inside (Figure 1I and 1J, arrows) the LYVE-1\(^+\) vascular structures, indicating that the lymphatic vessels in cardiac allografts are functional in transferring APCs.

**Macrophages and CD4\(^+\) T Cells Are the Major Sources of the Predominant Lymphangiogenic VEGFR-3 Ligand VEGF-C in Rat Cardiac Allografts**

We next investigated the presence of the potent lymphangiogenic cytokines VEGF-C and -D in transplanted rat hearts. VEGF-C mRNA levels were increased in chronically rejecting cardiac allografts compared with their syngeneic controls \((P=0.032; \text{Figure 2A})\). VEGF-C was expressed mainly in graft-infiltrating mononuclear cells (Figure 2C, arrows), and VEGF-C\(^+\) cell density was increased during chronic rejection \((P=0.016; \text{Figure 2B})\).

Subsets of ED1\(^+\) macrophages (Figure 2D, arrows) and CD4\(^+\) T cells (Figure 2E, arrows) were VEGF-C\(^+\), whereas CD8\(^+\) T cells were VEGF-C\(^-\) (Figure 2F), indicating that macrophages and CD4\(^+\) T cells are the major sources of VEGF-C during chronic rejection. VEGF-D mRNA levels were not significantly increased in acutely or chronically rejecting cardiac allografts (Figure 2G) compared with their syngeneic controls.

**VEGFR-3 Is Expressed in Lymphatic ECs and a Subset of DCs in Rat Cardiac Allografts**

The expression of VEGFR-3, the receptor for VEGF-C and -D, in transplanted rat hearts was then determined. We detected VEGFR-3 immunoreactivity in lymphatic vessels of nontransplanted (Figure 3A) and transplanted (Figure 3B; chronic rejection) hearts. Mononuclear cells were encountered inside the VEGFR-3\(^+\) vessels of cardiac allografts (Figure 3B, arrows); VEGFR-3\(^-\) vessel density was generally lower in the myocardium (Figure 3C) than in the epicardial area (Figure 3D) and was 3 times higher in chronically rejecting cardiac allografts than in syngeneic controls \((P=0.095; \text{Figure 3C})\).

Vessel VEGFR-3 immunoreactivity colocalized with LYVE-1 expression (Figure 3E), although not all LYVE-1\(^+\) vessels expressed VEGFR-3. In addition, lower
VEGFR-3 immunoreactivity was detected in allograft-infiltrating OX-62+ DCs (Figure 3F, arrow) and in very few ED1+ macrophages (Figure 3G, arrow). Macrophages were often encountered inside the VEGFR-3+ lymphatic vessels (Figure 3H, arrow). These findings indicate that VEGFR-3 is expressed in lymphatic vessels and a subset of APCs in cardiac allografts.

**Most VEGFR-3**+ **Lymphatic ECs in Chronically Rejecting Mouse Cardiac Allografts Are Donor Derived**

Circulating lymphatic endothelial progenitor cells contribute to lymphangiogenesis in inflamed cornea\(^{15}\) and in human kidney transplants.\(^{14}\) We next used 2 different marker gene mouse strains as cardiac allograft recipients to determine whether recipient-derived cells contribute to lymphangiogenesis in chronically rejecting cardiac allografts with tacrolimus immunosuppression at 8 weeks. In wild-type allografts transplanted to mice with GFP/H11001 cells, GFP/CXCR4/VEGFR-3 cells localized mainly around VEGFR-3+ lymphatic vessels (Figure 4A). Fewer than 4% of the BM-derived GFP+ cells colocalized with VEGFR-3+ lymphatic ECs (Figure 4B, arrows). Because these GFP+ cells may be BM-derived inflammatory cells migrating to the VEGFR-3+ lymphatic vessels, we next used mice that express LacZ under the VEGFR-3 promoter (VEGFR-3/LacZ) as cardiac allograft recipients. X-gal staining revealed lymphatic endothelial VEGFR-3 expression in the VEGFR-3/LacZ recipients’ own nontransplanted heart (Figure 4C). In contrast, no recipient-derived VEGFR-3+ lymphatic ECs were encountered in wild-type cardiac allografts transplanted to VEGFR-3/LacZ recipients (Figure 4D), indicating that the replacement of cardiac allograft lymphatic ECs with recipient-derived progenitor cells is very rare in this chronic rejection model.

**Treatment With Soluble VEGFR-3-Ig Improves Rat Cardiac Allograft Survival**

To investigate the effect of VEGFR-3 inhibition on cardiac allograft survival, recipient rats were injected intraperitoneally with Ad.GFP or the soluble form of VEGFR-3 (Ad.VEGFR-3-Ig, VEGF-C/D-trap) at the time of heart transplantation and treated with daily low-dose cyclosporine A immunosuppression. At 5 days, GFP immunoreactivity was found in the liver of Ad.GFP-perfused recipients (Figure 6A). The serum level of VEGFR-3-Ig reached 1 μg/mL at 5 days (Figure 6B) and...
remained elevated for at least 21 days in Ad.VEGFR-3-Ig–perfused recipients. Ad.VEGFR-3-Ig markedly prolonged cardiac allograft survival compared with Ad.GFP-perfused allografts ($P_{\text{H11005}}=0.026$; Figure 6C).

**VEGFR-3 Inhibition Decreases CD8$^+$ Effector Cell Recruitment to the Allograft and Alloimmune Response**

To clarify the underlying mechanisms behind the beneficial effect of VEGFR-3 inhibition on allograft survival, Ad.GFP- and Ad.VEGFR-3-Ig–treated allografts under cyclosporine A immunosuppression were harvested 5 days after transplantation. Ad.VEGFR-3-Ig significantly decreased the number of graft-infiltrating ED1$^+$ macrophages ($P_{\text{H11005}}=0.035$; Figure 6D) and CD8$^+$ T cells ($P_{\text{H11005}}=0.007$; Figure 6E), as well as alloimmune activation in the form of allograft interleukin (IL)-2R$^+$ ($P_{\text{H11005}}=0.026$; Figure 6H) and myosin heavy chain class II ($P_{\text{H11005}}=0.038$; Figure 6I) expression. No significant changes were seen in graft-infiltrating CD4$^+$ T cells (Figure 6F) or OX-62$^+$ DCs (Figure 6G) or in the density of LYVE-1$^+$ vessels.

**Figure 3.** Lymphatic ECs and DCs express VEGFR-3 in rat cardiac allografts. VEGFR-3 immunoreactivity was detected in epicardial lymphatic–like vessels of normal (A, arrows) and transplanted (B, chronic rejection) hearts and mononuclear cells inside the VEGFR-3$^+$ vessels of cardiac allografts (B, arrows). VEGFR-3$^+$ vessel density was generally higher in epicardium (D) than in myocardium (C), and VEGFR-3 immunoreactivity colocalized mainly with LYVE-1$^+$ vessels (E). A subset of OX-62$^+$ DCs (F, arrow) and few ED1$^+$ macrophages (G, arrow) were VEGFR-3$^+$, and ED1$^+$ macrophages were found inside VEGFR-3$^+$ lymphatic vessels (H, arrow). Mayer hemalum counterstaining (A and B). No immunoreactivity in IgG control (A, inset). Data are as in Figure 1. $n_{\text{H11005}}=5$ to 6 per group. Scale bars$=50$ μm.

**Figure 4.** Cardiac allograft VEGFR-3$^+$ lymphatic ECs are mainly donor derived. Balb/c cardiac allografts were transplanted to either C57BL/6J mice with GFP$^+$ BM cells (A and B, $n=3$) or VEGFR-3/LacZ mice with C57BL/6J background (C and D, $n=3$) using suboptimal tacrolimus immunosuppression to identify recipient-derived cells in chronically rejecting cardiac allografts. At 8 weeks, GFP$^+$ cells were found mainly around cardiac allograft VEGFR-3$^+$ lymphatic vessels (A), and very few GFP$^+$ cells colocalized with VEGFR-3$^+$ lymphatic ECs (B, arrows). VEGFR-3$^+$ lymphatic ECs were detected with X-gal staining in VEGFR-3/LacZ recipients' own nontransplanted heart (C) but not in wild-type (WT) cardiac allografts transplanted (TX) to VEGFR-3/LacZ recipients (D). Nuclear Fast Red counterstaining (C and D). Scale bars$=50$ μm.
VEGFR-3 Inhibition Decreased Allograft CCL21 Production

Real-time RT-PCR analysis of the transplanted hearts at 5 days was carried out to determine the effect of VEGFR-3 inhibition on the CCR7+ DC chemokines CCL21 and CCL19 and the phenotype of allograft T cells (Figure 6J). Ad.VEGFR-3-Ig decreased allograft CCL21 mRNA expression (P=0.026) but not CCL19 or CCR7 expression (Figure 6J). Ad.VEGFR-3-Ig increased Th2 transcription factor Gata-3 mRNA (P=0.038; Figure 6J), but no significant changes were observed in allograft mRNA levels of Treg transcription factor Foxp3, Th1 factors T-bet, IL-2, interferon-γ, Th2 cytokines IL-4 or IL-10, or Th17 cytokines IL-17 or IL-23 (Figure 6J). These results indicate that VEGFR-3 inhibition reduced allograft CCL21 production while not having a clear effect on allograft T-cell phenotype.

VEGFR-3 Inhibition Decreases DC Recruitment to the Spleen

We next investigated whether Ad.VEGFR-3-Ig treatment impaired APC recruitment to secondary lymphoid organs of the recipient at 5 days. Fluorescence-activated cell sorter analysis of spleen leukocytes showed that Ad.VEGFR-3-Ig decreased the recruitment of OX-62+ DCs to the recipient spleen (P=0.038; Figure 7A and 7B), suggesting that VEGFR-3 regulates APC traffic out from the allograft to secondary lymphoid organs.

VEGFR-3 Inhibition Regulates Chemokine Balance Between Cardiac Allograft and the Spleen

In contrast to the results in the transplanted heart (Figure 6J), treatment with Ad.VEGFR-3-Ig resulted in 1.5-times-higher CCL21 mRNA levels in the spleen (Figure 7C) and increased the ratio of spleen to allograft CCL21 mRNA from 9:1 to 23:1 at 5 days. Ad.VEGFR-3 also increased the levels of the Treg transcription factor Foxp3 mRNA (P=0.038; Figure 7C). No significant changes were observed in allograft mRNA levels of Th1, Th2, or Th17 transcription factors or cytokines (Figure 7C). These results suggest that Ad.VEGFR-3-Ig treatment regulates the CCL21 chemokine gradient between the allograft and secondary lymphoid organs in favor of attenuated immune response.

VEGFR-3–Neutralizing Monoclonal Antibody Decreases Lymphatic Vessel Activation, Intragraft Inflammation, and Arteriosclerosis in Chronically Rejecting Mouse Cardiac Allografts

Because VEGF-C and VEGF-D are ligands for both VEGFR-2 and VEGFR-3, Ad.VEGFR-3-Ig treatment may interfere with VEGFR-2 signaling in addition to VEGFR-3 signaling. To confirm that VEGFR-3 inhibition has direct effects in transplanted hearts, we used a mouse chronic rejection heart transplantation model with tacrolimus immunosuppression and treated recipients for 4 weeks with rat IgG or monoclonal neutralizing antibody against VEGFR-3. At 8 weeks, VEGFR-3 antibody treatment did not affect the number of LYVE-1+ lymphatic vessels (Figure 8A) or CD31+ capillaries (Figure 8E) but reduced the density of VEGFR-3− (P=0.014; Figure 8B) and CCL-21+ (P=0.051; Figure 8C) lymphatic vessels in the allograft. In addition, VEGFR-3 inhibition reduced the number of graft-infiltrating CD4+ cells (P=0.022; Figure 8F) and decreased arterial occlusion (P=0.037; Figure 8D). Our results thus show that VEGFR-3 inhibition reduced lymphatic vessel activation and chronic rejection without affecting lymphangiogenesis or angiogenesis.

Finally, because lymphoid neogenesis (organization of chronic inflammatory infiltrates into functional ectopic germinal centers or tertiary lymphoid organs) has been linked with chronic rejection,22,23 we investigated the presence of tertiary lymphoid organs in our chronically rejecting cardiac allografts. Immunohistochemical stainings revealed a characteristic pattern of peripheral node adressin-positive high endothelial venules (MECA-79) and discrete B- (B220) and T-cell (CD3) accumulation in only 1 allograft in the control group (Figure 8I through 8K). This implies that lymphoid neogenesis is not a critical phenomenon in our chronic rejection model and thus the effect of VEGFR-3 inhibition on TLO formation cannot be evaluated.

Discussion

Transfer of APCs from vascularized allografts to secondary lymphoid organs—both spleen and lymph nodes—is critical...
for the priming of alloreactive T cells and the development of alloimmune responses. Here, we investigated how lymphatic vessels of allografts participate in leukocyte traffic and whether the recently characterized lymphatic growth factors could modulate alloimmunity and thus offer a novel site for therapeutic intervention.

Active lymphangiogenesis has previously been reported in human kidney transplants with nodular inflammatory infil-
trates and in other inflammatory conditions. In addition, lymphatic vessels have been detected in association with subendocardial lymphonodular aggregates (Quilty lesions), and alteration of lymphatic EC markers has been observed during the first year after heart transplantation. Here, we found that chronic rejection increased lymphangiogenesis in rat cardiac allografts. The lymphatic vessels contained APCs and expressed the potent leuko-
cyte chemokine CCL21, also seen in kidney transplants, indicating that the allograft lymphatic vessels are func-
tional and active. In accordance with previous find-
ings, our results thus outline an emerging concept of transplantation-associated lymphangiogenesis.

It was recently shown that circulating recipient-derived lymphatic EC progenitor cells participate in lymphangiogenesis of human kidney allografts. Here, we found that recipient-derived cells only minimally contribute to the direct formation of VEGFR-3· lymphatic vessels in cardiac allografts. Kerjaschki et al found that ~13% of Prox-1· lymphatic vessels in rejected and nephrectomized kidney transplants originated from the recipient; thus, it is possible that the involvement of recipient-derived lymphatic progen-
itors is dependent on the severity of allograft injury. Simi-
larly, the degree of injury may determine whether allograft vascular ECs originate from the recipient, as in aortic trans-
plantation, or from the donor, as in cardiac allografts. In addition, the actual effect of lymphatic EC chimerism of transplanted organs on alloimmune responses remains unknown.

The existence of lymphangiogenesis raises a question about the presence of lymphatic EC growth factors in allografts and whether they modulate the function of lymphatic vessels. Interestingly, we found that transplantation-related lymphangiogenesis was accompanied with an increase in VEGF-C levels and that graft-infiltrating macrophages and CD4· T cells were the main source of VEGF-C. Because VEGF-D levels were not significantly changed, our results suggest an interplay of chronic rejection, VEGF-C/VEGFR-3 signaling, and growth of functional lymphatic vessels in cardiac allografts.

In adults, VEGFR-3 is found mainly in lymphatic ECs but also in DCs in corneal transplants. In the present study, VEGFR-3 was expressed mainly in cardiac allograft lymphatic ECs but also in a subset of DCs and macrophages. This suggests that VEGF-C could have effects on both VEGFR-3· APCs and VEGFR-3· lymphatic vessels in cardiac allografts.
Chen et al. have shown that VEGFR-3 inhibition impairs DC migration to draining lymph nodes and improves the survival of corneal transplants. These effects were considered to be mediated through direct inhibition of VEGFR-3 DC migration independently of lymphangiogenic effects. Here, we found that VEGFR-3 inhibition decreased DC recruitment to spleen and alloimmune response and improved cardiac allograft survival. In addition, treatment with neutralizing monoclonal VEGFR-3 antibody decreased allograft inflammation and development of arteriosclerosis in chronic rejection model. Because we found VEGFR-3 DCs in cardiac allografts, it is possible that VEGFR-3 inhibition had direct effects on DC migration in the present study similar to the corneal transplantation model observations.

In addition to these direct DC-mediated effects, our findings suggest that VEGFR-3 also regulates leukocyte traffic and alloimmunity through direct effects on allograft VEGFR-3 lymphatic vessels. Specifically, cardiac allograft VEGFR-3 lymphatic ECs produced CCL21, an important chemokine that orchestrates the homing of activated CCR7 TAPCs to secondary lymphoid tissue, and VEGFR-3 inhibition decreased allograft CCL21 production. Interestingly, CCL21 is produced by lymphatic ECs in human kidney transplants, and the CCL21/CCR7 pathway is important in leukocyte traffic and development of alloimmune response in experimental transplant models. Because we found that VEGFR-3 inhibition decreased allograft lymphatic vessel CCL21 production and DC recruitment to the spleen but did not decrease allograft CCR7 levels, it is tempting to speculate that VEGFR-3 inhibition reduced CCL21-mediated recruitment of CCR7 TAPCs to allograft VEGFR-3 lymphatic vessels and their homing to the secondary lymphoid organs. Recently, VEGF-C/VEGFR-3 signaling was shown to regulate CCL21-mediated recruitment of CCR7 tumor cells to lymphatic vessels. Therefore, VEGF-C/VEGFR-3–regulated lymphatic vessel CCL21 production may control both lymphatic spread of tumour cells and traffic of immune cells.

Surprisingly, in contrast to the reduced CCL21 levels in the cardiac allograft, VEGFR-3 inhibition did not decrease CCL21 mRNA in the spleen. In addition, the overall effect of VEGFR-3 inhibition was reduced alloimmunity and elevated spleen production of Foxp3, an important transcription factor in Tregs. Because recent reports show that CCL21 plays a vital role in homing, localization, and function of Tregs, it is possible that VEGFR-3 inhibition resulted in a suitable cytokine milieu for Treg recruitment to the spleen to inhibit alloimmune activation. The finding that VEGFR-3 inhibition simultaneously reduced cardiac allograft CCL21 levels and increased spleen CCL21 production suggests that CCL21 is differently regulated in peripheral tissues and secondary lymphoid organs. In support for this, it has been shown that of the 2 CCL21 genes, CCL21-Ser is dominant in the spleen and regulated by LT-α whereas CCL21-Leu is preferentially expressed in the lung in an LT-α–independent fashion.

**Conclusions**

Our present results suggest that VEGF-C/VEGFR-3 signaling has important effects on proximal events in cardiac allograft
allograft arteriosclerosis. This is possibly mediated through direct effects on VEGFR-3+ DCs, together with effects on allograft lymphatic vessel activation, CCL21 production, and leukocyte traffic. Therefore, VEGFR-3 inhibition could be used as a novel lymphatic vessel–targeted immunomodulatory therapy to regulate alloimmune activation after solid organ transplantation.

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Disclosures
Dr Pytowski is an employee of ImmClone Systems Inc. Drs Nykanen, Alitalo, and Lemström have filed a patent application on growth factor antagonists for organ transplant alloimmunity and arteriosclerosis. The other authors report no conflicts.

References
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**CLINICAL PERSPECTIVE**

Despite very good short-term results, the long-term survival of cardiac allograft recipients has not improved. Although the currently used immunosuppressive drugs efficiently inhibit the proliferation of alloreactive T cells, they have severe side effects such as infections, malignancies, and metabolic problems and do not prevent the development of cardiac allograft arteriosclerosis, a manifestation of chronic rejection. Here, we used rat and mouse heart transplantation models to investigate whether lymphatic vessel–targeted therapies modulate the exit of antigen-presenting cells from the allograft to secondary lymphoid organs and the development of acute and chronic rejection. Chronic inflammation increased the expression of allograft lymphatic vessel growth factor (VEGF-C) and induced lymphangiogenesis in rat cardiac allografts. Lymphatic vessels were active and functional in that they contained antigen-presenting cells and expressed dendritic cell chemokine CCL21 and VEGF-C receptor VEGFR-3. Inhibition of VEGFR-3 with gene therapy or monoclonal antibodies decreased allograft CCL21 production, dendritic cell traffic to the secondary lymphoid organs, and the development of acute and chronic cardiac allograft rejection. These results suggest VEGFR-3 inhibition as a novel lymphatic vessel–targeted immunomodulatory therapy for cardiac allograft rejection and indicate an active role for lymphatic vessels in inflammation-driven arteriosclerosis.
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SUPPLEMENTAL MATERIAL

Targeting lymphatic vessel activation and CCL21 production by VEGFR-3 inhibition has novel immunomodulatory and anti-arteriosclerotic effects in cardiac allografts

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Nykänen: VEGFR-3 in cardiac allograft rejection

Expanded Materials and Methods

Evaluation of lymphangiogenesis in acutely and chronically-rejecting cardiac allografts. Inbred male Dark Agouti (DA, RT1^av1) and Wistar Furth (WF, RT1^u) rats (Scanbur, Sollentuna, Sweden) 2-3 months of age were used. In the acute rejection model, no immunosuppression was used, and syngrafts (DA->DA) and allografts (DA->WF) were harvested at 5 days. In the chronic rejection model, recipients received cyclosporine A (CsA; Novartis, Basel, Switzerland) diluted in Intralipid (Fresenius Kabi, Bad Homburg, Germany) 2 mg/kg/d s.c. for the first week and 1 mg/kg/d thereafter, and the grafts were harvested at 8 weeks. Permission for animal experiments was obtained from the State Provincial Office of Southern Finland. All animals received care in compliance with the “Guide for the Care and
Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Academy Press (ISBN 0-309-05377-3, revised 1996). Lymphatic vessels and their growth factors were characterized and quantified with immunohistochemistry, and VEGF-C and VEGF-D mRNA levels also with quantitative RT-PCR.

**Origin of allograft lymphatic ECs.** To study recipient-derived VEGFR-3 expression in the transplanted heart, Balb/c hearts were transplanted to mice that express LacZ under VEGFR-3 promoter.\(^1\) Replacement of allograft lymphatic ECs with BM-derived cells was investigated using C57 mice that had received a BM transplant from GFP-expressing syngeneic mice as Balb recipients. Recipients received tacrolimus (Astellas Pharma, Tokyo, Japan) 3.0 mg/kg/d s.c. for the first week and 1.5 mg/kg/d thereafter.\(^2\) Grafts were harvested at 8 weeks, incubated in 2% paraformaldehyde for 30 min and in 20% sucrose overnight, embedded in TissueTek O.C.T. (Sakura Finetek Europe, Zoeterwoude, The Netherlands), and snap-frozen in liquid nitrogen.

**Effect of soluble VEGFR-3-Ig on rat cardiac allograft survival.** Allograft recipients were perfused intraportally with adenoviral vectors (1x10\(^9\) pfu in 1 ml) encoding green fluorescent protein (Ad.GFP) or soluble VEGFR-3 receptor (Ad.VEGFR-3-Ig) at the time of transplantation, and received low dose immunosuppression of CsA 1.0 mg/kg/d s.c. Recipient allografts and spleens were harvested at 5 days for mechanistic analysis, or in a separate experiment, at 8 weeks or if the graft function deteriorated, to determine the effect of VEGFR-3-Ig on allograft survival.

**Effect of VEGFR-3 mAb on mouse chronic rejection.** Inbred male BALB/c (B/c, H-2\(^d\)) and C57BL/6J (B6, H-2\(^b\)) mice (Harlan, Horst, The Netherlands) 2-3 months of age were used. Allograft recipients were treated with rat IgG (Sigma-Aldrich, St. Louise, MO) 800 µg
or rat anti-mouse VEGFR-3 neutralizing antibody (mF4-31C1; ImClone, New York, NY) 800 µg i.p. every third day for four weeks, and FK506 (Astellas Pharma) 3.0 mg/kg/d s.c. for the first week and 1.5 mg/kg/d thereafter. Allografts were harvested at 8 weeks.²

**ELISA.** ELISA (Quantikine-R&D Systems, Minneapolis, MN) was used to detect the presence of VEGFR-3-Ig in rat serum.

**Histology.** Allograft arteriosclerosis was determined from paraformaldehyde-fixed paraffin sections stained with hematoxylin-eosin and Resorcin fuchsin for internal elastic lamina using computer-assisted image processing (Axiovision 4.4; Carl Zeiss, Oberkochen, Germany) and measuring the area surrounded by the internal elastic lamina and vessel lumen. Arterial occlusion was determined as the ratio of neointimal area and internal elastic lamina area.

**Immunohistochemistry.** Cryostat sections were stained using the peroxidase ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) and the reaction was revealed by 3-amino-9-ethylcarbazole (AEC; Vector Laboratories). Immunofluorescence double stainings were performed using a sequential approach and Alexa Fluor 488 (green) and Alexa Fluor 568 (red; Promega, Madison, WI) secondary antibodies. Antibodies used were: anti-rat CD4 (22021D), CD8 (22071D), ED1 (22451D), and IL-2Rα (22090D), and anti-mouse CD4 (553043), CD8 (553027), CD11b (553308) from BDPharimingen, San Diego, CA; rabbit anti-mouse affinity purified LYVE-1, rabbit anti-human LYVE-1 (Molecular Cancer Biology Laboratory); VEGF-C (ab9546), anti-GFP (ab290) and CD31 (ab7388) from Abcam, Cambridge, UK; mouse anti-rat OX-62 (MCA1029G), RECA-1 (MCA970) and MHC class II (MCA46R) from Serotec, Oxford, UK; anti-mouse CCL-
21/6Ckine (AF 457) antibody and VEGFR-3 (AF743) from R&D Systems, Minneapolis, MN; PROX-1 (DP3501P) from Acris Antibodies, Hiddenhausen, Germany.

**Analyses of immunohistochemistry.** Graft-infiltrating inflammatory cells, CD31⁺ cross-sectional capillaries and LYVE-1⁺, VEGFR-3⁺ or CCL-21⁺ lymphatic vessels with a clear lumen, were counted from four random fields from each quadrant of the section’s parenchyme with 400x magnification and are given as the mean number of positive cells or vessels per mm². Lymphatic vessels were also counted from the epicardium of the section and the amount of vessels is given as the mean number of positive vessels per mm². All analyses were performed in blinded manner by two independent observers.

**RNA isolation and reverse transcription.** Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of mRNA was carried out using M-MLV reverse transcriptase, and random nonamers (Sigma-Aldrich).

**Real-Time PCR.** Real-time RT-PCR reactions were carried out on a Rotor-Gene 6000 (Qiagen, Venlo, Netherlands) using Dynamo Flash SYBR Green qPCR kit (Finnzymes, Espoo, Finland). Measurement of the PCR product was performed at the end of each extension period. The number of copies of the gene of interest was calculated from the corresponding standard curve using manufacturer's software (Qiagen) and is given in relation to 18S rRNA molecule numbers. The following primers were used:

CCL21 (NM_001008513)

5’-CATCCCAGCAATCCTGTTCG-3’

5’-CTTTCTTCCCAGACTTAGAGTTCC-3’
CCL19 (NM_001108661)
5'-TCAGGCTCATTCATTCTCTGTGG-3'
5'-CAGCAGTCTTCCGCATCGTTAG-3'

CCR7 (BC089762)
5'-TGGCTCTCCTGGTCATTTTCC-3'
5'-CTTGAAGCACACCGACTCATACAG-3'

FoxP3 (NM_001108250)
5'-GGAAGATGGCATTGACAAAAGC-3'
5'-TCTCCGCACAGCAAACAAGC-3'

T-box 21 (NM_001107043)
5'-CCAAGTTCAAACCAGCACCAGAC-3'
5'-CATCCACAAACATCCTGTAATGGC-3'

IL-2 (NM_053836)
5'-GCAGCGTGTGTTGGATTTGACTC-3'
5'-GAGATGATGCTTTGACAGATGCC-3'

IFN-γ (NM_138880)
5'-GCCATCAGCAAACAACATAAGTGTC-3'
5'-AGAATCAGCACCAGACTCCTTTTC-3'

IL-4 (NM_201270)
5'-GCAACAAAGGAACACCACCGGAGA-3'
Flow cytometry. Spleens were harvested to RPMI-1640 medium, homogenized, and 1x10^6 cells were incubated with FITC- and PE-conjugated antibodies at RT for 15 minutes. The cells were washed twice with PBS and analyzed with a FACScan (Becton Dickinson, San Jose, CA) flow cytometer. Antibodies used were CD45-FITC (MCA43FT) and OX62-RPE (MCA1029PE) from Serotec. IgG1-FITC (MCA43FT) and IgG-RPE (MCA1209PE, Serotec) were used as negative isotype controls.

Statistics. All data are given as mean ± SEM and analyzed by non-parametric Mann-Whitney U test, or by log-rank test using SPSS for Windows version 17.0 (SPSS inc., Chicago, IL). P<0.05 was regarded as statistically significant. The n-values of each experiment are given in the respective Figure texts.
References
