Lymphatic Neoangiogenesis in Transplanted Kidneys

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Transplantation and chronic dialysis are life saving renal replacement therapies, that, however, are not only associated with human suffering, but also with substantial financial burdens (1). The function of grafted kidneys is imperiled by rejection reactions, that are mediated by massive invasion of alloreactive mononuclear recipient cells into the cortical stroma, and destruction of tubules and/or endothelial cells. Aggressive, unselective immunosuppressive therapy usually results in rapid clinical improvement of the transplant function, however, each acute rejection episode leaves a permanent mark on the graft's function (2). Poorly defined, persistent low-grade alloimmune responses are thought to continue during the entire life span of the graft, and eventually contribute to the multifaceted process of chronic transplant rejection that is currently the major cause for long term graft failure, and is out of reach of therapy (3). Thus, detailed knowledge of the molecular mechanisms that determine influx and disappearance of the rejection infiltrate and the site(s) and mechanisms of continuous immune responses are required to design therapeutic strategies specifically targeted at the recurrence of acute rejection and development of chronic rejection.

Acute interstitial rejection is accurately defined as a special form of chronic inflammation, with a mixed infiltrate of mononuclear inflammatory cells ($CD4^+$ and $CD8^+$ T-lymphocytes, macrophages) that invade the cortical tubulo-interstitial spaces (4). Despite many years of experience with transplant biopsies little is known about the kinetics of infiltrate clearing after immunosupressive therapy, and it is not certain that the nodular infiltrates of mononuclear cells in the cortical stroma are residues of the diffuse infiltrate found in acute rejection. Disappearance of the rejection infiltrate is in part due to apoptosis that occurs in the renal cortex at a rate similar to that in the thymus (5). However, even this relatively high apoptotic rate is obviously too low to account for the disappearance of inflammatory infiltrate cells after immunosuppressive treatment, thus raising the necessity for additional mechanisms of elimination. This has raised the possibility that, similar to other inflamed tissues, cortical lymphatic vessels could serve as exit routes (6), as suggested by previous studies in experimental renal transplantat rejection in which lymphatic vessels drained large amounts of fluid and mononuclear cells (7). Therefore we have investigated here the distribution, density, and function of lymphatic vessels in acute rejection, taking advantage of recently developed specific markers for lymphatic endothelial cells (8). We have chosen to examine biopsies and explants from human renal transplants rather than experimental models that only imperfectly mirror the course of rejection and its corresponding morphological features in humans.

Angiogenesis of renal lymphatic vessels under pathological conditions is an uncharted territory, primarily due to the lack of reliable markers for lymphatic endothelial cells. Here we have compared the distribution of lymphatic vessels in normal kidneys and grafts with transplant rejection by immunohistochemistry, and have discovered the occurrence of massive lymphatic neoangiogenesis in transplants, as well as a novel function of lymphatic vessels in association with immunologically active, intrarenal nodular lymphatic infiltrates.

Critical for investigations in the nascent field of lymphangiogenesis is the reliability of the immunocytochemical markers used (10). Currently, four markers distinguish lymphatic from blood vessel endothelial cells: (i) The membrane mucoprotein podoplanin that qualifies as highly specific for lymphatic endothelial cells (9), and was instrumental for the first isolation of pure lymphatic endothelial cell lineages from human dermis (11, 12); (ii) VEGFR-3 that is also expressed in endothelial cells of newly formed blood vessels (13); (iii) LYVE-1, a CD44-related hyaluronate receptor (14); (iv) Prox-1, a transcription factor that controls the lymphatic phenotype of endothelial cells (15). In this study, the immunohistochemical results were obtained primarily with antibodies to podoplanin, and identical results were obtained by doublelabeling with antibodies against LYVE-1 and Prox-1.

In the cortex of normal human kidney, podoplanin/LYVE-1/Prox-1-expressing lymphatic vessels were confined to the adventitia of large and middle sized arteries, as described previously (16), and this pattern of distribution persisted in acute phases of transplant rejection with intense interstitial mononuclear infiltration. However, in biopsies containing nodular infiltrates there was about < 100 fold amplification of the lymphatic vessel density over controls, with lymphatic microvessels reaching deep into the tubulo-interstitial space. These lymphatic vessels were formed by lymphangiogenesis, as many of them expressed the nuclear proliferation marker Ki-67, and presumably sprouted from preexistent perivascular lymphatics. In the lumina of the newly formed lymphatics $CD2^+$ and fewer $CD2^-$ cells were frequently encountered, indicating that the lymphatic vessels contribute to the clearing of the rejection infiltrate from the renal cortex. Lymphatic neoangiogenesis apparently involved the appearance of $CD68^+CD23^+$ macrophages that produce VEGF-C and VEGF-D, similar to a recently discovered subset of tumor-associated macrophages that were related to peritumoral lymphatic vessel proliferation (11),

and to tubulo-interstitial mononuclear cells in the rat remnant kidney model of cortical fibrosis (20). These data provide further support for the hypothesis that VEGF-C-producing macrophages contribute to regionalized lymphatic neoangiogenesis.

A potentially important observation in all biopsies with nodular infiltrates is the colocalization of lymphatic vessels with the nodular mononuclear infiltrates that apparently are immunologically highly active organoid structures, with sometimes massive $Ki67^+$ T- and B-lymphocyte activation. Immunohistochemical typing of the nodular mononuclear infiltrates revealed clusters of $CD4^+$ and $CD8^+$ cells, as well as $CD20^+$ B-lymphocytes, and λ and κ chain-expressing plasmacytoid cells. $S\text{-}100^+$ dendritic cells were observed in association with lymphatic vessels. These results provide evidence that within the peri-lymphovascular nodular infiltrates, activation and maturation of T-lymphocytes occurs by antigen presentation by dendritic cells, and that $CD\text{-}20^+$ B-cells mature to immunoglobulin-producing plasmacytoid cells. Thus, nodular infiltrates resemble lymphatic organs in autoimmune diseases that locally perpetuate autoimmune reactions, and support autoantigenic epitope spreading (18). However, the precise positioning of nodular infiltrates and lymphatic neoangiogenesis in the chronology of resolution of acute transplant rejection remains to be determined, and a large scale systematic analysis of protocol biopsies is underways to clarify this issue (our unpublished observations).

The close association of nodular infiltrates with lymphatic vessels raises the possibility that the lymphatic endothelial cells actively recuit lymphocytes. A good candidate chemokine for this purpose is SLC/CCL21 that organizes lymphatic follicles when expressed ectopically in □-cells of mouse pancreas (19), and attracts mononuclear cells in inflammatory diseases (20), while mice with deletion of the SLC/CCL21 gene fail to develop certain lymphnodes (21). We have previously discovered that SLC/CCL21 is produced and polarly secreted by isolated lymphatic endothelial cells (11). Here we show that lymphatic vessels in transplants produce SLC/CCL21 in situ, and are surrounded by $CCR7^+$ cells, providing direct evidence for SLC/CCL21-mediated lymphocyte, and presumably also dendritic cell chemoattraction by lymphatic vessels. These findings assign a novel, active role to lymphatic vessels in the organization of the peri-lymphovascular nodular infiltrates in renal transplants, and perhaps in lymphoid organogenesis in general.

In tissues SLC/CCL21 gradients are required for the directed migration of $CCR7^+$ cells (22), and are established by charge interactions with proteoglycans in basement membranes (23), that, however, are not produced by lymphatic vessels. Here we provide evidence by double labeling immunoelectron microscopy that podoplanin contributes to the peri-lymphovascular SLC/CCL21 gradient formation, as SLC/CCL21-podoplanin complexes appear on the basal membrane of lymphatic endothelia, and are shed into the perivascular stroma. Also, surface plasmon resonance binding indicated a high affinity charge and carbohydrate side chain-dependent binding of podoplanin to SLC/CCL21. Collectively, these data provide evidence that SLC/CCL21-podoplanin complexes contribute to the perivascular gradient formation, and assign a novel function to podoplanin, as a binder of cationic chemokines in lymphatic endothelial cells.

In conclusion, the novel results of this study put newly formed lymphatic vessels and peri- and para-lymphovascular infiltrates center stage as foci of immunological activity in human renal transplants. By virtue of their cellular composition, nodular infiltrates have the potential to launch and perpetuate specific immune responses to graft alloantigens, and could thus contribute to recurrent episodes of acute rejection, support humoral rejection (24), pave the way for chronic rejection and eventual loss of transplant function, and thus could provide a novel therapeutic target.

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