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Sirolimus inhibits lymphangiogenesis in rat renal allografts, a novel mechanism to prevent chronic kidney allograft injury

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Summary

Lymphangiogenesis occurs in renal allografts and it may be involved in the maintenance of the alloreactive immune response and thus participate in the development of chronic kidney allograft injury. Sirolimus (SRL) has been shown to inhibit lymphangiogenesis. The aim of this study was to describe lymphangiogenesis and its regulation during the development of chronic kidney allograft injury and to investigate the effect of SRL on allograft lymphangiogenesis and chronic kidney allograft injury. A rat renal transplantation model was used. Allografts treated with cyclosporine A or with SRL were analyzed in various time points. Syngenic transplantations were used as controls. Kidney function was followed with serum creatinine. Histology was analyzed by Chronic Allograft Damage Index (CADI). Immunohistochemistry was used to detect lymphatic vessels, VEGF-C and VEGFR-3. In cyclosporine-treated allografts VEGF-C/VEGFR-3 pathway was strongly upregulated leading to extensive lymphangiogenesis 60 days after transplantation. Lymphangiogenesis correlated positively with the CADI score. Sirolimus efficiently inhibited lymphangiogenesis, improved graft function and attenuated the development of chronic kidney allograft injury when compared with cyclosporine. In conclusion, lymphangiogenesis is associated with chronic kidney allograft injury and SRL is a potent inhibitor of lymphangiogenesis in renal allografts. Inhibition of lymphatic proliferation could mediate the nephroprotective properties of SRL.

Introduction

Lymphatic vessel proliferation, lymphangiogenesis, occurs in renal allografts but its significance is unknown [1–3]. Lymphatic vessel formation has also been seen in other models of chronic inflammation such as psoriasis and rheumatoid arthritis [4]. At the sites of inflammation, lymphatic vessel proliferation is induced by macrophages and granulocytes, which produce VEGF-C [5,6]. VEGF-C and its receptor VEGFR-3 are key regulating molecules of lymphatic vessel growth [7,8]. Lymphatic vessels participate in inflammatory response by regulating lymphocyte traffic [4]. Afferent lymphatic vessels attract CC chemokine receptor 7 (CCR7) expressing immune cells to secondary lymphoid organs by producing CC chemokine ligand 21 (CCL21) [9]. Newly formed lymphatic vessels in kidney allografts also express CCL21, which implies that lymphatic vessels could be associated with the maintenance of inflammatory infiltrates in kidney transplants [1].

The mammalian target of rapamycin (mTOR) inhibitors sirolimus (SRL) and everolimus suppress interleukin (IL) -2 and IL-4 induced T-lymphocyte division and are therefore effective immunosuppressants. Similarly, mTOR inhibitors suppress the proliferation of many other cell types such as tumor cells which could explain why mTOR inhibitors may reduce the malignancy rates in transplantation patients [10]. However, the inhibitory effect of SRL on VEGF-A-mediated tumor angiogenesis is considered a

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more important mechanism in malignant processes [11]. Recently SRL has also been shown to inhibit lymphangiogenesis in a murine skin-flap model as well as in an experimental model of pancreatic tumor [12,13].

Only limited knowledge exists about the regulation, dynamics, and importance of lymphangiogenesis in renal transplants. The effect of SRL on lymphangiogenesis in kidney transplants is unknown. Inhibition of lymphangiogenesis could mediate the nephroprotective properties of SRL. The aim of this study was to describe the chronology of VEGF-C/VEGFR-3 pathway activation and lymphatic vessel proliferation during the development of chronic kidney allograft injury. In addition we investigated the effect of SRL on lymphatic vessel proliferation in kidney allografts.

Materials and methods

Animals and kidney transplantations

Permission for animal experiments was obtained from the State Provincial Office of Southern Finland. Inbred, male, fully major histocompatibility complex-mismatched Dark Agouti (DA) (AG-B4, $RT1^a$) and Wistar-Furth (WF) (AG-B2, $RT1^v$) rats (Harlan, Horst, the Netherlands) weighing 300–350 g were used. The animals received human care in compliance with the '*Principles of Laboratory animal care*' NIH publication Vol. 25, No. 28 revised 1996, as well as specific national laws. Kidney transplantations were performed as described before [14] using a modified microsurgical technique of Fisher and Lee. Right native kidney was removed at the time of the transplantation. To allow the graft to recover nephrectomy of the left kidney was performed 7 days after transplantation.

Experimental design

Allograft transplantations were performed from DA to WF rats and syngraft transplantations between DA rats. To analyze the precise chronology of VEGF-C/VEGFR-3 pathway activation and lymphatic vessel proliferation during the development of chronic kidney allograft injury, allografts treated with CsA 1.5 mg/kg/day and syngrafts without immunosuppression were recovered and examined 1, 3, 5, 7, 30, 60, and 90 days after transplantation. To investigate the effect of SRL on lymphatic vessel formation, allograft recipients were treated daily with SRL 2 mg/kg orally through an orogastric tube. In addition, SRL-treated animals were given CsA 1.5 mg/kg/d s.c. for the first 7 days after transplantation as SRL alone was not sufficient to overcome acute rejection. These grafts were recovered 3, 7, and 90 days after transplantation. Normal DA kidneys were used as additional controls. Numbers of animals in each group were : *n* (allo CsA 1d, 3d, 5d, 7d, 30d, 60d, 90d) = 5, *n* (syn 1d, 3d, 5d, 7d, 30d, 60d) = 3, *n* (syn 90d) = 5, *n* (allo SRL 3d) = 3, *n* (allo SRL 7d) = 5, *n* (allo SRL 90d) = 4, *n* (normal) = 5.

Serum creatinine was followed weekly during the experiment. As the transplant was recovered half of each sample was incubated in 4% paraformaldehyde for 24 h and then routinely fixed for paraffin blocks and the other half was embedded in O.C.T (Tissue-Tek; Miles Inc., Elkhart, IN, USA), snap-frozen in liquid nitrogen and stored at −70 °C.

Drugs used

Cyclosporine A (Novartis, Basel, Switzerland) was diluted in intralipid (Fresenius Kabi, Uppsala, Sweden) to a final concentration of 1.5 mg/ml and administered s.c. once a day. Sirolimus (kindly provided by Wyeth, Madison, NJ, USA) was diluted in polyethylene glycol and given 2 mg/kg once a day orally using an orogastric tube. SRL and CsA whole blood concentrations were followed during the experiment. CsA levels were measured using radioimmunoassay (Sandimmun-Kit; Novartis). SRL concentrations were determined using immunochemiluminometric assay.

Histopathology

Paraffin-embedded specimens were cut into 2-μm thick sections and stained with Mayer's hematoxylin-eosin, Masson's trichrome, diastase-periodic acid-schiff (D-PAS), methenamine silver PAS, and Unna-Pappenheim stains.

Chronic changes were scored according to the Chronic Allograft Damage Index (CADI). The CADI value is a sum of interstitial inflammation and fibrosis, tubular atrophy, glomerular mesangial matrix increase, glomerular sclerosis, and arterial intimal proliferation, scored from 0 to 3 and leading to CADI values from 0 to 18 [15]. The CADI grading of fibrosis and tubular atrophy from 1 to 3 are analogic with the BANFF-score (Banff 2010, category 5 grades I, II, and III) [16]. However, the CADI score is numeric unlike the BANFF-score which makes it possible to monitor statistical differences between study-groups both in individual histological parameters and also in total rejection-score and for this reason it is used here. All analyses were performed in a blinded manner.

Immunohistochemistry and immunofluorescence

Immunohistochemical stainings were used to detect VEGF-C, VEGFR-3, and lymphatic vessels. Lymphatic endothelial marker lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) was used for the detection of lymphatic vessels. To specify the location of these antibodies immunofluorescent double stainings were performed from 60-day samples using a sequential approach.

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Paraffin-embedded specimens were cut into 4-μm thick sections. For epitope retrieval, the paraffin sections were heated in a microwave oven for 20 min in a sodium citrate buffer (pH 6.0) and then allowed to cool down at room temperature for 20 min.

Lymphatic endothelial marker LYVE-1 was used for the detection of lymphatic vessels. The specificity of LYVE-1 antibody to lymphatic vessels was verified with a immunofluorescent double staining where LYVE-1 and rat endothelial cell antigen-1 (RECA-1) were expressed in distinct capillary structures. LYVE-1 was stained with the peroxidase ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) and the reaction was revealed by NovaRed (Vector Laboratories). Tyramide signal amplification (TSA) system was used to enhance the LYVE-1 signal (TSA indirect; PerkinElmer, Waltham, MA, USA). Endogenous peroxidase activity was blocked with a 20-min incubation with a 1% hydrogen peroxidase/phosphate-buffered saline solution.

All other immunohistochemical stainings were done using EnVision™ G|2 System/AP kit, Rabbit/Mouse (Permanent Red; DAKO, Glostrup, Denmark). Levamisole (DAKO) was used to reduce endogenous alkaline phosphatase activity. For specificity controls, the primary antibody was omitted. None of these specificity controls showed any immunoreactivity.

Immunofluorescence stainings were performed from frozen sections of 60-day allografts using a sequential approach with Alexa Fluor 488 (green) and Alexa Fluor 568 (red) fluorescent secondary antibodies (Invitrogen, Molecular Probes, Carlsbad, CA, USA). Slides were covered with a mounting medium including DNA stain 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield HardSet Mounting Medium with DAPI; Vector Laboratories). When bound to DNA, DAPI appears as blue fluorescence.

The following antibodies and dilutions were used: rabbit anti-human LYVE-1 (1:1000; Molecular Cancer Biology Laboratory, University of Helsinki, Helsinki, Finland), rabbit anti-human VEGF-C (1:100; sc-9047, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-mouse VEGFR-3 (1:200, sc-637, Santa Cruz), goat anti-mouse VEGFR-3 (1:50, AF743; R&D Systems, Minneapolis, MN, USA), mouse anti-rat ED1 (1:100, 554954; BD Pharmingen, San Diego, CA, USA), mouse anti-rat CD4 (1:100, 554835; BD Pharmingen), mouse anti-rat CD8 (1:100, 554854; BD Pharmingen), goat anti-mouse CCL21 (1:200, AF457; R&D Systems), mouse anti-rat RECA-1 (1:10, MCA970R; Serotec, Oxford, UK).

LYVE-1+ and VEGFR-3+ vessels with a clear lumen were counted from four random fields from each quadrant of the sections parenchyma with 400× magnification and are given as the mean number of vessels per square millimeter. VEGFR-3+ glomeruli were excluded from the capillary density analysis. The intensity of VEGFR-3 in glomeruli as well as the intensity of VEGF-C in tubuli, glomeruli, inflammatory cells and vessels was scored semiquantitatively from 0 to 3 as follows: 0, no visible staining; 1, cells with faint staining; 2, moderate intensity with multifocal staining and 3, intense diffuse staining. All analyses were performed in a blinded manner.

Statistical analyses

Statistical analyses were done with SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA). Semiquantitative measures (CADI, VEGF-C score and VEGFR-3 score in glomeruli) were analyzed using Mann–Whitney *U*-test. Quantitative measures (LYVE-1+ and VEGFR-3+ vessels/mm^2) were analyzed using Student's *t*-test. ANOVA for repeated measures was used to analyze serum creatinine results. Linear regression analysis was applied to evaluate a possible relation of lymphatic vessel density to the CADI score. Probability (*P*) -value <0.05 was accepted as significant.

Results

VEGF-C/VEGFR-3 pathway is upregulated in CsA-treated kidney allografts

The expression of VEGF-C is summarized in Fig. 1 and Table 1. In normal kidneys and syngrafts mild VEGF-C expression was detected in inflammatory, vascular, and tubular cells. Glomerular cells showed moderate immunoreactivity. In CsA-treated allografts the inflammatory cell VEGF-C expression intensified gradually and was moderate to strong 30 and 60 days after transplantation (syngrafts versus allografts; 30d $P < 0.05$; 60d $P < 0.05$) (Fig. 1a). In 90 days the expression had returned close to nonexistent (syngrafts versus allografts; 90d P = ns) (Fig. 1a). Tubular, vascular and glomerular VEGF-C expression remained mild to moderate in CsA-treated allografts during the whole study period. Double stainings at 60 days revealed that inflammatory cells expressing VEGF-C were mainly ED-1+ macrophages (Fig. 1d) and CD4$^+$ lymphocytes (Fig. 1e). Only few CD8$^+$ lymphocytes expressed VEGF-C.

The expression of VEGFR-3 is summarized in Fig. 2 and Table 1. Blood or lymphatic vessels showed no VEGFR-3 immunoreactivity in normal kidneys and syngrafts whereas mild VEGFR-3 immunoreactivity was found in glomeruli. In CsA-treated allografts, VEGFR-3 was detected in the endothelium of capillary structures (Fig. 2a). The capillary VEGFR-3 expression was at the highest 60 days after transplantation (syngrafts versus allografts; 60d $P < 0.01$) (Fig. 2a). Double stainings revealed that these capillaries were both blood and lymphatic vessels (Fig. 2d and e). VEGFR-3 was rarely recorded in inflammatory cells. In CsA-treated allografts glomerular VEGFR-3 immunoreactivity ranged from mild to moderate during the whole study period.

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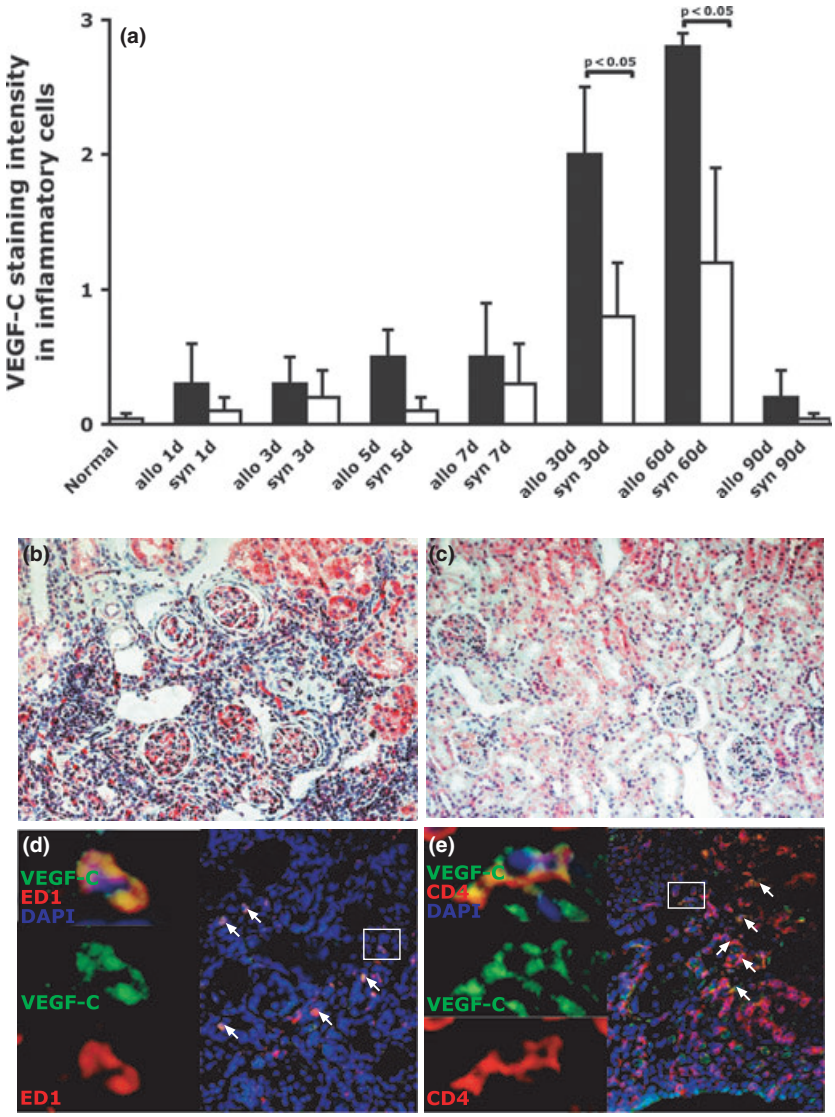

Figure 1 VEGF-C expression in normal kidneys, syngrafts, and CsA-treated kidney allografts. In CsA-treated allografts VEGF-C expression was significantly induced in graft-infiltrating inflammatory cells during the development of chronic kidney allograft injury. In normal kidneys and syngrafts the VEGF-C expression was mild. The histogram demonstrates the immunohistochemical VEGF-C staining intensity in normal kidneys, syngrafts, and CsA-treated allografts. Statistical comparisons were done between allografts and syngrafts using Mann–Whitney *U*-test. Data are presented as mean ± SEM (a). In a photomicrograph of an CsA-treated allograft 60 days after transplantation intense inflammatory cell VEGF-C expression can be detected (b). In normal kidneys, syngrafts, and CsA-treated allografts VEGF-C was expressed also in vascular structures, tubuli, and glomeruli. A typical staining pattern can be seen in a VEGF-C staining of a normal kidney (c). Immunofluorescent double stainings reveal that VEGF-C is expressed in ED1+ macrophages (d) and $CD4^+$ lymphocytes (e). In immunofluorescent pictures VEGF-C is marked with green and inflammatory cell markers with red (d & e). Blue DAPI stain was used to detect the nuclei of the cells. Original magnification used for photomicrographs was ×200 for immunohistochemistry and ×400 for immunofluorescence.

Extensive lymphatic vessel proliferation occurs in CsA-treated kidney allografts

In normal kidneys and syngrafts LYVE-1+ lymphatic vessels were detected around large vascular structures, but not in peritubular or periglomerular space. In contrast strong lymphatic vessel proliferation was recorded in CsA-treated allografts at 60 and 90 days after transplantation (syngrafts versus allografts; 60d $P < 0.05$; 90d $P < 0.01$) (Fig. 3a). Most of the lymphatic vessels were located close to inflammatory cell infiltrates. Newly formed lymphatic vessels expressed the inflammatory cell chemokine CCL21 as shown by immunofluorescence double stainings of CsA-treated allografts 60 days after transplantation (Fig. 3d).

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Table 1. The effect of sirolimus on VEGF-C and VEGFR-3 expression.

	3 days after transplantation		7 days after transplantation		90 days after transplantation	
Group	CsA	SRL	CsA	SRL	CsA	SRL
VEGF-C						
Tubuli score 0–3	**1.4 ± 0.2**	**0.2 ± 0.2***	1.0 ± 0.2	0.3 ± 0.2	0.3 ± 0.3	0.6 ± 0.4
Vessels score 0–3	**1.4 ± 0.2**	**0.2 ± 0.2***	1.3 ± 0.5	0.6 ± 0.2	0.6 ± 0.4	1.3 ± 0.3
Glomeruli score 0–3	**2.1 ± 0.1**	**1.2 ± 0.2***	2.1 ± 0.2	1.4 ± 0.3	1.9 ± 0.3	2.0 ± 0.0
Inflammation score 0–3	0.3 ± 0.2	0.1 ± 0.1	0.5 ± 0.4	0.1 ± 0.1	0.2 ± 0.2	0.5 ± 0.4
VEGFR-3						
Glomeruli score 0–3	0.1 ± 0.1	0.0 ± 0.0	0.8 ± 0.3	0.5 ± 0.3	0.4 ± 0.2	0.9 ± 0.3
Positive vessels/mm^2	4.6 ± 2.9	0.0 ± 0.0	12.2 ± 6.7	6.2 ± 4.4	2.4 ± 1.5	12.3 ± 6.5

The VEGF-C and VEGFR-3 staining intensities were scored 0–3. The VEGFR-3+ vessel density is given as mean number of vessels per square millimeter. All data are presented as mean ± SEM.

*$P < 0.05$ cyclosporine versus sirolimus, Mann–Whitney *U*-test

Sirolimus improves kidney graft function and inhibits chronic kidney allograft injury

No animals were lost before the end of the 90 day follow-up period. Both CsA and SRL were well tolerated at the used doses. The serum creatinine values of SRL-treated allografts were significantly lower compared with those of CsA-treated allografts ($P < 0.05$) (Fig. 4a). CsA blood trough levels were between 100–150 ng/ml and SRL blood trough levels were between 4–6 ng/ml throughout the study.

Sirolimus did not reduce acute inflammation 3 and 7 days after transplantation when compared with allografts receiving only CsA. No signs of chronic kidney allograft injury were seen in syngenic grafts 90 days after transplantation, CADI 1.1 ± 0.1 mean ± standard error of the mean (SEM) (Fig. 4b). In CsA-treated allografts moderate to intense chronic changes were seen, CADI 9.6 ± 0.4 (Fig. 4c). SRL significantly attenuated the development of chronic kidney allograft injury when compared with CsA, CADI 4.5 ± 0.3 (CsA versus SRL, $P < 0.05$) (Fig. 4d). According to the CADI score, SRL-treated allografts had significantly less inflammation ($P < 0.05$), fibrosis ($P < 0.05$), and arterial intimal proliferation ($P < 0.05$) than CsA-treated allografts (Fig. 4b). Inflammation was also reduced when defined by the number of nodular inflammatory infiltrates per square millimeter (infiltrates/mm^2; CsA 8.5/mm^2 vs. SRL 4.25/mm^2, $P < 0.05$).

Sirolimus reduces early VEGF-C expression and inhibits lymphatic vessel formation in kidney allografts

The immunohistology of VEGF-C and VEGFR-3 expression is summarized in Table 1. Treatment with SRL significantly reduced VEGF-C immunoreactivity in vessels, tubuli, and glomeruli 3 days after transplantation when compared with CsA treatment (vessels $P < 0.05$; tubuli $P < 0.05$; glomeruli $P < 0.05$). In the SRL group, vascular and tubular VEGF-C expression was nearly nonexistent whereas glomerular VEGF-C expression was mild. No significant differences in VEGF-C expression were seen between SRL and CsA treatment at 7 and 90 days after transplantation.

In SRL and CsA-treated allografts VEGFR-3 was found in glomeruli and vascular structures. Glomerular VEGFR-3 expression was low in both CsA and SRL groups at every time point of the study. Although not statistically significant 3 and 7 days after transplantation, the vascular VEGFR-3 expression seemed slightly lower under SRL-treatment than CsA-treatment. Vascular expression of VEGFR-3 fell in the CsA group 90 days after transplantation. In this time point, the vascular VEGFR-3 expression was somewhat higher in SRL group than in CsA group (P = NS).

Lymphangiogenesis was not present in either CsA or SRL-treated allografts 3 and 7 days after transplantation. In the 90 day samples SRL had inhibited lymphatic vessel proliferation efficiently. The lymphatic vessel density in SRL-treated allografts was only one-third of the density seen in to CsA-treated allografts (lymphatic vessel density ± SEM; CsA 21.0 ± 4.8 vessels/mm^2 vs. SRL 7.3 ± 1.8 vessels/mm^2, $P < 0.05$) (Fig. 5a). Linear regression analysis revealed a significant correlation between the CADI score and lymphatic vessel density ($r = 0.806$, $P < 0.01$) (Fig. 5b). SRL- and CsA-treated allografts at 3, 7, and 90 days after transplantation were included to the regression analysis.

Discussion

In the present study, we describe the dynamics of lymphatic vessel proliferation in kidney allografts and represent SRL as a potent inhibitor of allograft lymphangiogenesis. In our data, lymphangiogenesis was seen 60 days after transplantation in CsA-treated allografts. These lymphatic vessels

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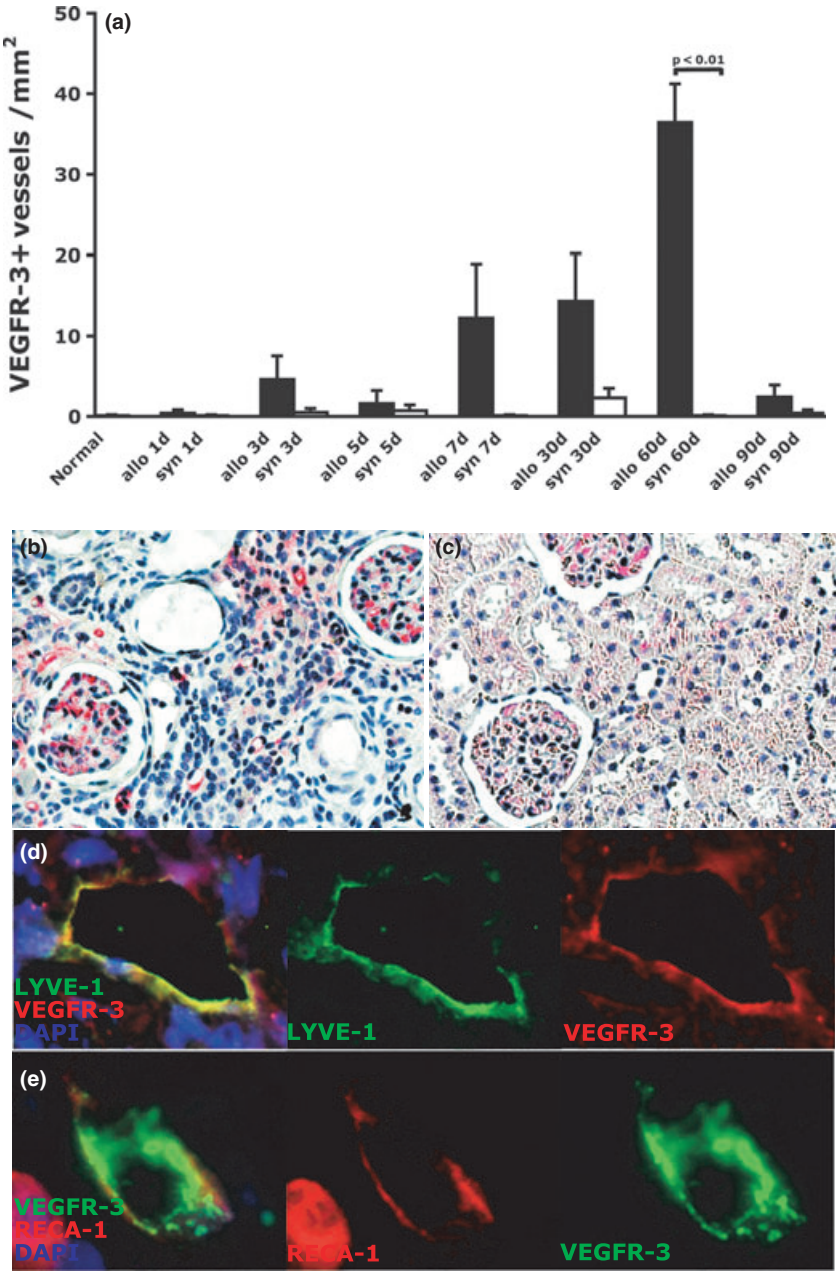

Figure 2 VEGFR-3 expression in normal kidneys, syngrafts, and CsA-treated kidney allografts. In CsA-treated allografts VEGFR-3 expression was increased in the capillary endothelium. VEGFR-3 capillary expression was not seen in normal kidneys or in syngrafts. The histogram demonstrates the density of VEGFR-3+ capillaries in the immunohistochemical stainings of normal kidneys, syngrafts, and CsA-treated allografts. Statistical comparisons were done between allografts and syngrafts using Student's *t*-test. Data are presented as mean ± SEM (a). Immunohistochemical staining of a CsA-treated allograft 60 days after transplantation demonstrates that VEGFR-3 is expressed in capillary endothelium and in glomeruli (b). Glomerular staining can be detected also in normal kidneys (c) and syngrafts. VEGFR-3+ capillaries were identified to be both lymphatic vessels (d) and blood capillaries (e) by immunofluorescent double stainings. VEGFR-3 is marked with red and lymphatic endothelium marker LYVE-1 with green (d). VEGFR-3 is marked with green and rat vessel endothelial cell marker RECA-1 with red (e). Blue DAPI stain was used to detect the nuclei of the cells. Original magnification used for photomicrographs was ×200 for immunohistochemistry and ×400 for immunofluorescence.

expressed the leukocyte chemokine CCL21 and were located close to inflammatory cell infiltrates. The production of VEGF-C was upregulated in the $CD4^+$ T cells and macrophages. VEGFR-3 expression was induced in blood and lymphatic vessel endothelial cells. Lymphatic vessel density correlated positively with the histological changes

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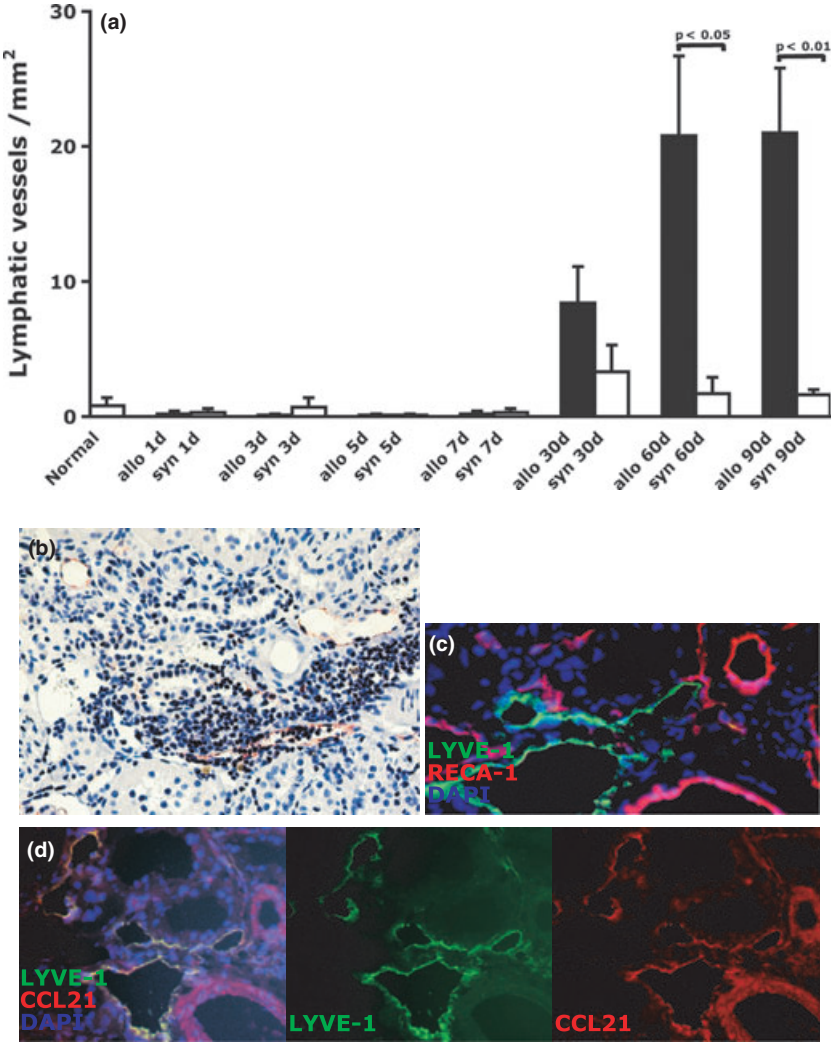

Figure 3 Lymphatic vessels in normal kidneys, syngrafts, and CsA-treated allografts. In CsA-treated allografts lymphatic vessel proliferation was seen 60 days after transplantation. In normal kidneys and syngrafts physiological lymphatic vessel network was detected. The histogram demonstrates lymphatic vessel density in the immunohistochemical stainings of normal kidneys, syngrafts, and CsA-treated allografts. Statistical comparisons were done between allografts and syngrafts using Student's *t*-test. Data are presented as mean ± SEM (a). A photomicrograph of a CsA-treated allograft 90 days after transplantation shows that newly formed lymphatic vessels are located close to inflammatory cell infiltrates (b). Lymphatic endothelial marker LYVE-1 and rat vessel endothelial marker RECA-1 were expressed in distinct capillary structures verifying the specificity of the LYVE-1 antibody to lymphatic vessels (c). Lymphatic vessels express CCL21, a potent inflammatory cell chemokine (d). Original magnification used for photomicrographs was ×200 for immunohistochemistry and ×400 for immunofluorescence.

of chronic kidney allograft injury. Treatment with SRL reduced early VEGF-C expression in vessels, tubuli and glomeruli and downregulated lymphatic vessel formation to one-third when compared with CsA. SRL also improved graft function and reduced the signs of chronic kidney allograft injury.

Newly formed lymphatic vessels are seen in 61–74% of renal protocol biopsies taken from kidney transplantation patients [2]. In a retrospective study with 47 patients almost all graft losses concentrated to patients with high lymphatic vessel density, but no statistical analysis was done because of the heterogeneity of the study population [1]. One-year follow-up study with 76 kidney transplant patients showed opposite results and indicated that high lymphatic vessel density was associated with better graft function and no association to chronic rejection was seen [2]. In our model of chronic kidney allograft injury lymphatic vessel proliferation was detected in all CsA-treated allografts 60 days after transplantation. Lymphatic vessel density also correlated positively with the CADI score suggesting that increased lymphangiogenesis is associated with poor graft outcome.

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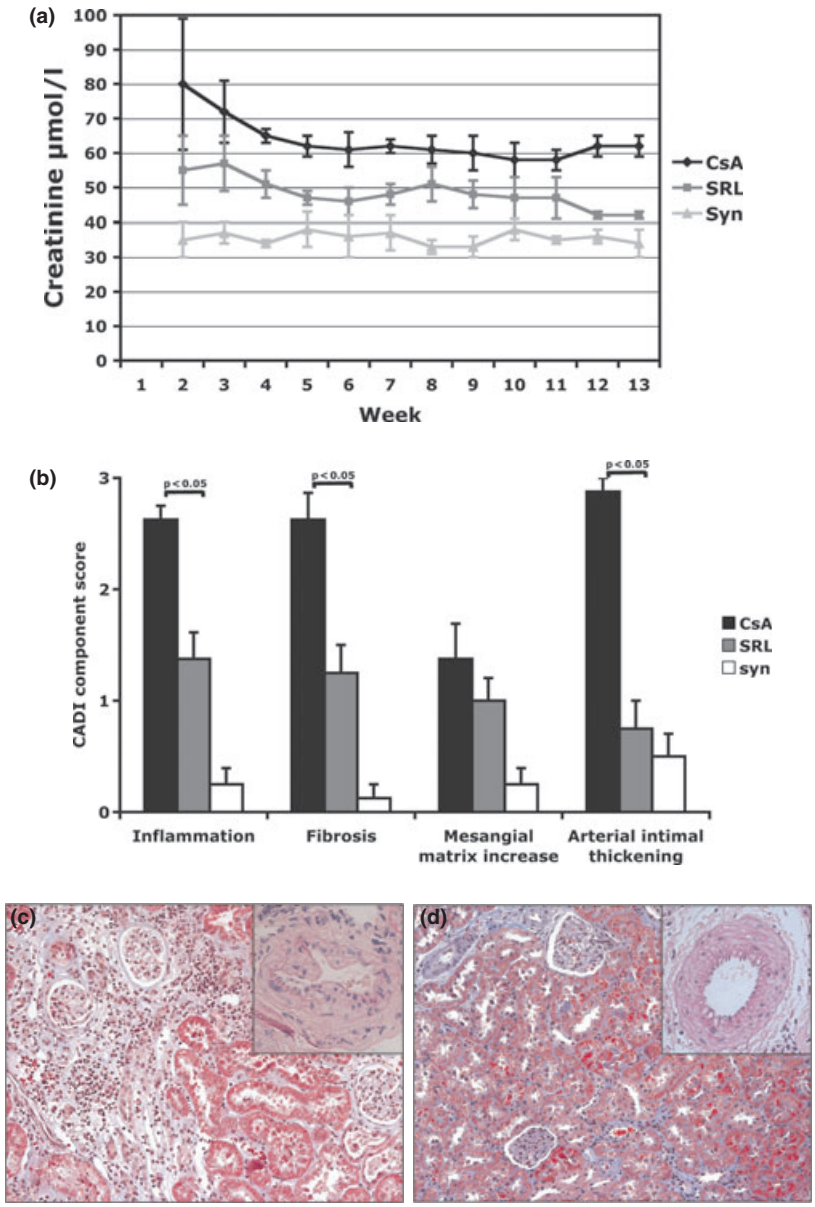

Figure 4 Sirolimus (SRL) improves kidney function and attenuates the development of chronic kidney allograft injury. Serum creatinine was measured weekly during the experiment. SRL significantly reduced the levels of serum creatinine when compared with CsA as tested with ANOVA for repeated measures ($P < 0.05$). CsA-treated allografts are marked with black, SRL-treated with dark gray, and syngrafts with light gray (a). Data are presented as mean ± SEM. Chronic allograft damage index (CADI) was used to determine the severity of chronic kidney allograft injury 90 days after transplantation. SRL treatment significantly reduced CADI score and its components inflammation, interstitial fibrosis, and arterial intimal proliferation when compared with CsA treatment. Statistical analysis was done using Mann–Whitney *U*-test. Data are presented as mean ± SEM (b). A photomicrograph of a CsA-treated allograft 90 days after transplantation where intense inflammation, fibrosis, and arterial intimal thickening can be detected (c). A photomicrograph of a SRL-treated allograft 90 days after transplantation where only mild signs of chronic allograft injury can be seen (d). Original magnifications used for photomicrographs was ×200.

Organized lymphoid structures that resemble secondary lymphoid organs are frequently observed in tissues that are targeted by chronic inflammatory processes. Formation of these tertiary lymphoid organs might have a role in maintaining local immune responses against persistent antigens [17]. In heart transplants organized inflammatory infiltrates are called Quilty lesions. Similar structures where inflammatory cells are segregated into different potentially functional regions are also seen in kidney allografts [1]. In renal protocol biopsies the newly formed lymphatic vessels

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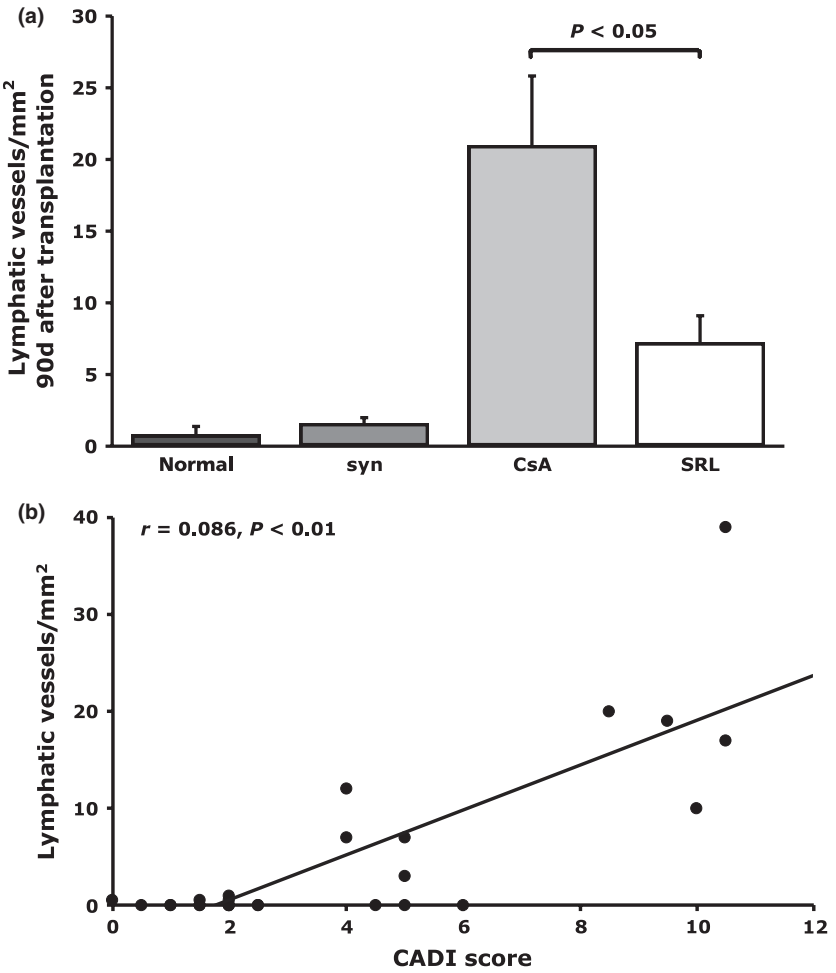

Figure 5 The effect of sirolimus (SRL) on lymphatic vessel proliferation. At 90 days after transplantation SRL-treated allografts had significantly less lymphatic vessels than CsA-treated allografts (a). All data are presented as mean ± SEM. Student's *t* test was used for lymphatic vessel density analysis. Significant correlation was found between the CADI score and lymphatic vessel density as analyzed by linear regression analysis suggesting that inhibition of lymphangiogenesis could be involved in the nephroprotective properties of SRL (b). SRL- and CsA-treated allografts at 3, 7, and 90 days after transplantation were included to the analysis. Correlation coefficient (*r*) and probability (*P*) value are given.

are closely associated with these nodular inflammatory infiltrates. These new lymphatic vessels express CCL21 and the surrounding inflammatory cells express CCR7 suggesting that lymphatic vessels attract lymphocytes to the graft [1]. In the present study, lymphatic vessels expressed CCL21 and in 90-day samples they were closely associated with nodular and non-nodular inflammatory infiltrates supporting the existing data.

The transplantation surgery disconnects the lymphatic vessels of the renal transplant. In canine kidney autografts, the reconnection of lymphatic vessels is seen 3 days after transplantation [18]. The connection between newly formed lymphatics and existing lymphatic network has not been examined in kidney transplants. New lymphatics could have a role in antigen-presenting cell (APC) transportation to secondary lymphoid organs as suggested in a study done with rat heart transplants where inhibition of VEGFR-3 resulted in reduced number of dendritic cells in the spleen [19]. The connection of lymphatics was not investigated in the present study. Future studies are required to clarify the functional role of newly formed lymphatics in APC transportation and lymphatic drainage in kidney transplants.

Lymphangiogenesis at sites of inflammatory insults is commonly induced by macrophages, which produce VEGF-C [5]. VEGF-C expression has also been seen in graft-infiltrating macrophages in kidney transplants [1]. In addition, $CD4^+$ lymphocytes have been reported to produce VEGF-C in an experimental heart transplantation model [19]. Here, VEGF-C production was upregulated in macrophages of CsA-treated allografts. Rich VEGF-C expression was also found in $CD4^+$ lymphocytes. Strong

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upregulation of VEGF-C, a key regulator of lymphangiogenesis, later followed by lymphangiogenesis suggests that VEGF-C has a regulatory role in kidney transplant lymphangiogenesis.

In postnatal period VEGFR-3 expression is commonly restricted to lymphatic endothelium and some fenestrated blood capillaries such as glomeruli [20]. There are studies, however, showing that VEGFR-3 may be expressed in the endothelium of blood vessels for instance in tumor angiogenesis, developing ovarian follicles of superovulated mice [21] and in chronic diabetic leg ulcers [22]. In the present study, VEGFR-3 expression was induced in the endothelium of both blood and lymphatic vessels of kidney allografts. Our findings suggest that VEGFR-3 is involved in transplant lymphangiogenesis and angiogenesis.

Sirolimus has inhibited lymphatic vessel growth in studies investigating tumor lymphangiogenesis and lymphatic metastasis as well as in a mouse skin-flap model [12,13]. Sirolimus could potentially reduce lymphangiogenesis by reducing VEGF-C or VEGFR-3 expression or alternatively by inhibiting VEGF-C driven phosphorylation of p70S6 kinase downstream of mTOR [12,13]. In this study, SRL efficiently inhibited lymphangiogenesis in the parenchyma of kidney allografts during the development of chronic kidney allograft injury. SRL reduced the production of VEGF-C below the normal level 3 days after transplantation. SRL did not alter the expression of VEGFR-3. Our results show for the first time that SRL inhibits lymphangiogenesis during alloimmune response. This strengthens the overall understanding that SRL is a potent antilymphangiogenic agent. The modest effect of SRL on VEGF-C and VEGFR-3 expression suggests that reduced lymphangiogenesis is more likely caused by the inhibition of VEGF-C-driven phosphorylation of p70S6 kinase.

At day 90, the expression of VEGF-C and VEGFR-3 fell in the CsA-treated group. In 90 days, the chronic allograft injury in our transplantation model has proceeded to a stage where the milieu has turned from proinflammatory and pro-proliferative to profibrotic and the grafts show remarkable chronic rejection changes. This is why the expression of VEGF-C and VEGFR-3 fell at the 90 days time point investigated in CsA-treated group. This is in line with SRL-treated group, where CADI score was significantly lower and hence, the expression of VEGF-C and VEGFR-3 expression somewhat, although not significantly, higher compared with CsA-treated group at day 90.

The CsA is known to increase the expression of many cytokines in allograft settings. This does not seem to be the case with lymphangiogenesis. Mice embryo that is exposed to CsA shows poorly organized lymphatic endothelial cell sprouts from the jugular lymph sac and diminished lymphatic function. In the same study, it was shown that inhibition of calcineurin activity reduces VEGFR-3 expression in a model of lung lymphangiogenesis in adult mice [23]. If CsA had distracting effect on lymphangiogenesis in our study, it definitely was not sufficient enough to prevent allograft lymphangiogenesis. Although we have not outlined the possibility that CsA increased lymphangiogenesis, the current knowledge implies that CsA does not have an activating effect on lymphangiogenesis.

In kidney transplantation patients conversion of CsA to SRL seems to improve their GFR at least when the conversion is done early after transplantation [10,24–26]. It is still unclear whether enhanced GFR is associated with improved histology as protocol biopsies were analyzed only in a few controlled studies [27,28]. Here, SRL reduced nodular inflammatory cell infiltrate formation, kidney fibrosis, arterial intimal proliferation, and enhanced kidney allograft function. Our results suggest that SRL ameliorates graft function and this finding is associated with improved histology. Lack of nephrotoxicity provides one explanation to the beneficial effects of SRL. Based on our findings SRL is a potent inhibitor of lymphangiogenesis in kidney allografts. As increased lymphatic vessel density seems to be associated with poor graft outcome, inhibition of lymphatic proliferation could be involved in the nephroprotective properties of SRL.

In conclusion, lymphangiogenesis is associated with poor graft outcome in kidney allografts. Lymphangiogenesis can be avoided by using SRL as immunosuppressant. In this study, SRL improved kidney function and attenuated the development of chronic kidney allograft injury. Inhibition of lymphangiogenesis is a possible mechanism mediating these protective effects of SRL.

Authorship

NP, JS: participated in research design, writing of the paper, performance of the research and data analysis. PK: participated in research design, provided intellectual input and edited the manuscript.

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Conflict of interest

The research group has received a research fund from Novartis for another study. Novartis produces mTOR inhibitor everolimus and may thus gain through this publication.

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