Inhibition of Neovascularization to Simultaneously Ameliorate Graft-vs-Host Disease and Decrease Tumor Growth

Olaf Penack, Erik Henke, David Suh, Chris G. King, Odette M. Smith, Il-Kang Na, Amanda M. Holland, Arnab Ghosh, Sydney X. Lu, Robert R. Jenq, Chen Liu, George F. Murphy, Theresa T. Lu, Chad May, David A. Scheinberg, Ding Cheng Gao, Vivek Mittal, Glenn Heller, Robert Beneza, Marcel R. M. van den Brink

Background. Blood vessels are formed either by sprouting of resident tissue endothelial cells (angiogenesis) or by recruitment of bone marrow (BM)-derived circulating endothelial progenitor cells (EPCs, vasculogenesis). Neovascularization has been implicated in tumor growth and inflammation, but its roles in graft-vs-host disease (GVHD) and in tumors after allogeneic BM transplantation (allo-BMT) were not known.

Methods. We analyzed neovascularization, the contribution of endothelial cells and EPCs, and the ability of anti-vascular endothelial-cadherin antibody, E4G10, to inhibit neovascularization in mice with GVHD after allo-BMT using immunofluorescence microscopy and flow cytometry. We examined survival and clinical and histopathologic GVHD in mice (n = 10–25 per group) in which GVHD was treated with the E4G10 antibody using immunohistochemistry, flow cytometry, and cytokine immunoassay. We also assessed survival, the contribution of green fluorescent protein-marked EPCs to the tumor vasculature, and the ability of E4G10 to inhibit tumor growth in tumor-bearing mice (n = 20–33 per group) after allo-BMT using histopathology and bioluminescence imaging. All statistical tests were two-sided.

Results. We found increased neovascularization mediated by vasculogenesis, as opposed to angiogenesis, in GVHD target tissues, such as liver and intestines. Administration of E4G10 inhibited neovascularization by donor BM-derived cells without affecting host vascularization, inhibited both GVHD and tumor growth, and increased survival (at 60 days post-BMT and tumor challenge with A20 lymphoma, the probability of survival was 0.29 for control antibody-treated allo-BMT recipients vs 0.7 for E4G10-treated allo-BMT recipients, 95% confidence interval = 0.180 to 0.640, P < .001).

Conclusions. Therapeutic targeting of neovascularization in allo-BMT recipients is a novel strategy to simultaneously ameliorate GVHD and inhibit posttransplant tumor growth, providing a new approach to improve the overall outcome of allogeneic hematopoietic stem cell transplantation.

in posttransplantation tumor growth in recipients of an allo-BMT. We hypothesized that inhibition of neovascularization could simultaneously ameliorate GVHD and tumor growth. To investigate vasculogenesis and angiogenesis during GVHD, we used murine models of allo-BMT, which provided several advantages: 1) Because GVHD and tumor relapse are the major complications of allogeneic hematopoietic stem cell transplantation, we were able to simultaneously study the role of neovascularization in inflammation and tumors in a clinically relevant model; 2) these models made it possible to identify BM–derived cells without genetically modifying them (and potentially affecting their function in vivo) because of the differences in the major histocompatibility complex (MHC) or minor histocompatibility antigens between donor and host; 3) the murine GVHD models were well described and characterized; and 4) inflammation was not restricted to a single organ and the pathophysiology had many similarities to other important inflammatory diseases, such as inflammatory bowel disease.

**Materials and Methods**

**Mice, BMT, and GVHD Models**

Female C57BL/6 (B6) (H-2b), C57BL/6 green fluorescent protein (GFP)+ (B6 GFP+–H-2b), B6D2F1 (H-2b/d), BALB/c (H-2d), B10BR (H-2k), and C57BL/6-CD45.1+ (B6-LyCD45.1) (H-2b) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice used in BMT experiments were between 8 and 12 weeks old. BM cells were isolated from femurs and tibias after the mice were killed as described previously (18–20). Briefly, donor mice were killed by asphyxiation using carbon dioxide, and spleens, femurs, and tibias were removed aseptically. Donor BM was obtained by flushing of tibia and femora with cold tissue culture media. Donor BM was T cell depleted (TCD) by incubation with 2.5 µg anti-Thy-1.2 (Monoclonal Antibody Core Facility, Sloan-Kettering Cancer Center, New York, NY) per 10^6 BM cells for 30 minutes at 4°C, followed by incubation with 10 µL of low-TOX-M rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) per 10^6 BM cells for 40 minutes at 37°C, so that GVHD could be reproducibly induced by simultaneous injection of T cell–depleted BM and donor splenic T cells in experimental mice. In control mice (transplantation without GVHD), T cell–depleted BM was injected without donor splenic T cells. The BM cells (5 × 10^6 per recipient) were transplanted by tail vein injection into lethally irradiated recipients (BALB/c: 850–900 cGy; B6: 1100 cGy; and B6D2F1: 1300 cGy total body irradiation from a 137 Cs source as a split dose with a 3-hour interval between doses). Recipients were treated by intraperitoneal (i.p.) injection on days 0, 2, 4, 6, 8, and 10 after allo-BMT with 1 mg of either E4G10 or rat IgG, a control antibody. In one experiment, recipients were treated i.p. on days 0, 2, 4, 6, 8, and 10 after allo-BMT with a combination of 800 µg of DC101 and MF1 or with 800 µg rat IgG. BMT protocols were approved by the Memorial Sloan-Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee. Mice were housed in sterilized microisolator cages and received normal chow and autoclaved hyperchlorinated drinking water (pH 3.0).

GVHD was induced by tail vein injection of purified splenic T cells that were obtained from the spleens of the same mice that were BM donors by nylon wool column passage as previously described (18–20). Briefly, splenocytes were mashed and strained through a 70-µm sterile strainer and incubated in nylon wool column for 1 hour at 37°C. After 1 hour, cells were eluted from column and stained with anti-CD3 antibodies conjugated to fluorescein isothiocyanate (FITC) so that T-cell purity could be analyzed by flow cytometry; results were expressed as the percentage of CD3+ cells per the number of total cells. Starting 1 week after injection of 1 × 10^6 splenic T cells, the severity of inflammation was assessed with a clinical scoring system as previously described (22). Briefly, ear-tagged mice in coded cages were individually scored every week for five clinical parameters (weight loss, posture, activity, fur, and skin) on a scale from 0 to 2. A clinical GVHD index was generated by summation of the five criteria scores (0–10), and moribund mice scoring more than five were killed. Survival was monitored daily. In some experiments, mice were killed at day 21 after BMT for blinded histopathologic analysis of GVHD target organs (small and large bowel, liver, and skin). Organs were harvested, formalin-preserved, and subsequently paraffin-embedded, sectioned, and stained with hematoxylin and eosin by K. La Perle (Cornell University Medical College, New York, NY). Gut and liver samples were analyzed by C. Liu, and a semiquantitative score was calculated that assessed 19–22 different parameters associated with GVHD (23). Skin samples were taken from the ears and were examined by G. F. Murphy, and the number of apoptotic cells per millimeter of epidermis was determined (24). The immunohistochemical detection of CD3+ T cells in GVHD target organs was performed using a rabbit polyclonal anti-CD3 antibody (DakoCytomation, Carpinteria, CA). Before the primary antibody incubation, the tissue sections were blocked for 30 minutes in 10%
normal goat serum and 2% bovine serum albumin (both from PAA Laboratories, Inc, New Bedford, MA) in phosphate-buffered saline (PBS, sodium chloride, 145 mM [0.85%] in phosphate buffer, 150 mM). Incubation with the primary antibody at 0.6 µg/mL was for 3 hours, followed by 60 minutes of incubation with biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA) at a 1:200 dilution. Secondary Antibody Blocker, Blocker D, Streptavidin–Horseradish Peroxidase, and 3,3’-diaminobenzidine detection kit (Ventana Medical Systems, Oro Valley, AZ) were used according to the manufacturer’s instructions. The protocol was established, and the experiments were performed at the Molecular Cytology Core Facility of the MSKCC using a Discovery XT automatic-processor from Ventana Medical Systems (Tucson, AZ).

**Tumor Models**

A20 mouse (BALB/c) B-lymphoma cells and RENCA mouse (BALB/c) renal cancer cells were transduced with retroviruses to stably express firefly luciferase (Promega, Madison, WI) as described previously (20,25,26). C1498 mouse (C57BL/6) myeloid leukemia cells were used without luciferase transduction. Cells were maintained in RPMI 1640 culture medium (Media Core Facility, Sloan-Kettering Cancer Center, New York, NY) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Inc), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO₂. Mice received tumor cells (2 x 10⁶ RENCA cells/mouse [n = 50 per group], 1 x 10⁶ C1498 cells/mouse [n = 20 per group] or 5 x 10⁶ A20 cells/mouse [n = 13 per group]) intravenously in a separate injection from the BM cells and T cells, on day 0. Bioluminescent signal intensity of tumor-bearing mice was determined weekly starting at 1 week after injection of tumor cells: 15 minutes after i.p. injection of 3 mg/mouse D-luciferin (Xenogen, Alameda, CA), mice were anesthetized and placed into the chamber of an IVIS 200 bioluminescence imaging system (Xenogen). grayscale photographic images of the mice were acquired first and then a low-level bioluminescent signal was recorded. Pseudo-color images showing the whole-body distribution of bioluminescent signal intensity were superimposed on the grayscale photographs, and total flux (photons/s) was determined for individual mice. The cause of death was confirmed by necropsy and histopathology.

**Histological Preparation of Tissues, Flow Cytometry, and Antibodies**

In experiments that were performed to quantify neovascularization during GVHD, livers from BALB/c or C57BL/6 allo-BMT recipients with or without GVHD (n = 10) were flushed in situ with PBS, minced, digested with 2 mg/mL collagenase D (Roche Diagnostics, Indianapolis, IN) for 45 minutes at 37°C and mashed through 40-µm cell strainers. Gradient centrifugation using 30% Histodenz (Sigma, St Louis, MO) was performed to enrich for ECs according to the manufacturer’s instructions. For analyses of EPCs in peripheral blood, mice that had been treated i.p. with 1 mg E4G10 on days 0, 2, 4, 6, 8, and 10 (n = 10) were anesthetized and 500 µL blood was withdrawn from the retro-orbital sinus. Mononuclear cells were isolated by gradient centrifugation using Histopaque 1083 (Sigma) according to the manufacturer’s instructions. Single mononuclear cells were washed in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% fetal bovine serum and 0.1% sodium azide) and incubated for 15 minutes at 4°C with 1 µg/mL anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA), an antibody that blocks nonspecific binding of antibodies to Fc receptors on monocytes and macrophages. Subsequently, mononuclear cells were incubated for 30 minutes at 4°C with one or more labeled primary rat anti-mouse antibodies from BD Biosciences Pharmingen (San Diego, CA) as follows: rat IgG2a, dilution and conjugated label according to controls; B220/CD45, phycoerythrin (PE)-Texas Red 1:800; CD117 (c-Kit), allophycocyanin (APC) 1:200; CD11b (MAC-1), APC-Cy7 1:400; CD11c, FITC 1:100; CD19, PE 1:1600; CD25, peridinin-chlorophyll (PerCP)-Cy5.5 1:100; CD25, PE-Texas Red; CD31 (PECAM-1), PE 1:100; CD34, FITC 1:200; CD3e, Pacific Blue 1:200; CD4, Alexa Fluor 700 1:800; CD4, Pacific Blue 1:400; CD45, PerCP/Cy5.5 1:800; CD45, APC 1:3200; CD62L, PE-Texas Red 1:400; CD8a, Pacific Blue 1:400; F4/80, FITC 1:200; F4/80, PE 1:50; H-2Dd, FITC 1:400; H-2kb, PE 1:200; IL-10, PE 1:50; NK-1.1, APC 1:400; VEGFR2 (Flk-1), PE 1:100; VEGFR2 (Flk-1), APC 1:100 and washed twice with FACS buffer. Rat anti-mouse E4G10 ImClone Systems Incorporated (New York, NY) was labeled to AF647 by the Monoclonal Antibody Core Facility (Sloan-Kettering Cancer Center) and was used in different concentrations as indicated. Stained cells were resuspended in FACS buffer and analyzed on a FACSCalibur or LSR II flow cytometer (BD Biosciences) with CellQuest or FlowJo software (Tree Star, Ashland, OR).

For experiments that included the adoptive transfer of EPCs, BM was harvested from B6 mice that expressed in all cells GFP for isolation of GFP+ EPCs (n = 10). Cells were stained with antibodies against mouse CD11b, TER119, c-Kit, and VEGFR2 for FACS using a BD FACS Aria cell sorter. Post–cell sorting analyses showed a purity of more than 95%. The TCD-BM and the T cells in these experiments were from B6 WT (GFP–) mice (n = 10).

The endotoxin levels of rat IgG (Innovative Research, Southfield, MI), which was used as control antibody in vivo, were <0.001 EU/µg rat IgG (Endosafe PTS Method, Charles River Laboratories, Wilmington, MA; performed by the Gene Transfer and Somatic Cell Engineering Core Facility, MSKCC).

**Immunofluorescence Staining, Microscopy, and Vessel Density Measurements**

Liver, small bowel, and large bowel from allo-BMT recipients with or without GVHD (n = 10) were cryosectioned in situ with PBS, minced, digested with 2 mg/mL collagenase D (Roche Diagnostics, Indianapolis, IN) for 45 minutes at 37°C and mashed through 40-µm cell strainers. Gradient centrifugation using 30% Histodenz (Sigma, St Louis, MO) was performed to enrich for ECs according to the manufacturer’s instructions. Single mononuclear cells were washed in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% fetal bovine serum and 0.1% sodium azide) and incubated for 15 minutes at 4°C with 1 µg/mL anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA), an antibody that blocks nonspecific binding of antibodies to Fc receptors on monocytes and macrophages. Subsequently, mononuclear cells were incubated for 30 minutes at 4°C with one or more labeled primary rat anti-mouse antibodies from BD Biosciences Pharmingen (San Diego, CA) as follows: rat IgG2a, dilution and conjugated label according to controls; B220/CD45, phycoerythrin (PE)-Texas Red 1:800; CD117 (c-Kit), allophycocyanin (APC) 1:200; CD11b (MAC-1), APC-Cy7 1:400; CD11c, FITC 1:100; CD19, PE 1:1600; CD25, peridinin-chlorophyll (PerCP)-Cy5.5 1:100; CD25, PE-Texas Red; CD31 (PECAM-1), PE 1:100; CD34, FITC 1:200; CD3e, Pacific Blue 1:200; CD4, Alexa Fluor 700 1:800; CD4, Pacific Blue 1:400; CD45, PerCP/Cy5.5 1:800; CD45, APC 1:3200; CD62L, PE-Texas Red 1:400; CD8a, Pacific Blue 1:400; F4/80, FITC 1:200; F4/80, PE 1:50; H-2Dd, FITC 1:400; H-2kb, PE 1:200; IL-10, PE 1:50; NK-1.1, APC 1:400; VEGFR2 (Flk-1), PE 1:100; VEGFR2 (Flk-1), APC 1:100 and washed twice with FACS buffer. Rat anti-mouse E4G10 ImClone Systems Incorporated (New York, NY) was labeled to AF647 by the Monoclonal Antibody Core Facility (Sloan-Kettering Cancer Center) and was used in different concentrations as indicated. Stained cells were resuspended in FACS buffer and analyzed on a FACSCalibur or LSR II flow cytometer (BD Biosciences) with CellQuest or FlowJo software (Tree Star, Ashland, OR).

For experiments that included the adoptive transfer of EPCs, BM was harvested from B6 mice that expressed in all cells GFP for isolation of GFP+ EPCs (n = 10). Cells were stained with antibodies against mouse CD11b, TER119, c-Kit, and VEGFR2 for FACS using a BD FACS Aria cell sorter. Post–cell sorting analyses showed a purity of more than 95%. The TCD-BM and the T cells in these experiments were from B6 WT (GFP–) mice (n = 10).

The endotoxin levels of rat IgG (Innovative Research, Southfield, MI), which was used as control antibody in vivo, were <0.001 EU/µg rat IgG (Endosafe PTS Method, Charles River Laboratories, Wilmington, MA; performed by the Gene Transfer and Somatic Cell Engineering Core Facility, MSKCC).
1:200 dilution. Five micrograms per milliliter rat IgG2a, κ was used as an appropriate isotype-negative control. The protocol was established, and the experiments were performed at the Molecular Cytology Core Facility, MSKCC, using the Discovery XT automatic-processor from Ventana Medical Systems. Positive cells were detected by secondary antibodies conjugated to different Alexa Fluor dyes (488, 568, 647) or by intrinsic GFP signals. For nuclear counterstaining, the fluorescent DNA-binding stain, 4',6-diamidino-2-phenylindole (DAPI), was used. Digital fluorescent images were obtained using an Axiovert 200M computerized microscope (Zeiss, Jena, Germany). For confocal laser scanning microscopy, Leica TCS SP2 AOBS microscopes were used (Leica, Germany). Images were analyzed by using Axiovision 4.5, Velocity, and MetaMorph 7.5 software. Z-stack resolutions were 23 μm. To detect blood vessels in tissue sections, similar results were obtained with anti-CD34, anti-Flik-1 (VEGFR2), and MECA-32 antibodies. To determine the vessel density, pixel areas representing CD34+ vessels were measured with a pre-determined threshold set as cutoff. Ten sections per sample were investigated, and the mean value was calculated. Vessel density was reported as percentage of the aggregated pixel vessel area to total area.

Measurement of Cytokines and Chemokines
Serum samples were obtained from allo-BMT recipients with GVHD that had been treated with 1 mg E4G10 at days 0, 2, 4, 6, 8, and 10 (n = 5) by retro-orbital collection. Serum levels were determined by using the Milliplex Mouse Cytokine/Chemokine Immunoassay (Millipore, Billerica, MA) according to the manufacturer’s instructions.

Gene Expression Profiling in Liver Tissue During GVHD
To investigate expression of proangiogenic genes during GVHD, we compared gene expression in allo-BMT recipients (generated from transfer of B6 [H-2b] BM into BALB/c [H-2d] hosts) with that of syn-BMT recipients (generated from transfer of BALB/c BM into BALB/c hosts) at day 14 after BMT. Lethally irradiated recipients received 5 × 10⁶ cells from TCD-BM and 1 × 10⁶ enriched donor splenic T cells. Livers were flushed in situ with PBS, and RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Quality of RNA was ensured before labeling by analyzing 20–50 ng of each sample using the RNA 6000 NanoAssay and a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Samples with a 28S to 18S ribosomal peak ratio of 1.8–2.0 were considered suitable for labeling. For samples meeting this standard (n = 8), 200 ng of total RNA was labeled using the Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Three micrograms of labeled and fragmented cRNA was then hybridized to the mouse-ref8 array ( Illumina, San Diego, CA), which assesses approximately 22,000 transcripts of known genes. The normalization was performed in R from the Bioconductor statistical package (http://www.bioconductor.org/open access software). The normalized value was log-transformed, and the fold change was calculated between the two groups (GVHD vs control, n = 4 per group). An average change in gene expression of twofold or more was considered to be biologically meaningful.

Blinding
Evaluator blinding was performed when microscopy of tissue sections was performed. The blinded experiments included the histological scoring of GVHD, quantification of CD3+ cells in GVHD target organs, and the quantification of vascular density in GVHD target organs by immunofluorescence microscopy. Briefly, the slides were blindly labeled by one person before evaluation, evaluated by another person, and treatment conditions were revealed after the evaluation had been completed. Clinical GVHD scoring and killing of very sick mice was not blinded. However, persons directly involved in the project (eg, the principal investigator, postdocs, and students) were excluded from scoring and killing to minimize bias.

Statistics
All values shown in graphs represent the mean with 95% confidence intervals (CIs). Comparisons with P values less than .05 were considered to be statistically significant. Groupwise comparisons were done with either the Wilcoxon rank sum statistic or the t statistic. The Wilcoxon rank sum test was used to assess the differences in clinical GVHD scores between treatment groups because of the ordinal nature of the GVHD scores. For all other comparisons, the t test was used. A stratified t test (stratified by day posttransplant) was used to determine whether the number of host or donor-derived ECs differed between transplant recipients with GVHD and control mice. Recipients that died before day 21 were given a GVHD score equal to 5 in the group comparisons. Survival data were analyzed with the log-rank test. Survival experiments were designed using group sizes of n = 5–10. Experiments were repeated at least once to assess reproducibility, and data from the experiments were combined. All statistical tests were two-sided.

Results
Quantification of Neovascularization During GVHD
To determine whether neovascularization plays a role in inflammation during GVHD, we first used an MHC-disparate allo-BMT model, specifically, the transplantation of B6 (H-2b) marrow into BALB/c (H-2d) mice. Lethally irradiated recipients received TCD-BM, and GVHD was induced by the addition of donor splenic T cells to the allograft. We quantified neovascularization during inflammation in GVHD target organs (small bowel, large bowel, and liver) by immunofluorescence microscopy after staining with antibodies to CD34, a marker of ECs and hematopoietic stem cells. Vessel density was higher in the intestine and the liver of allo-BMT recipients with GVHD than in control mice that received TCD-BM, but not donor T cells, and thus did not have GVHD (Figure 1, A and B). Similar results were obtained when intestine and liver sections were stained with antibodies to Flik-1, a flt-related receptor tyrosine kinase that is a marker for EPCs, or to MECA-32, another marker specific for mouse endothelium (data not shown).

To investigate whether neovascularization during GVHD originated from the host’s resident tissue ECs or from the donor’s BM-derived ECs, we harvested livers from allo-BMT recipients at different time points after BMT, disrupted the tissue with collage-nase, and quantified ECs by flow cytometric analyses of suspensions containing dissociated single cells. We could distinguish donor and host ECs by the MHC disparity (H-2b vs H-2d). Recipients with
GVHD had a greater number of donor-derived ECs than control mice in which GVHD had not been induced, whereas the number of host-derived ECs was not different (Figure 1, C). These results indicate that inflammation during GVHD is characterized by neo-vascularization due to recruitment of donor BM-derived ECs as opposed to proliferation of host resident tissue ECs.

Figure 1. Neovascularization by donor bone marrow (BM)-derived endothelial cells (ECs) during graft-vs-host disease (GVHD). Lethally irradiated BALB/c mouse recipients were transplanted with $5 \times 10^6$ T cell–depleted BM (TCD-BM) cells, and in some mice, but not others, $1 \times 10^6$ T cells from B6 donors were simultaneously injected to induce GVHD. A) Representative image of increased neovascularization in inflamed intestine during GVHD (right) compared with noninflamed intestine 7 days after BM transplantation (BMT) (left). Sections of the liver, ileum, and colon of both groups of mice were stained with a fluorescent antibody to CD34 (green) to detect blood vessels and counterstained with a nuclear stain, 4′,6-diamidino-2-phenylindole (DAPI) (blue). B) Quantification of the neovascularization (vascular area/total area) in GVHD target organs by microscopy (as in A) at 7 and 14 days after allogeneic BMT, $n = 10$ per group, for statistical comparisons two-sided $t$ tests were used. Means and 95% confidence intervals are shown. C) Quantification of host resident tissue ECs and donor BM-derived ECs by flow cytometric analysis. Donor H-2kb (B6) TCD-BM was transplanted into host H-2kd (BALB/c) mice with or without addition of donor T cells as above ($n = 5$ per group). Livers were excised from recipient mice on days 7 and 14, disaggregated, and (VEGFR2+/CD45$^-$/CD11b$^-$/TER119$^-$) ECs were sorted and quantified according to H2k type. A stratified $t$ test (stratified by day posttransplant) was used to for statistical comparisons.

BM-Derived EPCs and Neovascularization of GVHD Target Organs

Next, we sought to analyze the role of EPCs in inflammation during GVHD. We defined EPCs as c-Kit$^+$/VEGFR2$^+$/CD11b$^-$/TER119$^-$ cells (Figure 2, A) that do not express other hematopoietic cell surface markers, as described previously (10,12). We found a substantially increased number of donor BM-derived (H-2kb+) EPCs in peripheral blood and BM (Table 1) of allo-BMT recipients with GVHD as compared with control mice. To distinguish whether EPCs or other cells of donor origin (in the BM or elsewhere) contributed to neovascularization, we adoptively transferred selected GFP$^+$ EPCs from C57BL/6 GFP$^+$ (B6 GFP$^+$) (H-2b) mice into lethally irradiated BALB/c (GFP$^-$) (H-2d) allo-BMT recipients at the day of BMT. Using high-resolution confocal microscopy, we observed that GFP$^+$ EPCs were recruited to the neovasculature of the inflamed intestines and liver during GVHD (only the EPCs were GFP$^+$, other cells were GFP$^-$) (Figure 2, B–D, and Supplementary Figure 1, available online). In three-dimensional reconstructions of high-resolution Z-stack pictures, we found that the GFP$^+$ EPCs coexpressed the endothelial antigen MECA-32 (Supplementary Movies 1 and 2, available online). We conclude that inflammation during GVHD is characterized by neovascularization in target tissues and that (donor BM-derived) vasculogenesis plays an important role in this process.
Use of the Anti–VE-Cadherin Antibody E4G10 to Deplete EPCs During GVHD

Based on the previous findings, we hypothesized that depletion of EPCs might decrease neovascularization and inflammation in GVHD target organs. To inhibit neovascularization during GVHD, we used an antibody, E4G10, that was found to specifically bind EPCs in previous studies (10,12). E4G10 recognizes the vascular endothelial adhesion molecule VE-cadherin, which is an endothelial-specific adhesion molecule localized at junctions between ECs with ubiquitous expression on vasculature in the entire body (27,28). E4G10 binds to an N-terminal epitope that is exposed on VE-cadherin monomers but is masked upon VE-cadherin trans-dimerization (29). EPCs express the monomeric form of VE-cadherin, whereas established vasculature expresses VE-cadherin dimers. Therefore, E4G10 recognizes VE-cadherin on EPCs but not on ECs in established vasculature. E4G10 does not bind to preformed vasculature throughout the body and, therefore, does not cause vascular leakage, in contrast to anti–VE-cadherin antibodies directed at other epitopes, which disrupt normal vessels (10,12,14).

In flow cytometric analyses, we found that E4G10 specifically binds to EPCs, as opposed to hematopoietic stem cells (Figure 3, A). We detected no E4G10 binding to hematopoietic cells in the spleen, BM, as well as the peripheral blood by flow cytometric analyses (data not shown) and found no negative effects of E4G10 on hematopoietic reconstitution in recipients of TCD-BMT without GVHD (Supplementary Tables 1–4, available online). These results illustrate that E4G10 therapy is safe and has low toxicity.

Next, we administered E4G10 to lethally irradiated allo-BMT recipients with GVHD and found reduced numbers of donor BM-derived EPCs in the peripheral blood and BM at different time points after allo-BMT (Figure 3, B), indicating that E4G10 depletes EPCs.

Effect of E4G10 Administration on Donor BM-Derived vs Host Vessels During GVHD

To determine whether E4G10 binds to host vessels during GVHD, we intravenously administered different doses (50–200 µg) of fluorescently labeled E4G10 antibody or rat IgG control antibody to normal BALB/c mice or to allo-BMT recipients with GVHD. We transfected 20 000 GFP+ EPCs (from C57BL/6 GFP+ [B6 GFP+] [H-2b] mice) along with GFP− bone marrow and GFP− T cells (from C57BL/6 GFP− [B6 GFP−] [H-2b] mice) into lethally irradiated H-2kd (BALB/c) allo-BMT recipients on the day of bone marrow transplant. Colon sections from recipient mice were stained with a fluorescent antibody to the endothelial marker CD34 (red) and with the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI) (blue). The location of GFP+ cells was evaluated by high-resolution confocal microscopy. Scale bar: 50 µm. C) Overlay of GFP+ ECs with CD34 staining, in experiments described in (B). Scale: 1 U = 2.5 µm. D) Overlay of GFP+ ECs (indicated by white arrows in the left lower panel) in colon sections from similar mice stained with an alternative endothelial marker, Meca-32 (red) and DAPI (blue). Scale bar: 100 µm.

Figure 2. Contribution of bone marrow–derived endothelial progenitor cells (EPCs) to neovasculature in graft-vs-host disease (GVHD) target tissues. A) Identification of EPCs by flow cytometric analysis. CD11b−/TER119− cells in peripheral blood and in bone marrow of B6 mice are shown. The c-Kit+/VEGFR+/CD11b−/TER119− cell population (red square) contains EPCs. B) Incorporation of GFP+ EPC-derived endothelial cells (ECs) surrounding the luminal space in neovasculature of the colon during inflammation. We transfected 20 000 GFP+ EPCs (from C57BL/6 GFP+ [B6 GFP+] [H-2b] mice) along with GFP− bone marrow and GFP− T cells (from C57BL/6 GFP− [B6 GFP−] [H-2b] mice) into lethally irradiated H-2kd (BALB/c) allo-BMT recipients on the day of bone marrow transplant. Colon sections from recipient mice were stained with a fluorescent antibody to the endothelial marker CD34 (red) and with the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI) (blue). The location of GFP+ cells was evaluated by high-resolution confocal microscopy. Scale bar: 50 µm. C) Overlay of GFP+ ECs with CD34 staining, in experiments described in (B). Scale: 1 U = 2.5 µm. D) Overlay of GFP+ ECs (indicated by white arrows in the left lower panel) in colon sections from similar mice stained with an alternative endothelial marker, Meca-32 (red) and DAPI (blue). Scale bar: 100 µm.
GVHD on day 7 or 14 after allo-BMT. We harvested livers and intestines at 2, 8, 24, and 48 hours after antibody administration and performed immunofluorescence staining with Meca-32 or CD34 antibodies to identify vessels. Early (2 or 8 hours) after injection of either E4G10 or the control antibody, a similar pattern of nonspecific fluorescent staining appeared in the liver and intestinal vessels of both normal BALB/c mice and allo-BMT recipients with GVHD (data not shown). However, at later time points (24 and 48 hours), we did not detect fluorescence signals in either the E4G10-treated or control antibody-treated groups. In further experiments, we administered 1 mg of unlabeled E4G10 antibody or rat IgG control antibody i.p. to normal mice or allo-BMT recipients with GVHD at day 7 or 14 after allo-BMT. We harvested livers and intestines after 24 hours and performed immunohistochemistry with an Alexa Fluor 647-labeled secondary antibody against rat IgG. Again, we found no differences in binding of either the E4G10 antibody or the rat IgG control antibody in allo-BMT recipients vs in normal mice (data not shown). These data suggest that there is no specific binding of the E4G10 antibody to the vasculature of GVHD target organs in our models.

We next quantified neovascularization in GVHD target organs at different time points after allo-BMT by immunofluorescence microscopy followed by measurement of the percentage increase in vessel area vs total area in pixels. We detected statistically significantly lower vascular density in the liver and colon of E4G10-treated allo-BMT recipients compared with control antibody-treated recipients during inflammation (E4G10-treated vs control-treated mice, vascular density in liver, day 7: 3.90% vs 4.76%, difference = 0.86%, 95% CI = 0.04% to 1.69%; day 14: 4.78% vs 6.31%, difference = 1.53%, 95% CI = 1.01% to 2.06%; vascular density in colon, day 7: 2.60% vs 3.61%, difference = 1.01%, 95% CI = 0.50% to 1.51%; day 14: 2.53% vs 3.07%, difference = 0.54%, 95% CI = 0.06% to 1.01%) (Figure 4, A). We then performed flow cytometric analyses after antibody staining to count donor-derived ECs vs host-derived ECs from disaggregated liver tissues and found that E4G10 treatment resulted in inhibition of donor BM-derived neovascularization but did not affect host-derived vasculature (Figure 4, B). These findings demonstrate that administration of E4G10 inhibits vasculogenesis during GVHD. A stratified \( t \) test that used a weighted average of the data from days 7 and 14 was performed to assess these comparisons. The observed difference in the mean number of donor ECs with weights determined by the number of subjects within strata was \( -27.556 \) (95% CI = \(-50.818\) to \(-42.93\), \( P = .002 \)). The observed difference in the mean number of host ECs with weights determined by the number of subjects within strata was \(-64.35 \) (95% CI = \(-39.628\) to \(-26.759\), \( P = .70 \)).

### Table 1. Quantification of endothelial progenitor cells (EPCs) in peripheral blood and bone marrow during graft-vs-host disease (GVHD)*

<table>
<thead>
<tr>
<th>Source of cells and time after transplant</th>
<th>No GVDH, Mean No. (95% CI)</th>
<th>GVDH, Mean No. (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EPCs in peripheral blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>23.4 (20.1 to 26.8)</td>
<td>36.1 (29.5 to 42.7)</td>
<td>.004</td>
</tr>
<tr>
<td>Day 14</td>
<td>28.0 (23.0 to 33.0)</td>
<td>53.6 (40.1 to 67.1)</td>
<td>.003</td>
</tr>
<tr>
<td>Day 21</td>
<td>33.2 (24.2 to 42.2)</td>
<td>132.9 (103.1 to 162.7)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td><strong>EPCs in bone marrow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>44.0 (32.0 to 56.0)</td>
<td>72.5 (56.4 to 88.6)</td>
<td>.012</td>
</tr>
<tr>
<td>Day 14</td>
<td>107.6 (97.6 to 117.6)</td>
<td>181.2 (150.8 to 211.6)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Day 21</td>
<td>152.1 (125.3 to 179.0)</td>
<td>249.6 (201.2 to 298.0)</td>
<td>.003</td>
</tr>
</tbody>
</table>

* Lethally irradiated H-2kd (BALB/c) recipient mice were transplanted with \( 5 \times 10^6 \) T cell–depleted bone marrow cells from H-2kb (B6) donor mice with or without the additional transfer of \( 1 \times 10^6 \) T cells from B6 donors (in \( n = 10 \) per group). Donor bone marrow–derived EPCs (c-Ki+/VEGFR2+/H-2k+/CD11b−/TER119−) in 500 \( \mu \)L peripheral blood and the bone marrow one tibia were quantified in mice with GVHD compared with control mice by flow cytometric analysis. Combined data from two experiments are shown; for statistical comparisons, two-sided \( t \) tests were used.

### Effect of E4G10 Administration on Organ-Specific GVHD

We analyzed the effect of E4G10 administration on GVHD in two different MHC-mismatched murine models with various doses of donor T cells. In the second model, cells from B6 (H-2b) donors were transplanted into B6D2F1 (H-2b/d) hosts. In both models and at all doses tested, we found that E4G10-treated recipients had statistically significantly better survival 60 days after transplantation than similar mice treated with the control antibody (probability of survival at day 60 after transplant for B6 to BALB/c transplanted mice treated with E4G10 = 0.28 vs 0.13 with control antibody, difference = 0.15, 95% CI = 0.006 to 0.294, \( P = .002 \)) [by log-rank test]; among B6 to B6D2F1 [1 \( \times 10^7 \) T cells] transplanted mice, the probability of survival was 0.67 for E4G10-treated mice vs 0.47 for control antibody-treated mice, difference = 0.20, 95% CI = -0.140 to 0.540, \( P = .060 \) [by log-rank test]; among B6 to B6D2F1 [2 \( \times 10^7 \) T cells] transplanted mice, probability of survival was 0.33 for E4G10-treated mice vs 0.08 for control antibody-treated mice, difference = 0.25, 95% CI = -0.059 to 0.559, \( P = .018 \) [by log-rank test]; among B6 to B6D2F1 [3 \( \times 10^7 \) T cells] transplanted mice, the probability of survival was 0.13 for E4G10-treated mice vs 0 for control antibody-treated mice, difference = 0.13, 95% CI = -0.042 to 0.302, \( P = .001 \) [by log-rank test]) (Figure 5, A). Within each model, the GVHD score at day 21 after transplantation was lower for the population treated with E4G10 (Figure 5, B) (among B6 to BALB/c transplanted mice, GVHD score for E4G10-treated mice = 2.5 vs 4.0 for control antibody-treated mice, difference = 1.5, 95% CI = 0.5 to 2.0, \( P < .001 \); among B6 to B6D2F1 [1 \( \times 10^7 \) T cells] transplanted mice, GVHD score for E4G10-treated mice was 1.5 vs 2.5 for control antibody-treated mice, difference = 1.0, 95% CI = 1.0 to 1.1, \( P \leq .001 \); among B6 to B6D2F1 [2 \( \times 10^7 \) T cells] transplanted mice, GVHD score for E4G10-treated mice = 1.8 vs 2.5 for control
Reduction of Tumor Growth and GVHD by E4G10 Administration

To test whether EPC depletion by the administration of E4G10 antibody could help to prevent both tumor growth and inflammation, we first assessed the contribution of EPCs to neovasculature in mice transplanted with 5 × 10^4 T cells from B6MF1- and DC101-treated mice (10 of 10 died by day +12) compared with rat IgG-treated controls (three of 10 died by day 12), indicating an inhibitory effect of these antibodies on hematopoietic reconstitution (data not shown). Next, we analyzed the expression of 200 genes that were known to be involved in EC biology using hepatic tissue that exhibited GVHD. Expression of several proangiogenic genes, which did not include VEGF, was substantially (more than twofold) increased, but expression of no such proangiogenic genes was decreased, illustrating that a proangiogenic tissue environment that is not dominated by VEGF was partially (more than twofold) increased, but expression of no such proangiogenic genes was decreased, illustrating that a proangiogenic tissue environment that is not dominated by VEGF was thereby eliminated the development of GVHD. We used three well-characterized mouse cell lines that represent two common antibody-treated mice, difference = 2.5, 95% CI = 0.0 to 2.5, P ≤ .001).

To analyze whether E4G10 diminishes inflammation in GVHD target organs, we performed blinded, semiquantitative, histopathological analyses and found substantially less GVHD in the terminal ileum, colon, liver, and skin of E4G10-treated allo-BMT recipients compared with rat IgG-treated controls (Figure 5, C). Also, the numbers of tissue-infiltrating CD3+ T cells in GVHD target organs were diminished in the presence of E4G10 treatment, indicating suppressed inflammation (Figure 5, D, and Supplementary Figure 2, available online). In allo-BMT recipients with GVHD, we found that the numbers of circulating inflammatory cells (Supplementary Figure 3, available online) and serum cytokine levels (Supplementary Table 5, available online) were not statistically significantly reduced as a result of E4G10 treatment. We conclude that therapeutic targeting of VE-cadherin monomers to inhibit neovascularization can ameliorate GVHD.

**Effect of Vascular Endothelial Growth Factor Receptor–Blocking Antibodies on Hematopoietic Reconstitution in allo-BMT Recipients**

To assess the safety and efficacy of “non-EPC-specific” antiangiogenic therapies in the prevention of GVHD, we used a combination of MF1 and DC101, which are vascular endothelial growth factor receptors (VEGFR1 and VEGFR2) blocking antibodies, to treat mice after allo-BMT. Similar to the experiments with the E4G10 antibody BALB/c (H-2d) mice were lethally irradiated and transplanted with 5 × 10^4 BM cells and with 1 × 10^6 T cells from C57BL/6 (H-2b) mice. In these experiments, 800 µg of MF1 and DC101 or control rat IgG antibody were administered i.p. to lethally irradiated BALB/c (H-2d) allo-BMT recipients on days 0, 2, 4, 6, 8, and 10 after allo-BMT. We found reduced numbers of hematopoietic cells in the peripheral blood and early death in all MF1- and DC101-treated mice (10 of 10 died by day +12) compared with rat IgG-treated controls (three of 10 died by day 12), indicating an inhibitory effect of these antibodies on hematopoietic reconstitution (data not shown). Next, we analyzed the expression of 200 genes that were known to be involved in EC biology using hepatic tissue that exhibited GVHD. Expression of several proangiogenic genes, which did not include VEGF, was substantially (more than twofold) increased, but expression of no such proangiogenic genes was decreased, illustrating that a proangiogenic tissue environment that is not dominated by VEGF was induced during GVHD (Supplementary Table 6, available online). These results suggest that the use of anti-VEGF strategies for prevention of GVHD after allo-BMT may not be effective and even have the potential to inhibit hematopoietic reconstitution.

antibody-treated mice, difference = 0.7, 95% CI = 0.0 to 1.0, P ≤ .001; among B6 to B6D2F1 [3 × 10^5 T cells] transplanted mice, GVHD score for E4G10-treated mice = 3.5 vs 5.0 for control
hematological malignancies (C1498 [C57BL/6] mouse acute myeloid leukemia and A20 [BALB/c] mouse B-lymphoma cells) as well as a solid tumor (RENCA [BALB/c] mouse renal carcinoma cells). C1498 leukemia and A20 lymphoma cell lines were chosen because leukemia and lymphoma are the most frequent indications for allogeneic stem cell transplantation in humans. We chose to use the RENCA cell line because allogeneic stem cell transplantation is performed in patients with renal carcinoma (but not in those with most other solid tumors) and because the lung metastases of RENCA allowed us to study tumor neovascularature. We adoptively transferred selected GFP+ EPCs from C57BL/6 GFP+ (B6 GFP+) (H-2b) mice into lethally irradiated BALB/c (GFP−) (H-2b) allo-BMT recipients that were challenged intravenously with mouse renal carcinoma (RENSA) cells at the day of BMT. We harvested the lungs at day +14 after allo-BMT and found that GFP+ EPCs were recruited to the neovascularature of RENCA lung metastases (Figure 6, A). In further experiments, we performed allo-BMTs in which C57BL/6 BM cells and C57BL/6 T cells were transplanted into lethally irradiated BALB/c mice, and A20 or RENCA cells were also transferred on the day of BMT. As in the GVHD experiments, 1000 μg of E4G10 antibody was administered i.p. on every other day from day 0 to 10 after allo-BMT. We monitored tumor growth by weekly monitoring of the in vivo bioluminescent signal intensity in tumor-bearing mice, starting at 10 days after injection of tumor cells. We detected a moderate inhibitory effect of E4G10 administration on tumor growth, in both tumors tested (RENSA + A20 lymphoma) (17 days after BMT in RENCA-transplanted mice, signal intensity in photons s−1 cm−2 sr−1 = 8.67 × 107 with vs 24.25 × 107 without E4G10 antibody, difference = 11.91 × 107, 95% CI = 5.44 × 107 to 35.39 × 107, P = .002; 17 days after BMT in A20-transplanted mice, signal intensity = 19.91 × 107 with vs 32.35 × 107 without E4G10 antibody, difference = 12.44 × 107, 95% CI = −0.31 × 107 to 29.90 × 107, P = .121) (Figure 6, B). This effect was more pronounced during the early phase of tumor growth (before day 21). Moreover, we observed slightly prolonged survival in tumor-bearing mice that were treated with E4G10 as compared with controls treated with rat IgG in the RENCA and C1498 (AML) models but not in the A20 model (at 40 days after BMT, survival of RENCA-transplanted mice with E4G10 = 0.462 vs 0.300 with control antibody, difference = 0.162, 95% CI = −0.05 to 0.37, P = .900; 40 days after BMT in A20-transplanted mice with E4G10 = 0.300 vs 0.294 with control antibody, difference = 0.006, 95% CI = −0.22 to 0.23, P = .821) (Figure 6, B). By flow cytometry of the tumor cell lines A20, C1498 and RENCA using AF647-conjugated E4G10 or AF647-conjugated rat IgG2a control antibody, we could not detect any binding of E4G10 to any of the tumors, which makes it unlikely that E4G10 would have a direct effect on tumor cells (Supplementary Figure 4, available online). We conclude that EPCs contribute to neovascularature in tumors after allo-BMT and that administration of E4G10 results in modest inhibition of tumor growth in different tumor models.

Finally, we performed experiments in which GVHD and tumors were induced simultaneously. In these experiments, tumor-bearing allo-BMT recipients received allogeneic T cells, which can not only mediate beneficial graft-vs-tumor activity but also cause inflammation in GVHD target organs. We determined the presence of tumors by in vivo bioluminescence imaging as well as by postmortem histopathology, and we monitored GVHD by clinical signs. Compared with control antibody-treated mice, mice that had been administered E4G10 had a
statistically significantly higher rate of tumor-free survival in all three models; however, the differences were more pronounced in the A20 model as compared with the C1498 and Renca models (at 60 days after BMT, survival of Renca-transplanted mice with E4G10 = 0.35 vs 0.05 with control antibody, difference = 0.30, 95% CI = 0.069 to 0.530, P = .015 [by log-rank test]; survival of C1498-transplanted mice with E4G10 = 0.25 vs 0.0 with control antibody, difference = 0.25, 95% CI = 0.060 to 0.440, P < .001 [by log-rank test]; survival of A20-transplanted mice with E4G10 = 0.70 vs 0.29 with control antibody, difference = 0.41, 95% CI = 0.180 to 0.640, P < .001 [by log-rank test]) (Figure 6, C). Interestingly, the survival benefit seemed to be because of both attenuation of GVHD and inhibition of tumor growth (Figure 6, C). These results illustrate that E4G10 therapy improves survival of tumor-bearing allo-BMT recipients by exhibiting beneficial effects on neovascularization in inflammation as well as in tumors.

**Figure 5.** Treatment of graft-vs-host disease (GVHD) with E4G10 antibody. In the first two panels (A and B), lethally irradiated BALB/c or B6D2F1 mice were transplanted with 5 × 10⁶ T cell–depleted bone marrow (TCD-BM) cells plus variable doses of T cells (as indicated in the figure) from B6 mice. Recipients were treated intraperitoneally with 1 mg E4G10 or rat IgG control antibody on days 0, 2, 4, 6, 8, and 10 after allogeneic BM transplantation (allo-BMT). A) Survival of E4G10- vs control-treated allo-BMT recipients in different GVHD models with variable T-cell doses, as shown by Kaplan–Meier plots. Combined data from five experiments (A1) or two experiments (A2, A3, and A4) are shown. Sample sizes: A1 group 1+2 n = 25, A1 group 3+4 n = 60; A2 group 1+2 n = 10, A2 group 3+4 n = 15; A3 group 1+2 n = 10, A3 group 3+4 n = 20; A4 group 1+2 n = 10, A4 group 3+4 n = 15. Survival data were analyzed with the log-rank test. The numbers of mice at risk on days 0, 20, 40, 60, and 80 post-BMT were as follows: for A1 control, 60, 40, 60, 38, 12; for A1 E4G10, 60, 48, 38, 16, 12; for A2 control, 15, 15, 13, 7, 3; for A2 E4G10, 15, 13, 13, 10, 9; for A3 control, 12, 12, 4, 1, 0; for A3 E4G10, 12, 11, 4, 3; for A4 control, 15, 13, 0, 0; for A4 E4G10, 15, 14, 6, 3, 1. B) Clinical GVHD scores of E4G10- vs control-treated allo-BMT recipients at 21 days post-transplantation in the experiments shown in (A). Group sizes: B1 n = 60; B2 n = 15; B3 n = 20; B4 n = 15. The Wilcoxon rank sum test was used to assess the differences in clinical GVHD scores between treatment groups. In the second two panels (C and D), lethally irradiated BALB/c recipients were transplanted with 5 × 10⁶ TCD-BM cells and 1 × 10⁶ T cells from B6 donors. Organs were harvested at day +21 after allo-BMT, fixed in formalin, embedded in paraffin, stained with hematoxylin and eosin, and investigated microscopically to assess organ damage due to GVHD. C) Histopathologic GVHD scores showing inflammation in multiple organs after E4G10 vs control treatment (liver and intestine: n = 10 per group; skin: n = 5 per group); for statistical comparisons, two-sided t tests were used. Means and 95% confidence intervals are shown. D) T-cell infiltration of liver and intestine during GVHD after E4G10 vs control treatment, as quantified by immunohistochemistry with anti-CD3 (n = 5 per group); for statistical comparisons, two-sided t tests were used.

**Discussion**

This article demonstrates that GVHD is characterized by neovascularization, which is mainly driven by vasculogenesis, as opposed to angiogenesis. We identify inhibition of neovascularization as a novel therapeutic concept to simultaneously reduce inflammation and the growth of tumors: the VE-cadherin monomer–specific antibody E4G10 simultaneously inhibited GVHD and tumor growth, resulting in reduction of both GVHD-related mortality and tumor-related mortality, leading to improved long-term survival of tumor-bearing allo-BMT recipients. We conclude that targeting of neovasculature provides a unique means to address the two major complications of allogeneic hematopoietic stem cell transplantation (GVHD and tumor relapse) with a single therapeutic strategy.

A key feature of our study is that it used the MHC disparity of donor and host to separately quantify vasculogenesis and angiogenesis by flow cytometry (Figure 1, C). Our MHC class
I–mismatched model (B6→BALB/c), in which C57BL/6 BM cells and C57BL/6 T cells were transferred into lethally irradiated BALB/c recipients, enabled us to easily distinguish vasculogenesis from angiogenesis by H-2b (C57BL/6) and H-2d (BALB/c) expression. We found that only the number of donor BM-derived ECs increased during GVHD, whereas the number of host resident tissue ECs remained constant. The predominant role that donor ECs play in the formation of neovascularature after allo-BMT is not surprising because of the negative effect that lethal doses of irradiation have on host EC function. The inhibition of angiogenesis by irradiation is well known and has been demonstrated by many groups (30–37). Even relatively low doses of irradiation, such as 2 and 4 Gy, potently inhibit EC proliferation (38–40). It has been shown that irradiation doses similar to those used clinically are sufficient to inhibit EC function in allo-BMT recipients (41). Transplanted tumors grow more slowly when the tissue around them becomes irradiated (42–44). This effect is termed the “tumor bed effect” and is explained by the vascular damage caused by the radiation leading to inhibition of host angiogenesis (42–44). The tumor bed effect is used experimentally to block angiogenesis, favoring vasculogenesis, in experimental tumor models (44–46).

The role of vasculogenesis and EPCs in neovascularization has been a topic of considerable discussion over the past decade (1,2,21,17,47–49). Some controversy regarding the contribution of EPCs to neovascularature was based on the use of nonselected BM cells in previous studies, which could have resulted in the contribution of non-EPC hematopoietic cells of donor origin. We therefore performed experiments with purified GFP-expressing EPCs of donor origin and found recruitment to neovascularure (Figures 2, C, and 6; A; Supplementary Figure 1 and Supplementary Movies 1 and 2, available online). Furthermore, we quantified vasculogenesis with another method: using donor–host markers in flow cytometry, we found that donor BM–derived ECs contribute to host neovascularure during GVHD (Figure 1, C). Thus, we used two independent methods and consistently found contribution of donor BM-derived EPCs to host neovascularure during GVHD.

We have demonstrated that the inhibition of neovascularization with an antibody (E4G10) against the vascular endothelial adhesion molecule VE-cadherin can be used therapeutically to inhibit inflammation during GVHD. The main mechanism is likely to be the inhibition of vasculogenesis in GVHD target organs, which leads to a reduced recruitment of proinflammatory cells migrating via the blood vessels to inflammatory sites. We cannot exclude that donor BM-derived EPCs exhibit some paracrine proinflammatory functions, which could explain inhibition of inflammation after depletion of EPCs. However, since serum cytokine and chemokine levels as well as the number of circulating inflammatory cells were not reduced as a result of E4G10 treatment, this possibility appears less likely. There are potential clinical implications of our findings for a broad variety of inflammatory diseases. Previous findings have shown that EPCs are recruited from peripheral blood to neovascularure during inflammation in several inflammatory diseases, including asthma (50) and rheumatoid arthritis (51), so justify studies on the efficacy of this therapeutic approach in inflammatory diseases, other than GVHD.

E4G10 specifically targets VE-cadherin monomers on EPCs as opposed to VE-cadherin trans-dimers in established vasculature. We know of no direct evidence that cell surface VE-cadherin...
monomers are more abundant on inflamed vessels compared with normal vessels. This is in keeping with our findings that E4G10 did not bind specifically to vasculature in GVHD target organs. However, we cannot completely rule out that E4G10 binds to host endothelium at sites of inflammation and, therefore, could have some effects on host angiogenesis. In tumor vasculature, Liao et al. (52) found that E4G10 stains a subset of tumor vessels. However, because angiogenesis was not increased in our GVHD models with lethally irradiated ECs (Figure 1, C) and administration of E4G10 had no effect on angiogenesis (Figure 4,B), a major effect of E4G10 on host vasculature appeared to be very unlikely in our study. In addition, the findings of Liao et al. are in contrast to later studies by Nolan et al. (12), who published compelling data showing that E4G10 recognized only monomeric VE-cadherin expressed exclusively on EPCs and not the dimerized form in host vessels. Importantly, we observed 1) that GVHD is characterized by neovascularization and 2) that the administration of E4G10 leads to simultaneous benefits with respect to GVHD and tumor growth. This observation—and its clinical relevance—is independent of the question of whether E4G10 binds specifically to EPCs or whether it also binds to host vasculature.

In our study, the inhibition of neovascularization with E4G10 reduced the intensity of GVHD but did not completely prevent it. It would be of great interest to determine whether the dosing schedule of anti–VE-cadherin antibodies could be further optimized to enhance GVHD prevention. In the clinical setting, the inhibition of neovascularization most likely would be combined with current standard therapies to prevent or treat GVHD, such as T-cell depletion and immunosuppression.

We found that VEGF gene expression was not increased during GVHD (Supplementary Table 6, available online), which suggests that VEGF might not be the dominant proangiogenic factor during GVHD in our models. Interestingly, Min et al. (53) found in a clinical study that low VEGF serum levels after allo-SCT are associated with high mortality and with an exacerbated severity of acute GVHD. These results are in agreement with a recent publication that links VEGF gene polymorphisms that lead to lower production of VEGFA with increased incidence of acute GVHD (54). 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. In another GVHD model, a higher and more rapid mortality was seen in allo-BMT recipients treated with anti-VEGF peptide (53). These results suggest that the use of anti-VEGF strategies for prevention of GVHD after allo-BMT may not be effective and may potentially inhibit hematopoietic reconstitution. Taken together, it is currently not possible to draw final conclusions regarding the role of VEGF in the regulation of vasculogenesis and angiogenesis during GVHD.

Recently, we and other investigators demonstrated that vasculogenesis contributes to tumor vasculature and that depletion of EPCs leads to inhibition of primary as well as metastatic growth of solid tumors in certain models (8–15). This study extends these findings to tumors after allo-BMT and to hematological malignancies: We found that E4G10 administration resulted in inhibition of lymphoma and AML growth in vivo. Our results are in agreement with recent clinical data suggesting a critical role for neovascularization not only in solid tumors but also in hematological malignancies (55–57). However, the inhibition of tumor growth that we observed in hematological malignancies as a result of E4G10 administration was rather modest and model-dependent. A possible approach to enhance the efficacy of E4G10 toward hematological malignancies in future studies would be the optimization of the dosing schedule (eg, a longer treatment duration) and the use of E4G10 bound to alpha particles (eg, [225]Ac-E4G10), which deliver potent short-ranged radiation and have been successfully used in solid tumor models (14). Interestingly, we found that the benefit of E4G10 on tumor growth seemed to be stronger in the presence of donor T cells than in their absence. One possible explanation is that administration of E4G10 may lead to a normalization of tumor vasculature, increasing the blood flow, and leading to a more effective recruitment of tumor-reactive T cells to the tumor tissue (1,3,14,58).

Our study has some limitations. First, we showed that administration of the E4G10 antibody leads to depletion of EPCs and to inhibition of neovascularization during inflammation as well as tumor growth. However, with the methods applied, it is not possible to prove that the mechanism of the E4G10 action is exclusively mediated by its effects on EPCs (as opposed to an unknown effect of E4G10 on other cells or structures in the body). Second, several endpoints in our experiments, such as the histological scoring and clinical scoring of GVHD, are subjective. Therefore, we blinded the evaluators who performed the histological scoring. For practical reasons, it was not possible to perform blinded clinical scoring. However, to minimize bias, those persons with direct interests in the project were excluded from clinically scoring and killing the mice.

In conclusion, this study demonstrates that GVHD is characterized by increased neovascularization and identifies the inhibition of neovascularization with an antibody against VE-cadherin monomers as a novel therapeutic concept to simultaneously ameliorate GVHD and tumor growth.

Supplementary Data

Supplementary data can be found at http://www.jnci.oxfordjournals.org/.

References


**Funding**

National Institutes of Health award numbers R01 HL084815 (to G.F.M.), R01-HL069929 (M.R.M.v.d.B.), R01-CA105706 (M.R.M.v.d.B.), R01-AI080455 (M.R.M.v.d.B.), P01-CA102049 (M.R.M.v.d.B.), and R01-HL095075 (M.R.M.v.d.B.). Support was also received from the US Department of Defense: USAMRAA Award W81XWH-09-1-0294 (M.R.M.v.d.B.), the Ryan Gibson Foundation, the Elsa U. Pardee Foundation, the Byrne Foundation, the Emerald Foundation, and the Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center funded by Mr William H. Goodwin and Mrs Alice Goodwin, the Commonwealth Foundation for Cancer Research, the Bobby Zucker Memorial Fund (M.R.M.v.d.B.), the Lymphoma Foundation, the Deutsche Forschungsgemeinschaft (to O.P.), and the Deutsche Krebshilfe (to I.-K.N. and A.G.).

**Notes**

The content is solely the responsibility of the authors. C. May is an employee of ImClone Systems Incorporated. The other authors have no relevant conflicts of interest to declare.

O. Penack designed the study, collected data, analyzed data, and wrote the paper; E. Henke collected and analyzed digital fluorescent images; D. Suh, C. G. King, O. M. Smith, I.-K. Na, A. M. Holland, A. Ghosh, S. X. Lu, and R. R. Jenq performed experiments and collected data; C. Liu and G. F. Murphy performed histopathological analyses; T. T. Lu, D. A. Scheinberg, D. C. Gao, V. Mittal, C. May, and R. Benezra were involved in study design and gave technical assistance; G. Heller was involved in study planning and performed statistical analyses, M. R. M. van den Brink designed the study, analyzed data, and wrote the paper. All authors discussed the results and commented on the article.

**Affiliations of authors:** Department of Immunology (OP, DS, CGK, OMS, I-KN, AMH, AG, SXL, RRJ, MRvMB), Department of Cancer Biology and Genetics (EH, RB), Department of Medicine (RRJ, MRvMB), Molecular Pharmacology and Chemistry Program (DAS), and Department of Epidemiology and Biostatistics (GH), Memorial Sloan-Kettering Cancer Center, New York, NY; Department of Hematology and Oncology, Charité, Campus Benjamin Franklin, Berlin, Germany (OP, I-KN); Department of Immunology and Microbial Pathogenesis (AMH) and Autoimmunity and Inflammation Program, Hospital for Special Surgery (TTL), Weill Cornell Medical College, New York, NY; Department of Pathology, Immunology, and Laboratory Medicine, University of Florida College of Medicine, Gainesville, FL (CI); Program in Dermatopathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA (GFM); ImClone Systems Incorporated, New York, NY (CM); Lung Cancer Center, Cornell University Medical College, New York, NY (DCG, VM).