Perspective

The importance of neovascularization and its inhibition for allogeneic hematopoietic stem cell transplantation

Olaf Penack¹, Gerard Socié² & Marcel R M van den Brink³

1. Department of Hematology and Oncology, Charité, Campus Benjamin Franklin, 12200 Berlin, Germany
2. Department of Hematology, Hôpital Saint-Louis, AP-HP, 1, avenue Claude Vellefaux, 75010 Paris, France
3. Departments of Immunology and Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

Short title: Neovascularization and its inhibition during HSCT

Corresponding author:
Olaf Penack, MD
Charité University Hospital,
Department of Hematology and Oncology,
Hindenburgdamm 30, 12200 Berlin, Germany
Phone: +493084454713, Fax: +493084454468
E-mail: olaf.penack@charite.de
Abstract

Graft-versus-host disease (GVHD) and tumor relapse are fundamental problems in allogeneic hematopoietic stem cell transplantation (HSCT). Recent research has linked neovascularization to GVHD, tumor growth and graft-versus-tumor activity (GVT). Damage of the endothelium by the conditioning regimen provides the initiation stimulus for recruitment of donor-derived endothelial cells and its progenitors. During the early inflammatory phase of GVHD there is considerable neovascularization facilitating migration of inflammatory cells to target organs. In the course of GVHD, however, the vasculature itself becomes a target of alloreactive donor T cells. As a consequence, later stages of GVHD are characterized by fibrosis and rarefaction of blood vessels. Importantly, the inhibition of tumor-neovascularization by activated donor T cells that release anti-angiogenic substances contributes to GVT and may be enhanced by pharmacological inhibition of neovascularization. Furthermore, the therapeutic inhibition of neovascularization may improve immunotherapy for cancer by enhancing leukocyte infiltration in tumor tissue due to normalization of tumor vessels and stimulation of leukocyte-vessel wall interactions. These insights identify important mechanisms underlining the importance of neovascularization for allogeneic immune responses and move therapeutic approaches targeting neovascularization into the spotlight. This perspective covers current knowledge of the role of neovascularization during GVHD as well as GVT and its implications for HSCT.
The vasculature during graft-versus-host disease

Graft-versus-host disease (GVHD) is a potentially lethal complication in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT). It is characterized by damage of predominantly epithelial tissues in target organs caused by allo-activated T-cells recognizing host tissue antigens. However, vascular pathological processes, such as neovascularization and endothelial damage, play important roles during GVHD. The vasculature is sequentially affected during GVHD (Figure 1): 1) Initial endothelial damage is caused by the conditioning regimen; 2) In the second phase neovascularization and recruitment of inflammatory cells occur; 3) in the third phase alloreactive T cells target the endothelium and blood vessels are destroyed. Table 1 summarizes studies on neovascularization during GVHD.

Initial Endothelial Damage by the Conditioning Regimen

Radiation and chemotherapy are used as conditioning regimens and both cause endothelial damage in many organs including the lung, the intestines and the brain.\(^1\)\(^2\) In murine models, chemotherapy regimens that are often used in clinical HSCT, e.g. cyclophosphamide (60mg/Kg, days 1+2) or methotrexate (15mg/m\(^2\), days 1,3,6+11), were found to increase the number circulating endothelial cells, which are measured to estimate endothelial damage.\(^3\) Both cyclophosphamide and methotrexate cause significant apomorphosis, hydropsia and cytomembrane damage in endothelial cells.\(^3\) Radiation activates endothelial cells *in vitro* and *in vivo* in doses that are clinically applied as HSCT conditioning (2 Gy to 12 Gy).\(^4\)\(^5\) Radiation with 7.5 Gy, which is lower than the standard ‘full dose’ conditioning with 12 Gy total body irradiation (TBI), was found to induce persistent anatomic changes in the endothelium, including intracellular edema and occlusion of microvascular lumens by edematous endothelial cells.\(^6\) Human studies show that the intensity of the conditioning regimen positively correlates with endothelial damage, as assessed by plasma levels of VWF, ADAMTS-13 activity, sVCAM-1, and sTNFRI.\(^7\) The level of cyclic GMP, which is also an indicator for severe endothelial damage, was found to be increased after total body irradiation (TBI) in a subset of patients undergoing HSCT. An elevated cyclic GMP level was a negative predictive factor for survival after HSCT, suggesting that endothelial damage plays a significant role in post-transplant morbidity and mortality.\(^8\) Calcineurin inhibitors, in particular cyclosporine A, may further aggravate endothelial
damage caused by the conditioning regimen.\textsuperscript{9} Taken together these findings demonstrate that the conditioning regimen (irradiation or/and chemotherapy) as well as cyclosporine A may damage host endothelial cells. The early endothelial damage probably contributes to the initiation of processes that lead to neovascularization and inflammation characterizing GVHD.

Neovascularization during GVHD
The new formation of blood vessels in adults is termed neovascularization. Neovascularization is either mediated by angiogenesis, the proliferation of resident tissue endothelial cells, or by vasculogenesis, the incorporation of vascular endothelial progenitor cells (EPCs). It was discovered in the early 1970s that angiogenesis by capillary sprouting of host vessels is important for growth of malignant tumors.\textsuperscript{10} During capillary sprouting, vessels dilate and become leaky in response to several factors including vascular permeability factor (VPF) and vascular endothelial growth factor (VEGF).\textsuperscript{11} Angiopoetin-2 is involved in the detachment of pericytes and loosening of the matrix. Various factors stimulate endothelial proliferation during angiogenesis, including VEGF, fibroblast growth factor, transforming growth factor (TGF)-\(\beta\)1, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), platelet-derived growth factor (PDGF) and several chemokines.\textsuperscript{11} The importance of angiogenesis for malignant diseases and for a variety of inflammatory diseases is underlined by the clinical efficacy of substances that inhibit angiogenesis to treat cancer and inflammation.\textsuperscript{12-15} Vasculogenesis by bone marrow-derived EPCs plays a role during embryogenesis and recent data suggest that it is also important for tumor vasculature in adults, although there is controversy regarding this issue.\textsuperscript{16-18} EPCs are a subset of bone marrow resident cells, which are probably derived from hematopoietic stem cells and express progenitor markers as well as endothelial antigens. Mobilization of EPCs from the bone marrow to the peripheral blood is regulated by various factors and can occur during inflammation, tumor growth, ischemia and vascular trauma.\textsuperscript{19} EPCs preferentially home to activated endothelium with a high level of adhesion molecule expression. After homing to the endothelium, EPCs are inserted into the monolayer of surrounding mature vascular endothelial cells, which may lead to the formation of new blood vessels.

It was discovered early that graft-versus-host (GVH) reactions are associated with increased neovascularization. In the late 1960s / early 1970s Brent\textsuperscript{20,21} and
Billingham\textsuperscript{22,23} injected allogeneic lymphocytes and found local GVH reactions characterized by local swelling, induration of the skin and erythema. In the mid 1970s Sidky and Auerbach analyzed in detail the host local vascular response after irradiation and intracutaneous allogeneic lymphocyte transfer.\textsuperscript{24} This work was performed in the context of a lack of reliable methods to predict and measure the strength of GVH reactions. The authors hypothesized that the assessment of the host local vascular response can serve as a quantitative measure of GVH reactions. They irradiated HalCr mice (8 Gy) and intracutaneously injected allogeneic BALB/c splenocytes or syngeneic splenocytes. Using a dissecting microscope the vascular density was assessed by counting the number of vessels per field. When immunocompetent cells were injected into histoincompatible hosts, the scar region became surrounded by a network of blood vessels as early as 48 hrs after injection. Interestingly, the number of allogeneic splenocytes that were injected correlated directly with the amount of neovascularization. Intracutaneous injection of syngeneic splenocytes did not result in neovascularization. Despite these early studies on vascular proliferation during local GVH reactions, the role of neovascularization during GVHD has not been studied experimentally until very recently. We used murine GVHD models to assess neovascularization\textsuperscript{25} following the hypothesis that neovascularization plays an important role during GVHD and can be used as therapeutic target. In lethally irradiated HSCT recipients we found higher vessel density in the liver, illeum and colon during GVHD. Using flow cytometry we found that neovascularization was due to donor-derived endothelial cells. We next adoptively transferred selected green-fluorescence-protein positive (GFP+) EPCs and observed incorporation into the neo-vasculature of the inflamed intestines and liver during GVHD. Taken together, these data shows that GVHD is characterized by neovascularization, which is mainly driven by vasculogenesis, as opposed to angiogenesis. The predominant role that donor ECs play in the formation of neovasculature after HSCT in our models is not surprising because of the negative effect that lethal doses of irradiation have on host EC function.\textsuperscript{26-33} It has been shown that irradiation doses similar to those used clinically are sufficient to inhibit EC function in allo-BMT recipients.\textsuperscript{6} Future experimental studies should determine the role of vasculogenesis vs. angiogenesis during GVHD in HSCT models using chemotherapy, as opposed to lethal irradiation.
Results from human studies are in line with the findings in murine models demonstrating the presence of neovascularization during GVHD. Several studies have shown that GVHD is associated with neovascularization in target organs, such as the intestines and the skin. The vascular density in samples from gastric biopsies was found to be greater in patients with GVHD as compared with samples from patients with gastritis and to normal controls. In the histopathological analysis of skin biopsies signs of vascular proliferation were significantly more common in acute cutaneous GVHD than in control skin biopsies from HSCT recipients free of GVHD. Regarding the importance of vasculogenesis vs. angiogenesis in human GVHD a number of studies support the hypothesis that vasculogenesis contributes to neovascularature in GVHD target organs. Hebbel and colleagues investigated circulating endothelial cells in HSCT recipients. In peripheral blood, they found host-derived endothelial cells as well as donor-derived endothelial cells. However, only the donor-derived circulating endothelial cells had a high capacity to proliferate in cultures. These findings suggest that circulating donor-derived endothelial cells and its progenitors, as opposed to host-derived endothelial cells, contribute to blood vessel growth after HSCT.

In line with these results, there is a series of clinical studies that showed that donor bone marrow derived vasculogenesis contributes to neovascularization in the skin and intestines during GVHD. These studies used a combination of XY fluorescence in situ hybridization (FISH) and immunostaining in skin and/or gut biopsies from sex-mismatched female transplant recipients (with male donors). In skin biopsy samples endothelial cells of donor origin were considerably increased in patients with GVHD. Our group also studied the donor-versus recipient origin of endothelial cells in the skin of sex-mismatched HSCT recipients. Combining FISH 3D tissue Z-stack analysis of double immunostaining, we found endothelial cells of donor origin, but only in patients with GVHD in areas of severe GVHD tissue damage.

There are two possible mechanisms how bone marrow-derived EPCs may directly contribute to vasculogenesis after HSCT: 1) fusion between donor EPCs and host endothelial cells; or 2) differentiation of donor EPCs to endothelial cells. To clarify the mechanism, Fleming and coworkers performed XY FISH and immunohistochemistry in gut and skin biopsies of sex-mismatched transplant recipients. Donor-derived endothelial cells were detected in the skin and gut of transplant recipients with a
mean frequency of 2%. None of the >4,000 endothelial cells examined had more than two sex chromosomes, consistent with an absence of cell fusion. This finding is in line with our own data (unpublished): In a MHC mismatched murine HSCT model (donor H2kB and recipient H2kD) we specifically looked for cell fusion events, that would lead to co-expression of the MHC molecules H2kB and H2kD on endothelial cells detectable, using flow cytometry. Endothelial cells in GVHD target organs were always single positive (either H2kB or H2kD) for the donor-host markers. Therefore, differentiation of EPCs to ECs, rather than cell fusion, appears to be the main mechanism of vasculogenesis during GVHD.

In conclusion the human studies support the experimental data showing that acute GVHD is associated with increased neovascularization (Table 1). Experimental data suggest that neovascularization in GVHD target organs after HSCT is mediated primarily by donor-derived vasculogenesis, as opposed to host-derived angiogenesis. Clinical studies confirm that donor-derived vasculogenesis contributes to neovascularization during GVHD. However, clinical studies did not permit any quantitative assessment of the relative contributions of vasculogenesis vs. angiogenesis.

These findings in experimental models as well as in humans may be clinically significant because of their potential implications for therapies targeting neovascularature after HSCT. To date most anti-neovascularization therapies, which are used clinically or pre-clinically against cancer or inflammatory diseases, inhibit angiogenesis. The potential effect of drugs targeting VEGF, such as bevacizumab, on vasculogenesis warrants further experimental data and clinical studies. One could hypothesize that anti-VEGF treatment inhibits vasculogenesis because VEGF is highly expressed on circulating EPCs; however, this has not been studied experimentally. The predominant role of vasculogenesis in the formation of neovascularature during GVHD makes it an suitable target for selective therapies. Since many physiological processes, e.g. wound healing and tissue regeneration, are dependent on angiogenesis it is reasonable to believe that the specific inhibition of vasculogenesis has fewer unwanted effects as compared with the inhibition of angiogenesis. The perception that neovascularization plays a role in GVHD pathophysiology prompted several studies investigating vascular endothelial growth factor (VEGF) levels and VEGF single nucleotide polymorphisms (SNPs). A positive correlation between a low VEGF level and the occurrence of GVHD was found in
patients undergoing HSCT.\textsuperscript{40} Another study correlated SNPs leading to a lower VEGF production with a higher incidence of GVHD.\textsuperscript{41} In line with these results it was demonstrated that high VEGF levels after HSCT were associated with a trend towards less severe acute graft-versus-host disease.\textsuperscript{42} These clinical results suggest a correlation between low VEGF production and the severity of GVHD in HSCT recipients. However, the mechanism of this connection is unclear and the clinical results are currently not supported by experimental data. In murine HSCT models, we found that VEGF genes were neither upregulated nor downregulated during GVHD.\textsuperscript{25} Further experimental studies in animal models are needed to clarify the mechanism of the correlation between VEGF production and GVHD in patients undergoing HSCT. In future experiments it will be particularly important to investigate the effect of monoclonal antibodies that target murine VEGF (e.g. G6-31) and to use VEGF-deficient mice as allo-HSCT donors and/or recipients.

Vasculogenesis also plays a role in solid organ transplantation: several investigators have demonstrated that bone marrow-derived EPCs participate in the formation of neovasculature in allografts.\textsuperscript{43} After human cardiac transplantation\textsuperscript{44} and after human renal transplantation\textsuperscript{45} as many as 20\% of donor vascular endothelial cells were found in the allograft. The percentage of bone marrow-derived endothelial cells was highest after acute vascular allograft rejection.\textsuperscript{45}

Neovascularization as therapeutic target in GVHD

The inhibition of neovascularization has been successfully used therapeutically in inflammatory diseases, such as inflammatory bowel disease, arthritis and dermatitis.\textsuperscript{12-14} As mentioned earlier, vasculogenesis, as opposed to angiogenesis, plays a predominant role in the formation of neovasculature during GVHD after lethal irradiation. However, whether donor endothelial cells simply reflect a wound healing process or an active pathological process is currently unknown. Only in the latter case it would be logical to use inhibitors of neovascularization as a GVHD therapy. We hypothesized that the inhibition of neovascularization could prevent the development of GVHD suggesting that neovascularization during GVHD is an active pathological process. To specifically inhibit vasculogenesis, we used an antibody (E4G10), which recognizes vascular endothelial cadherin (VE-cadherin) monomers on EPCs.\textsuperscript{25} We observed that administration of E4G10 was associated with a significant inhibition of donor bone marrow-derived neovascularization in the liver,
ilium and colon during GVHD. E4G10 treated HSCT recipients had better survival, less target organ damage, reduced numbers of tissue-infiltrating CD3+ T cells and lower clinical GVHD scores in different murine GVHD models. The main mechanism of the therapeutic efficacy of the inhibition of neovascularization to reduce inflammation is likely to be the impaired recruitment of pro-inflammatory cells migrating via the blood vessels to inflammatory sites. However, endothelial cells have many in vivo functions and further evidence from animal studies during GVHD with particular focus on the role of different cell types during vasculogenesis, such as EPCs vs. myeloid cells, are needed to gain knowledge regarding the mechanisms of the interplay between neovascularization and inflammation. In animal models there are several established methods to genetically or pharmacologically deplete circulating EPCs, including the use of ID deficient mice, ID antagonism and the use of antibodies against VE-cadherin monomers. Furthermore it will be important to investigate the effects of the adoptive transfer of selected donor EPCs after allo-HSCT on the development of GVHD. Aggravation of GVHD as a result of EPC transfer would support the hypothesis that EPCs are mediators of vasculogenesis during GVHD. The investigation of the specific contribution of myeloid cells to vasculogenesis in GVHD animal models might proof to be more difficult because a global depletion of myeloid cells during GVHD, e.g. with liposomal clodronate, has multiple negative effects, including a higher susceptibility to infections, and may lead to shorter survival. There are, however, several substances/pathways that could be useful to specifically target the migration and/or function of myeloid cells in preclinical GVHD models: 1) Prokineticin-2 (Bv8) is an important pro-angiogenic factor which is produced by myeloid cells. Monoclonal antibodies against Bv8 lead to impaired recruitment of myeloid cells to tumor neovasculature and to inhibition of neovascularization; 2) Matrix Metalloproteinase 9 (MMP9) is another pro-angiogenic factor produced by myeloid cells. Genetic or pharmacological antagonism of MMP9 leads to impaired neovascularization during tumor growth and inflammatory diseases, such as bronchial asthma and inflammatory bowel disease; 3) The tyrosine kinase receptor 'colony stimulating factor receptor-1' (CSF-1R, CD115) regulates the recruitment of myeloid cells to tumors as well as to inflammation sites and can be used as a therapeutic target to inhibit myeloid-cell mediated vasculogenesis.
To test whether VEGF could be used as a therapeutic target during GVHD we used anti-VEGFR1/anti-VEGFR2 antibodies after allo-BMT and found an inhibitory effect of on hematopoietic reconstitution leading to early death of allo-BMT recipients. Furthermore, we found that VEGF was not overexpressed during GVHD in target tissues. These results suggest that the use of anti-VEGF strategies for prevention of GVHD may not be effective and may potentially inhibit hematopoietic reconstitution.

However, one recently published small clinical study used bevacizumab (Anti-VEGF-A mAb) in sarcoma patients undergoing autologous HSCT without apparent negative impact on reconstitution. Sixteen patients received 7.5 or 10 mg/Kg bevacizumab at day –5 of HSCT in combination with ICE (ifosfamide, carboplatin and etoposide) and no delay of hematopoietic reconstitution was seen. The use of bevacizumab prior to HSCT, as opposed to using bevacizumab after HSCT, could explain the discrepancies in the inhibition of reconstitution between the preclinical models and the clinical study.

Future studies should investigate neovascularization not only in GVHD target organs but also in lymphoid organs because the inhibition of lymphatic vessel growth may impact the activation and proliferation of immune cells, such as alloreactive T cells, during GVHD. A recent report shows that the specific inhibition of lymphangiogenesis with a monoclonal antibody against vascular endothelial growth factor receptor 3 (VEGFR3 mAb) increases the severity of inflammation in a mouse model of chronic inflammatory arthritis. In line with these results, another study found that activation of the VEGFR-3 pathway by VEGF-C attenuates skin inflammation by promoting lymphangiogenesis. On the other hand, treatment with VEGFR-3 mAb reduced the level of tissue infiltrating alloreactive T cells in a cardiac allograft model, suggesting that inhibition of lymphangiogenesis may lead to reduced inflammatory reactions in the setting of histoincompatibility. Taken together, it is currently hard to predict if lymphangiogenesis could be a therapeutic target during GVHD.

Another approach to inhibit GVHD with substances that inhibit neovascularization, is the administration of proteasome inhibitors. Bortezomib has been shown to be a potent inhibitor of angiogenesis. Two different groups reported that the early administration of bortezomib, a proteasome inhibitor, protects against the development of acute GVHD in murine HSCT models. These reports are in line with a promising clinical report investigating bortezomib as GVHD prophylaxis in patients undergoing HSCT. However, proteasome inhibitors have multiple in vivo
effects, in particular the proteasome has been shown to play a role in T cell activation, proliferation, and apoptosis.\textsuperscript{64} The above mentioned studies on bortezomib in experimental GVHD have not assessed neovascularization as a possible effect of bortezomib efficacy. Therefore, it is not clear if the inhibition of neovascularization by bortezomib is relevant to its positive effects on GVHD. Further studies on bortezomib in GVHD mouse models, specifically designed to investigate neovascularization, are needed to clarify if the activity of bortezomib is mainly – or in part - based on the inhibition of neovascularization.

Currently, there are no clinical studies available investigating the efficacy of drugs specifically targeting neovascularure in the prevention or treatment of GVHD. However, several established drugs for GVHD prophylaxis, such as cyclosporine A (CSA), methotrexate and mycophenolate mofetil (MMF), inhibit neovascularization besides having multiple other effects \textit{in vivo}. In murine models of corneal neovascularization CSA inhibited the migration of primary endothelial cells and reduced angiogenesis induced by vascular endothelial growth factor (VEGF).\textsuperscript{65} Furthermore, CSA inhibits endothelial cell function \textit{in vitro}\textsuperscript{66} and was found to cause endothelial dysfunction in animal studies using capillary tube assays.\textsuperscript{67} Methotrexate also inhibited endothelial cell proliferation \textit{in vivo} in a model for corneal neovascularization.\textsuperscript{68} Several groups have found that MMF reduces endothelial cell proliferation \textit{in vitro} as well as \textit{in vivo}.\textsuperscript{69,70} These data suggest that the inhibition of neovascularization might contribute to the inhibitory activity of CSA, methotrexate and MMF in the development of GVHD.

Endothelial damage during GVHD

Endothelial damage is a pathological hallmark of vascular complications after HSCT, such as veno-occlusive disease of the liver, thrombotic microangiopathy and capillary leak syndrome. Although acute GVHD is classically considered to be an ‘epithelial’ disease, both the presence of cutaneous erythema and gastrointestinal bleeding led to the hypothesis that the vasculature may be directly or indirectly damaged during GVHD.\textsuperscript{95} Disseminated endothelial cell apoptosis was the first detectable lesion in a murine model of acute tissue damage induced by systemic transfer of allogeneic lymphocytes suggesting that vascular lesions play an important role in the pathogenesis of allogeneic immune responses.\textsuperscript{71} In another murine model of acute GVHD that did not involve any conditioning treatment, the earliest detectable oral
mucosa lesion was apoptosis of the endothelial cells from chorionic capillaries, which precedes basal keratinocyte apoptosis. Moreover, endothelial cell death and lymphocytic inflammation preceded epithelial injury during the development of acute GVHD. These findings collectively demonstrate that endothelial cells are damaged by activated alloreactive donor T cells. During GVHD host hematopoietic antigen presenting cells play an important role in the activation of donor T cells. Alloantigen presentation by hematopoietic professional antigen-presenting cells is, however, not required for activation of allogeneic T cells. Vascularized cardiac allografts are acutely rejected via CD8+ direct allore cognition even if the alloantigen is not presented by hematopoietic antigen presenting cells. This can happen because endothelial cells are able to present antigens to T cells potently through different pathways. Through a direct pathway and through an indirect pathway liver sinusoidal endothelial cells are capable of cross-presenting soluble exogenous antigen to CD8+ T cells. Of note, endothelial cells do not always effectively activate alloreactive T cells. There is the possibility that antigen in the vasculature can be immunologically ignored. Lakkis et al. found in cardiac allograft models that alloimmune responses to a vascularized organ transplant were not initiated in the graft itself. In recipients lacking secondary lymphoid organs they demonstrated that the permanent acceptance of allografts was due to immunologic 'ignorance'. Another group found in a GVHD-like model and in solid organ transplant models that CD8+ T-cell responses against minor antigens were not initiated by endothelial cells in the absence of dendritic cells. In a human study of intestinal GVHD, pericapillary hemorrhage was demonstrated in areas with endothelial cell lesions, and this severe form of intestinal GVHD was associated with severe hemorrhagic enterocolitis. The endothelial damage during GVHD may be intensified by prophylaxis with calcineurin inhibitors which cause injury to endothelial cells. In later stages of GVHD, the destruction of vasculature leads to rarefaction of blood vessels in target organs. In murine models of GVHD, we found that the vascular density in the intestines decreased after day +30 after HSCT (unpublished observation). This finding is in line with a report on patients with sclerotic chronic GVHD of the skin. Here, cutaneous microvessel loss was identified as a hallmark feature and was associated with an infiltration of CD8 T lymphocytes into the upper dermis. In line with these results it was demonstrated that during chronic GVHD the

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number of circulating EPCs is decreased. Patients with chronic sclerodermatous GVHD also had impaired endothelial-forming ability compared to patients after HSCT without chronic sclerodermatous GVHD. However, the rarefaction of vessels in the skin during chronic GVHD seems to be less pronounced as compared with systemic sclerosis. Areas of microvascular endothelial proliferation were present in the biopsies taken at relatively early time points during chronic GVHD but not in late biopsies.

Since neovascularization and endothelial damage occur during GVHD, markers of endothelial biology may be helpful in diagnosis of GVHD. Circulating endothelial cells, endothelial microparticles and endothelial cell markers, are rarely found in the peripheral blood of healthy individuals and increase when endothelial injury occurs. In patients after myeloablative HSCT with Busulfan/Cylophosphamide circulating endothelial cells were found to continuously increase till day +21. Kolb and colleagues analyzed endothelial cell derived microparticles in HSCT recipients. Microparticles were not significantly influenced by the conditioning regimen or by infectious complications. However, in patients with GVHD significantly higher levels of microparticles were detected as compared to the controls after HSCT without GVHD. Endothelial microparticles may contribute to inflammation because they induce maturation and activation of plasmacytoid dendritic cells. The same group also demonstrated that the endothelial cell markers von Willebrand factor (VWF) and thrombomodulin (TM) are elevated in the peripheral blood of HSCT patients with GVHD. These results suggest that markers of endothelial cell biology might serve as a diagnostic test for differentiation of GVHD from other transplanted related complications. This underlines the importance of vascular processes for the pathophysiology of GVHD.

Activation, targeting and damage of endothelial cells are not only critical for GVHD – these processes are also important mechanisms during allograft rejection in solid organ transplantation. Allograft rejection involves recruitment and activation of circulating leukocytes in response to activated microvascular endothelial cells. During rejection class I MHC molecules on graft endothelial cells are recognized by host alloactivated T cells leading to endothelial damage. The replacement of graft endothelium by recipient bone marrow-derived endothelial cells was found to be required for allograft rejection by CD4+ T cells, underlining the significance of vasculogenesis for alloimmunity.
The vasculature and graft-versus-tumor (GVT) activity

It is well accepted that the inhibition of angiogenesis as well as of vasculogenesis can inhibit tumor growth and several inhibitors of angiogenesis are already in clinical use as cancer therapies. More recently, knowledge has been gained regarding the inhibition of neovascularization as mechanism of action of T cell therapies against cancer. There is an increasing body of evidence showing that T cells not only directly interact with tumor cells, but also target tumor vasculature during allogeneic immune responses against malignancies.

To investigate the role of neovascularization in malignancies in HSCT recipients we used murine HSCT models with acute myeloid leukemia, B-lymphoma and renal carcinoma. We found that neovascularization mediated by donor-derived EPCs played a significant role in tumor growth after HSCT and lethal radiation. In HSCT models without GVT activity (without donor T cells) we found a moderate inhibitory effect of the pharmacological inhibition of vasculogenesis on tumor growth. We found inhibition of tumor growth in a solid tumor model (renal carcinoma) as well as in hematologic malignancies (lymphoma and acute myeloid leukemia). Our results are in agreement with recent clinical data suggesting a role for neovascularization not only in solid tumors, but also in hematological malignancies. In patients after HSCT relapse is more likely to involve vasculogenesis than angiogenesis, because angiogenesis is compromised due to damage to the vasculature from conditioning. This situation could be similar to malignant glioma where primary tumors induce angiogenesis, whereas relapse after radiation therapy induces vasculogenesis.

In contrast to the rather moderate effects on tumors in HSCT models without GVT, we found a stronger therapeutic effect of the inhibition of neovascularization on survival in HSCT models with GVT. We are currently performing studies to find out the main mechanism of the enhancement of GVT effects by inhibitors of neovascularization. One possible explanation for the enhancement of GVT activity through inhibitors of neovascularization is a normalization of tumor vasculature that increases the blood flow and leads to a more effective recruitment of tumor-reactive T cells to the tumor tissue. Another explanation is the enhancement of leukocyte infiltration in tumors after anti-angiogenic therapy. Several inhibitors of neovascularization including anginex, endostatin and angiostatin, were found to stimulate leukocyte-vessel wall
interactions and increase leukocyte infiltration in tumor tissues.\textsuperscript{94} These results suggest that immunotherapy strategies including HSCT may be improved by combination with reagents that inhibit neovascularization.

As mentioned before, several studies have shown that the inhibition of neovascularization contributes to the anti-tumor effects of T cell therapies. In animal models using syngeneic and allogeneic tumors it was demonstrated that tumor rejection depends on stromal events affecting the tumor environment.\textsuperscript{95} The damage of tumor neovasculature, mediated by host leukocytes, was a prerequisite to tumor rejection. Blankenstein and Qui showed that CD4+ immunity against MHCII- tumor depends on the inhibition of tumor angiogenesis as a result of IFN\textgamma\textsuperscript{ release.}\textsuperscript{96} They used different primary tumors from IFN\textgamma\textsuperscript{R}+/− as well as from IFN\textgamma\textsuperscript{R}−/− mice. For tumor rejection IFN\textgamma\textsuperscript{R} expression was necessary only in the effector phase on nonhematopoietic cells. In IFN\textgamma\textsuperscript{R}−/− mice, tumor blood vessels were observed at early time points. In contrast, in IFN\textgamma\textsuperscript{R}+/− mice, blood vessels within the tumor mass were completely absent and the tumor mass became necrotic. The authors concluded that CD4+ T cell–dependent tumor immunity involves tumor destruction indirectly by inhibition of angiogenesis. Another group demonstrated that T antigen (Tag)- specific CD4+ T cells homed selectively into the tumor microenvironment in an animal model for pancreatic carcinoma.\textsuperscript{97} CD4+ T cells inhibited tumor neovascularization through release of antiangiogenic chemokines. Combined TNFR1 and IFN-γ signaling was found to be involved in the anti-angiogenic activity. Results of another study are clinically very relevant to GVT reactions in patients undergoing HLA-matched HSCT.\textsuperscript{98} Transferred CD8+ T cells primed against a minor antigen (H7a) lead to tumor rejection in melanoma bearing mice. Tumor rejection was initiated by preferential extravasation at the tumor site of IFN\textgamma producing H7a-specific T cells leading to inhibition of tumor neovascularization.

Taken together these studies support the hypothesis that inhibition of neovascularization contributes to the beneficial GVT activity after HSCT.

**Conclusions and future directions**

Results from animal models as well as clinical data show that neovascularization is involved in GVHD, tumor growth and GVT activity after HSCT. The initiation phase of GVHD is characterized by increased neovascularization and by recruitment of inflammatory cells. In the course of the disease the vasculature is targeted by
alloreactive donor T cells and vascular destruction occurs. Tumor growth after HSCT is dependent on neovascularization and the inhibition of neovascularization contributes to the GVT activity of HSCT. Alloreactive donor T cells infiltrate the tumor stroma and secrete anti-angiogenic substances consequently leading to inhibition of tumor neovascularization and tumor cell death.

The therapeutic concept of the inhibition of neovascularization is promising because of its simultaneous beneficial effects on GVHD and GVT. A key feature after HSCT is that vasculogenesis by donor bone marrow-derived cells contributes to neovascularization, which influences therapeutic concepts to inhibit neovascularization. First results of animal studies demonstrate that amelioration of GVHD and inhibition of tumor growth is achievable by therapeutic targeting of neovascularization, in particular by targeting vasculogenesis. However, the optimal compounds as well as the best time points to inhibit neovascularization after HSCT have not been determined and clinical studies are not yet available. Approaches targeting neovascularization in HSCT recipients could provide novel strategies to prevent or treat GVHD and decrease post-transplant relapse.

Author contributions and conflict of interest:
Olaf Penack wrote the paper and declares no conflicting financial interest.
Gerard Socié wrote the paper and declares no conflicting financial interest.
Marcel van den Brink wrote the paper and declares no conflicting financial interest.
References


Table 1. Studies on neovascularization during GVHD.

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<tr>
<td>Donor bone marrow derived vasculogenesis contributes to neovascularization in the intestines during GVHD.</td>
<td>37</td>
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<tr>
<td>Donor-derived endothelial cells are more numerous and preferentially distributed in the areas of severe acute GVHD damage.</td>
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<tr>
<td>In gastric biopsies the vascular density is greater in patients with acute GVHD as compared with normal controls.</td>
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<tr>
<td>In skin biopsies from patients with acute GVHD, there are areas with high vascular density.</td>
<td>35</td>
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<tr>
<td>There is a positive correlation between a low VEGF serum level and the occurrence of GVHD.</td>
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<tr>
<td>Single nucleotide polymorphisms leading to low VEGF production are associated with a higher incidence of GVHD.</td>
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<tr>
<td>High VEGF serum levels after HSCT are associated with less severe GVHD (there was a trend, however, the association was statistically not significant).</td>
<td>42</td>
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<tr>
<td>Treatment with bevacizumab (Anti-VEGF-A mAb) prior to autologous HSCT has no major negative impact on hematopoietic reconstitution.</td>
<td>56</td>
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</tbody>
</table>
Figure 1: The endothelium is sequentially affected during GVHD. Initial endothelial damage is caused by the conditioning regimen; During the second phase neovascularization and recruitment of inflammatory cells occur; During the later stages of GVHD alloreactive T cells target the endothelium and blood vessels are destroyed. Figure by Terry Helms from Medical Graphics at Memorial Sloan-Kettering Cancer Center.
The importance of neovascularization and its inhibition for allogeneic hematopoietic stem cell transplantation

Olaf Penack, Gerard Socié and Marcel R M van den Brink