Effect of vascular endothelial growth factor and its receptor KDR on the transendothelial migration and local trafficking of human T cells in vitro and in vivo

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Running Title: VEGFR expression on T cells and transendothelial migration

Abbreviations used: VEGF, Vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; EC, Endothelial cell; TNF, tumor necrosis factor.
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Shortened Title: VEGF-KDR interactions mediate T cell migration
ABSTRACT

In these studies, we demonstrate that the vascular endothelial growth factor (VEGF) receptor KDR is expressed on subset(s) of mitogen-activated CD4+ and CD8+ T cells in vitro. We also find that KDR co-localizes with CD3 on mitogen-activated T cells in vitro and on infiltrates within rejecting human allografts in vivo. To evaluate whether VEGF and KDR mediate lymphocyte migration across endothelial cells (EC), we used an in vitro live-time transmigration model and found that both anti-VEGF and anti-KDR antibodies inhibit the transmigration of both CD4+ and CD8+ T cells across TNFα-activated, but not unactivated EC. In addition, we found that interactions among CD4+ or CD8+ T cells and TNFα-activated EC result in the induction of KDR on each T cell subset, and that KDR-expressing lymphocytes preferentially transmigrate across TNFα-activated EC. Finally, using a humanized SCID mouse model of lymphocyte trafficking, we found that KDR-expressing lymphocytes migrate into human skin in vivo, and that migration is reduced in mice treated with a blocking anti-VEGF antibody. Collectively, these observations demonstrate that induced expression of KDR on subsets of T cells, and locally expressed VEGF, facilitate EC-dependent lymphocyte chemotaxis, and thus, the localization of T cells at sites of inflammation.
INTRODUCTION

Vascular Endothelial Growth Factor (VEGF), an angiogenesis factor, is established to function in the migration, proliferation and survival of endothelial cells. It is well known to function in wound healing, organ development and in tumor growth, and it serves to promote tissue protection and repair following acute injury. VEGF is also expressed in association with cell mediated immune inflammation and acute and chronic inflammatory reactions. In chronic inflammatory disease processes, VEGF fails to elicit effective tissue repair, and rather may induce a pathological form of angiogenesis that has been proposed to augment disease activity. Indeed, several studies have demonstrated that blockade of VEGF may attenuate the progression of chronic diseases such as arthritis, atherosclerosis and allograft rejection.

Although relatively underappreciated, VEGF has potent proinflammatory properties including an ability to mediate leukocyte trafficking into sites of cell-mediated immunity. The proinflammatory properties of VEGF are reported to be dependent on its ability to interact directly with monocytes resulting in chemotaxis, its ability to induce the expression of endothelial adhesion molecules and chemokine production, and its ability to enhance vascular permeability. Furthermore, VEGF has been reported to have direct chemoattractant effects on murine and human T cells, and blockade of VEGF in vivo has been found to inhibit lymphocyte trafficking into skin and rejecting cardiac allografts. However, the mechanism underlying the ability of VEGF to interact with T cells is not known, and the molecular basis for its ability to facilitate lymphocyte chemotaxis in vitro or in vivo is poorly understood.
Several recent studies have determined that the VEGF receptors Flt-1 (VEGF receptor 1), KDR (VEGF receptor 2) and neuropilin-1 may be expressed on subsets of T cells. Murine effector T cells express both Flt-1 and KDR, and murine populations of CD4^+CD25^+FoxP3^+ T regulatory cells selectively express neuropilin-1. Human T cell lines express all VEGF receptors, and purified subsets of human T cells including CD4^+CD45RO^+ cells express both Flt-1 and KDR. In addition, KDR, like neuropilin-1, has been found to be expressed by human FoxP3^+CD4^+ T regulatory cells. Neuropilin-1 has also been reported to be expressed on populations of human naïve T cells, where it functions in the initiation of T cell activation, and in primary immune responses. Classically, neuropilin-1 serves as an accessory co-receptor to bind VEGF and mediate crosslinking to KDR. However, VEGF has never been implicated as a ligand for T cells, or to function in T cell-APC interactions.

Nevertheless, VEGF has been reported to be associated with T helper (Th) type 1, Th2, and Th17 responses in vitro and in vivo, and VEGF-KDR interactions may qualitatively and quantitatively regulate memory CD4^+ T cell reactivation, including the costimulation of IFNγ production. Also, while VEGFRs have been reported to be expressed by T regulatory cells, the function of VEGF-VEGFR interactions on immunoregulatory cell activity is currently unknown. Collectively, these studies indicate that VEGF may have direct effects on different subsets of T effector and T regulatory cells via interactions with its receptor(s). Further, since KDR is a dominant receptor on both subsets of T cells, it is possible that VEGF-inducible signaling via KDR may be most important in human immunological responses.
In this study, we find that KDR is induced in expression on activated CD4$^+$ and CD8$^+$ T cells \textit{in vitro}, and further, we find that it is expressed on T cell infiltrates within human allografts \textit{in vivo}. In addition, we demonstrate that KDR is induced in expression on T cells following interactions with TNF$\alpha$-activated endothelial cells, and that VEGF and KDR function in transendothelial migration. Together, these observations identify T cell KDR as an important molecule in immunity, and suggest that VEGF-KDR interactions facilitate transendothelial migration of lymphocytes and their localization at sites of inflammation.
MATERIAL AND METHODS

**Reagents:** See Supplementary Material and Methods describing the antibodies and reagents used in these studies.

**Human Subjects:** Human peripheral blood mononuclear cells (PBMCs) was obtained from healthy volunteers. Informed consent was obtained in accordance with the guidelines of the Committee on Clinical Investigation at Children's Hospital Boston. The protocol for the collection of neonatal foreskin and the protocol for the use of cardiac transplant biopsy tissue in our research studies was approved by the Human Research Committee at the Brigham and Women’s Hospital, Boston, MA. Endomyocardial biopsies were collected from cardiac transplant recipients as part of routine post-transplant care. Diagnostic specimens were processed for clinicopathological analysis according to standard care and specimens were used for research when clinical diagnosis was complete. Renal biopsies were obtained for clinical indications from renal transplant recipients at Children's Hospital Boston. Research studies were also performed after clinicopathological diagnoses and clinical care was completed. Both cardiac and renal biopsy specimens were obtained over 5 years ago and were stored at −80°C until use in this study.

**Endothelial Cell Culture:** Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described. Briefly, HUVEC were serially passaged at 37°C in 5% CO₂ in M199 medium (BioWhittaker, Walkersville, MD) supplemented with 20% heat-inactivated fetal calf serum (FCS; Gibco-BRL Products, Gaithersburg, MD), 50μg/ml endothelial cell...
growth supplement (ECGS; Biomedical Technologies, Stoughton, MA), and 100 μg/ml heparin (Sigma Chemical, St. Louis MO), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco/Invitrogen, Carlsbad, CA). Confluent HUVEC were harvested using 0.025% trypsin/ 0.01% EDTA (Sigma Chemical, St Louis, MO).

Transfection: A validated KDR siRNA (Sense-r (CGC UGA CAU GUA CGG UCU A) dTdT Antisense-r (UAG ACC GUA CAU GUC AGC G) dTdT) and control (AllStars negative control siRNA, #1027280) were purchased from Qiagen (Valencia, CA), and were transfected (25 nM) into HUVEC using Hiperfect Transfection Reagent (Qiagen, Valencia, CA), according to the manufacturer's instructions. The efficiency of siRNA for knockdown was assessed by Western Blot analysis.

Leukocyte Isolation and coculture experiments: CD4+ and CD8+ T cells were isolated from PBMCs by positive selection using magnetic beads (Dynal® CD4 and CD8 Kits, Dynal Inc., Lake Success, NY) according to the manufacturer's instructions. Purified cells were cultured overnight in RPMI 1640 medium supplemented with 10% FCS (Sigma, St. Louis, MO) and penicillin (100U/ml), streptomycin (100mcg/ml), L-glutamine (2mM) at 37° in 5% C02. For some experiments, CD4+ T cells and CD8+ T cells were stimulated with plate bound anti-CD3 (0.5-1μg/ml) and anti-CD28 (0.5-1μg/ml) for 3 days prior to their use in transmigration assays. Also, for some experiments, untreated or TNF-α-treated HUVEC (100U/ml for 6 h) were harvested and cocultured with CD4+ or CD8+ T cells (1x10^5 cells, ratio of 1:1 in round bottom 96 well plates).
Lymphocyte migration assays: Briefly, 3μm pore membrane Falcon® FluoroBlok™ transwell inserts (Becton Dickenson, Franklin Lakes, NJ) were coated with 0.01% gelatin and subsequently with 3μg/well of human fibronectin (Sigma-Aldrich, Saint Louis, MO), similar to that described 35. Subsequently, 3x10^4 HUVEC (subculture 2-3) were seeded onto the membranes. After 5-6 days of culture, and prior to each experiment, the integrity of confluent EC monolayer was assessed by microscopy and by Coomassie stain. In addition, the confluency of the EC monolayer was assessed occasionally using the FITC-labeled dextran permeability assay, as described 35. siRNA-transfected EC were plated onto Falcon® FluoroBlok™ transwells (Becton Dickenson, Franklin Lakes, NJ) ~24 hrs following transfection, and were cultured for an additional ~18hrs prior to transmigration assays.

For transmigration assays, confluent EC monolayers were used untreated or following treatment with TNF-α (100U/ml) for 6 hours and were washed prior to transmigration assays. CFSE-labeled (2.5uM) CD4^+ or CD8^+ T cells (5x10^5 cells) were added into the upper chamber of the transwell in the absence or presence of blocking antibodies (anti-VEGF (1μg/ml), anti-KDR (2μg/ml)) or control IgG antibodies, as indicated. Antibodies were added to the transwell prior to addition of the lymphocytes. In some experiments, mitogen-activated CD4^+ or CD8^+ T cells were used, untreated or following preincubation with anti-KDR (2μg/ml) or mouse IgG for 3h at 37°C. After pretreatment, T cells were washed in culture medium prior to use in the assay.

Lymphocyte migration was monitored by the assessment of increasing fluorescence in the lower chamber of the transwell using an automated plate reader (Victor, Perkin Elmer, Wallac Inc, Turku, Finland). Automated fluorometric readings were assessed ‘real time’ every 15 minutes and increases in fluorescence intensity were reflective of increases in transendothelial migration. The number of cells transmigrating across into the lower chamber was calculated
using a standard curve generated by assessing the relative fluorescence intensity of increasing numbers of cells in control wells.

Chemotaxis assays were also performed across type 1 collagen coated 5μm pore polycarbonate filters using the standard Boyden Chamber, according to the manufacturer's instructions (Neuro Probe Inc., Gaithersburg, MD) as previously described 15 (see the Supplementary Material and Methods section)

**FACS analysis:** FACS was performed using standard techniques 14 with FITC-, PE-, APC- and Fluorescein-conjugated mAbs, or appropriate isotype control antibodies. After incubation with antibodies at 4°C for 30 min, the cells were washed and fixed in 1% paraformaldehyde and were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and CellQuest and FlowJo software.

**Western Blot Analysis:** Cultured cells were washed in PBS, were lysed with ice-cold RIPA buffer (Boston Bioproducts, MA) and were separated on a SDS-polyacrylamide gel, and transferred onto a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA). Using standard methodology as described 36, membranes were blocked with 5% milk in TBS-Tween 20 for one hour and incubated overnight with the primary antibody. Membranes were washed and incubated with a secondary peroxidase-linked antibody and the reactive bands were detected by chemiluminescence (Pierce, Rockford, IL). (Pierce, Rockford, IL).

**Real-Time PCR:** Total RNA was prepared using the RNeasy isolation kit (Qiagen). cDNA synthesis and PCR were performed using the SuperScript one-step RT-PCR kit (Invitrogen) and cDNA was synthesized using cloned AMV first-strand synthesis kit (Invitrogen, Carlsbad, CA).
Quantitative real-time PCR was performed using the 7300 real-time PCR system and the Assays-on-Demand Gene Expression Product (TaqMan, MGC probes, Applied Biosystems, Foster City, CA). Gene-specific primers for the analysis of human KDR and GAPDH by real-time PCR were obtained from Applied Biosystems. Ct values for the evaluation of KDR expression were calculated. Change(s) in the mRNA expression of KDR following T cell activation was evaluated as fold change relative to untreated cells as follows: fold change = $2^X$ (where $X = \text{Ct value for the control group} - \text{Ct value for each experimental group}$)

**In vivo humanized SCID mouse model of lymphocyte trafficking:** Human neonatal foreskin grafts were transplanted onto SCID mice as described $^{8,37,38}$ and were allowed to heal for 6 weeks. Prior to humanization, the mice were treated with 100 µl of anti-asialo G$_{M1}$ antibody (Wako Chemicals, Richmond, VA) by i.p. injection to neutralize host natural killer cells. After ~24 hrs, 3 x 10$^8$ human PBMC were injected i.p as described $^{37,38}$. The mice were untreated or were treated with humanized anti-human VEGF (5mg/kg every other day by i.p injection, Genentech, CA). After 14 days, the skin grafts were harvested from the mice and divided into two. One portion was frozen in OCT for cryosectioning, immunostaining and analysis of cellular infiltrates. The other portion was fixed in formalin, embedded in paraffin and processed for H&E staining. All studies were performed in accordance with protocol approval by the Animal Care and Use Committee, Children’s Hospital Boston, Massachusetts.

**Immunostaining/Immunofluorescence Microscopy:** Four micron cryostat sections were fixed in acetone, and were blocked in 5% goat serum and/or endogenous peroxidase activity was quenched with hydrogen peroxide in phosphate buffered saline (PBS). Subsequently the sections were incubated with the primary antibodies anti-KDR (55B11) and anti-CD3 (UCHT1) in
increasing dilutions in 5% goat serum in PBS overnight at 4°C. Following three washes in PBS, sections were incubated with a secondary biotinylated antibody or with Alexa Fluor 488 and Alexa Fluor 594 secondary antibodies (Molecular Probes Eugene, OR, USA), diluted according to the manufacturers recommendations. After washing, immunohistochemical staining was performed using the Vectastain ABC avidin biotin-peroxidase enzyme complex kit (Vector Laboratories, CA, USA); and immunofluorescence stained sections were mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes). Immunofluorescence microscopy was performed using a Nikon eclipse 80i microscope (MVI Instruments, Avon MA). Confocal laser scanning microscopy was carried out with a LSM 510 META NLO microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) and each image was collected, processed and analyzed using the LSM Image Brower Software.

For single cell staining, T cells or EC were harvested, and 2x10^5 cells were cytospun at 500 RPM for 7 min onto non-coated Shandon slides and mounted for confocal microscopy. The cells were fixed in 3%, formaldehyde, washed three times in PBS, and blocked in 5% goat serum/0.3% Triton X for 1h, prior to incubation with the primary antibodies anti-KDR (55B11, Cell Signaling) and anti-CD3 (UCHT1, BD Pharmingen) or anti-CD31 (BD Pharmingen). After washing, the cells were incubated with goat anti mouse Alexa Fluor 488 and goat anti rabbit Alexa Fluor 594 secondary antibodies, diluted in 5% goat serum in PBS. The slides were washed four times in PBS and mounted over ProLong Gold antifade reagent with DAPI (Molecular Probes Eugene, OR, USA). Confocal microscopy was performed using the LSM 510 META NLO microscope and LSM Image Brower Software (Zeiss) using standard techniques.

**Statistical analysis:** Statistical analysis was performed using the Mann Whitney test.
RESULTS

Expression of VEGF receptor-2 (KDR) on human T cells in vitro and in vivo: We initially isolated pooled populations of CD4\(^+\) and CD8\(^+\) T cells from PBMC by positive selection, and we evaluated the expression of KDR at the protein level by FACS, Western blot analysis and confocal microscopy, and at the mRNA level by PCR. As illustrated in Figure 1A-C, we find that KDR is not detectable, or is minimal, on unactivated T cells. In contrast, following activation with anti-CD3/anti-CD28, expression on both CD4\(^+\) and CD8\(^+\) T cells was induced at the mRNA and protein levels. By quantitative real time PCR, we find an 8-20 fold increase in KDR mRNA expression in both CD4\(^+\) and CD8\(^+\) populations following mitogen-dependent activation (Figure 1B). And, by confocal microscopy (Figure 1D), we find that KDR co-localizes with CD3 on mitogen-activated T cells, and its expression appears to be concentrated within focal areas of the cell surface membrane. In contrast in endothelial cells, KDR is diffusely expressed on the cell membrane, where the overall intensity of expression is high (Supplementary Figure 1). These initial observations clearly indicate that KDR may be expressed on purified populations/subsets of activated CD4\(^+\) and CD8\(^+\) T cells.

To next determine if this observation is of pathophysiological significance, we analyzed KDR expression within human allografts undergoing rejection and questioned whether it co-localizes with CD3 on T cells. A total of 19 endomyocardial biopsies, previously collected from 10 cardiac transplant recipients, were used for this analysis. We found that the mean number of CD3\(^+\) T cell infiltrates in these biopsies ranged from 4 to 79 cells per high power field (400X magnification). By immunofluorescence staining, as expected, KDR was expressed on endothelial cells diffusely throughout all biopsies including those without CD3\(^+\) infiltrates or
rejection (*Supplementary Figure* 2A), as well as those with infiltrates and evidence of rejection (*Figure* 2A). Furthermore, we observed that KDR co-localized with CD3 on monocuclear cells within infiltrated cardiac allograft biopsy specimens (*Figure* 2A, Mid-Lower Panels and *Figure* 2B). We also examined five kidney allograft biopsies and similarly observed co-expression of KDR on CD3+ T cell infiltrates (*Supplementary Figure* 2B-C). By grid counting, we found KDR expression on a mean (± 1 SEM) of 29 ± 3.3% of all CD3-stained cells/cardiac biopsy and 30 ± 2.6% of CD3-stained cells/renal biopsy (*Figure* 2C). Of note, in both kidney and heart allografts, consistently, KDR was present at high levels on endothelial cells throughout all grafts. In contrast, we observed that the expression of KDR on T cells was not identical within each biopsy. KDR was expressed at high levels on T cell infiltrates in some biopsies, regardless of whether the infiltrates were focal or diffuse (see *Supplementary Figure* 2). However, occasional biopsies with diffuse infiltrates had low levels of co-expression. Nevertheless, in all biopsies examined, KDR was expressed on isolated subsets of T cells under pathophysiological conditions. We interpret these observations to suggest that there may be circumstances or mechanisms that facilitate/augment expression at the local inflammatory site.

*Function of VEGF in the transendothelial migration of T cells*: Our observations suggest that local overexpression of VEGF, for instance, within allografts39-41, may mediate biological responses within VEGFR-expressing T cells. Indeed, VEGF-inducible responses in lymphocytes have been previously suggested to result in recruitment in vivo.8 To evaluate the effect of VEGF on chemotaxis responses in T cells, we developed a real-time migration model in which T cells transmigrate across endothelial cells in FluoroBlok™ transwells. Briefly, endothelial cells were cultured to confluency on 3µm pore FluoroBlok™ transmembranes in the upper chamber of
transwells. CFSE-labeled T cells were subsequently placed in the upper chamber in the absence or presence of a blocking anti-VEGF antibody, and migration was assessed every 15 mins using an automated assay, as previously described\textsuperscript{15}. As illustrated in \textit{Figure} 3, we consistently found two patterns of transmigration, an early phase, characterized by a rapid rate of transmigration, and a later phase, when the rate of transmigration slowed and stabilized. Furthermore, we found that the addition of our blocking anti-VEGF antibody (1-10\(\mu\)g/ml) into cocultures failed to have a significant effect on T cell transmigration across unactivated endothelial cells (\textit{Figure} 3A-B).

We next treated confluent cultures of EC with TNF\(\alpha\) for six hours prior to the transmigration assay. This treatment resulted in an increase in the early rate of transmigration, as well as the total number of T cells that transmigrated into the lower chamber, as compared to untreated endothelial cells (\textit{Figure} 3C-D). Moreover, as illustrated in \textit{Figure} 3C-F, we found that anti-VEGF (1 \(\mu\)g/ml) significantly inhibited both CD4\(^+\) and CD8\(^+\) T cell transmigration across TNF\(\alpha\)-activated endothelial cells \((P<0.01)\) at times >2 hrs. At earlier times (0-2hrs) blockade with anti-VEGF failed to inhibit transmigration. Collectively, these \textit{in vitro} observations suggest that interactions among T cells and activated endothelial cells (over a period of 0-2 hrs) results in the induction of molecule(s) that facilitate responsiveness to VEGF.

\textit{Blockade of KDR inhibits the transmigration of T cells across activated endothelial cells in vitro:}\textbf{

Next, we assessed the function of KDR in the transendothelial migration of T cells. Using FluoroBlo\textsuperscript{TM} transwells, as above, we found that saturating concentrations of a blocking anti-KDR antibody (R&D Systems) failed to inhibit CD4\(^+\) or CD8\(^+\) T cell migration across untreated EC (\textit{Figure} 4A, white bars). However, similar to our observations using anti-VEGF, we found
that anti-KDR (2µg/ml) significantly inhibited T cell migration across cocultures of TNF-α-activated EC ($P<0.01$, Figure 4A, black bars and Figure 4B).

We next used a siRNA approach to determine the cell specific function of KDR in VEGF-dependent migration. Since knockdown with siRNA is inefficient in purified populations of human T cells, but is very efficient in EC (Figure 4C), we evaluated transmigration across KDR siRNA-transfected EC. Confluent control and KDR siRNA transfected EC were treated with TNFα for 6hrs, and after washing, CD4+ or CD8+ T cells were placed into the upper chamber, as above. We found no difference in the pattern of T cell transmigration across control siRNA-transfected EC vs. non-transfected EC (Figure 4D). However, the transmigration of both CD4+ and CD8+ T cells across TNFα-activated KDR siRNA transfected EC was reduced as compared to control siRNA-transfected EC (Figure 4D). This observation indicates that KDR-induced response(s) in EC alone function in transendothelial migration, as we previously reported 14. However, notably, anti-KDR (2µg/ml) further increased the inhibition of T cell transmigration across KDR siRNA transfected EC (Figure 4 F and Figure 4 G-H). This observation suggests that KDR expression on T cells is also functional in the migratory response.

In order to characterize the functional effect of VEGF on KDR-induced chemotaxis of T cells, we next preincubated mitogen-activated CD4+ or CD8+ T cells with anti-KDR (or control IgG) prior to placement in the upper chamber of transwells. As illustrated in Figure 5A, we found that mitogen-activated T cells had an increased rate of transmigration, but preincubation with anti-KDR resulted in a weak but significant ($P<0.05$) inhibition of transmigration across both untreated as well as TNF-α activated EC (inhibition ~9% in $n \geq 5$ experiments). Furthermore, and consistent with our earlier findings, we observed that the addition of anti-KDR into cultures for the entire period of the assay resulted in a greater and more persistent inhibition
of transendothelial migration \((Figure\ 5B)\). These observations further support the possibility that T cell expression of KDR is functional in the migratory response.

Finally, we also performed Boyden chamber assays (in the absence of EC) to evaluate the effect of VEGF on KDR-dependent chemotaxis of T cells. We found that VEGF elicited a marked chemotaxis response in both untreated as well as in mitogen-activated T cells \((Figure\ 5C)\), and further, we observed that this response was similar to IP-10, a well established T cell chemoattractant which was used as a positive control. This observation alone is highly suggestive that VEGFRs expressed on T cells function in migration. Next, we treated unactivated or mitogen-activated CD4\(^+\) T cells with a pharmacological KDR signaling inhibitor (SU5416, 1 and 5 \(\mu\)M) prior to and during the assay. As illustrated in \(Figure\ 5C\), we found that SU5416 resulted in a significant inhibition of the VEGF-induced chemotaxis response. However, SU5416 failed to inhibit the VEGF-inducible chemotaxis response to baseline, suggesting that additional VEGFRs expressed on T cells may also mediate migration. Nevertheless, we found that the inhibitory effect of SU5416 was greater in mitogen-activated T cells (which express higher levels of KDR) as compared to unactivated T cells. Collectively, these observations indicate that VEGF has direct effects on human T cells to elicit chemotaxis, and further, migratory responses in T cells involve interactions between VEGF and T cell VEGFRs, including KDR.

\textit{Interaction(s) with endothelial cells result in the induction of KDR on human T cells:} We have previously reported that cell surface molecules expressed by activated T cells induce VEGF expression in EC \(^{42}\). Thus, T cell-mediated induction of VEGF by EC may result in a VEGF-dependent amplification loop to facilitate transmigration events, as evidenced by our functional observations above in \textit{Figures 3 and 4}. However, it is possible that this interaction is
bidirectional in as much as activated EC may also induce the expression of KDR on T cells. To test this possibility, we cocultured CD4+ or CD8+ T cells with unactivated EC or with TNFα-activated EC, and we subsequently performed FACS analysis to examine the co-expression of KDR on either CD4+ or CD8+ T cells. As illustrated in Figure 6A, coculture with untreated EC had minimal effects on KDR expression. However, coculture with TNFα-activated EC resulted in a notable induction of expression of KDR on both CD4+ and CD8+ cells, whereas the expression of KDR on EC was essentially unchanged in the absence or presence of T cells (Figure 6B). EC-induced expression of KDR on each T cell subset was evident after 4 hrs, and peaked in expression 6 hrs following coculture (Figure 6C). In addition, after 6 hrs of coculture with TNFα-activated EC, we found noticable expression of KDR on each T cell subset by confocal microscopy (Figure 6D). The temporal pattern of induction of KDR expression following coculture with activated EC is consistent with our functional analyses illustrated in Figures 3 and 4, where we observed that blockade of VEGF or KDR had functional inhibitory effects only at times >2hrs following interactions. Thus, EC-mediated induction of KDR on T cells may function to elicit a VEGF-dependent chemotaxis response.

Finally, we evaluated whether KDR-expressing T cells are primed to migrate across EC. Endothelial cells were cultured to confluency in transwells, as above, and were treated with TNFα for 6 hrs prior to each assay. After washing, CD4+ or CD8+ T cells were placed in the upper chamber of transwells, and subsequently, the cells were harvested either from the upper chamber (non-transmigrated T cells) or from the lower chamber (transmigrated cells) for FACS analysis. As illustrated in Supplementary Figure 3, we found that the expression of KDR was significantly higher in cells that transmigrated across TNFα-activated EC. Collectively, these
data indicate that contact between T cells and TNFα-activated EC results in the induction of KDR, which subsequently facilitates migration.

Blockade of VEGF inhibits the recruitment of human KDR-expressing lymphocytes in vivo: To test the in vivo relevance of our in vitro observations, we made use of a humanized SCID model of human skin inflammation, previously established in the laboratory. Following i.p. injection of human PBMC into SCID mice, it has been shown that there is a transient emergence of human memory T cells within the mouse circulation. Also, it has been found that these circulating human T cells have the potential to infiltrate human tissues that engraft onto the SCID mice. To evaluate whether VEGF mediates the trafficking of KDR-expressing T cells in vivo, human neonatal foreskin grafts were transplanted onto SCID mice and were allowed to heal for four-six weeks. Subsequently, human PBMC were transferred into the mouse by i.p. injection. One group of mice received control human IgG, and a second group received a blocking anti-human VEGF (Avastin, Genentech, San Francisco, CA; 5mg/kg every other day). After 14 days, the skin grafts were harvested and were analyzed by routine histology for leukocytic infiltrates, and by immunofluorescence for the colocalization of KDR and CD3. As illustrated in Figure 7, grafts harvested from untreated animals had marked infiltrates including significant numbers of CD3+ T cells. There was notable expression of KDR on T cell infiltrates within grafts harvested from untreated mice. In contrast, we found reduced numbers of T cells in the grafts harvested from mice treated with the blocking anti-human VEGF antibody, and we found that KDR was essentially absent on these infiltrates. We interpret these findings to indicate that VEGF may functionally interact with KDR-expressing T cells in vivo to facilitate their migration and/or localization at sites of inflammation.
DISCUSSION

In this study, we demonstrate that the VEGF receptor KDR is expressed at the mRNA and protein level on subsets of mitogen-activated CD4+ and CD8+ T cells \textit{in vitro}. Furthermore, we show that the interaction between T cells and activated EC results in KDR expression, and that blockade of VEGF or KDR inhibits transendothelial migration \textit{in vitro}. We also find that blockade of VEGF inhibits the localization of KDR-expressing T cells within inflammed tissues \textit{in vivo}. These new observations indicate that induced expression of KDR on T cells and locally expressed VEGF serve as direct mediators of immune inflammation.

We focused our analyses on the expression and function of KDR, but other VEGF receptors including Flt-1 and neuropilin-1 may be expressed by select T cell subsets\textsuperscript{13,15,20,21,26}. Ectopic expression of FoxP3 in T cells has been found to result in the induced expression of neuropilin-1\textsuperscript{20,22}, which is well established to function in motility responses\textsuperscript{45}. Also, while Flt-1 is expressed on T cells\textsuperscript{13,15}, our observations indicate that its level of cell surface expression is low, even following mitogen-activation\textsuperscript{15}. Thus, while VEGF likely mediates motility responses through several VEGF receptors expressed on T cells, our findings in this report indicate that signaling via KDR is sufficient to mediate chemotaxis.

Nevertheless, we find that anti-VEGF alone (in the absence of KDR blockade) is potent to inhibit T cell migration \textit{in vitro} and \textit{in vivo}. This observation suggests that the local production of VEGF, and not the induction of T cell KDR, may be critical for directed lymphocyte migration. In our \textit{in vitro} studies, the effect of anti-VEGF was not evident for 2-4 hrs following lymphocyte-endothelial contact, suggesting induction in expression (and thus function) following coculture. Consistent with this possibility, we have demonstrated that cell surface expression of
CD40L, known to be expressed by activated T cells \(^{46}\), may interact with CD40 on endothelial cells to mediate VEGF overexpression \(^{36,42}\). Thus, an interpretation of our observations is that the interaction between T cells and endothelial cells increases the production of local VEGF (by endothelial cells), which in turn facilitates the migratory response.

VEGF-KDR interactions in EC result in the induced expression of adhesion molecules and chemokines (including MCP-1 and IP-10) \(^{8,9,11,12,14}\). Furthermore, in previous studies, we defined a major role for VEGF in the inducible expression of the T cell chemoattractant chemokine IP-10, which subsequently mediates T cell trafficking \textit{in vivo} \(^8,14\). Consistent with these observations, using siRNA, we find that knockdown of KDR expression in EC alone inhibits T cell transendothelial migration. However, we also find that the addition of anti-KDR into assays using knockdown cells has an additive effect on the inhibition of T cell transmigration. In addition, using the Boyden chamber assay in the absence of EC, we find that VEGF mediates chemotactic effects on T cells via KDR-induced signals. Collectively, these observations, as well as our previously published reports \(^8,14,15\) point to non-endothelial mechanisms whereby VEGF may interact directly with T cells to promote chemotaxis. The studies outlined in this report indicate that these additional mechanisms in part involve direct interactions between VEGF and KDR expressed on subsets of T cells. Therefore, local tissue expression of VEGF may interact with KDR on EC and on T cells to mediate T cell trafficking within inflamed tissues.

In our studies, we find that T cell-endothelial cell interaction(s) result in the induction of KDR expression on interacting T cells; and further, we observed that induced T cell expression of KDR, and KDR-induced signals function in transendothelial migration. Signaling via KDR is well established to mediate migratory responses in endothelial cells, in part involving the PI-3
Kinase and MAP Kinase pathways. Consistent with this possibility, PI-3K-inducible signals and MAPK-inducible signals are potent for the chemotaxis of T cells, including the chemotaxis response to VEGF. Thus, there are several mechanisms and distinct signaling pathways by which VEGF and KDR may elicit a T cell motility response.

Finally, we suggest that the findings defined in this report have significant clinical implications in transplantation medicine. For instance, in chronic allograft rejection, ongoing inflammation as well as local tissue ischemia results in persistent overexpression of VEGF. Our findings outlined in this report are consistent with the possibility that circulating allosensitized T cells (expressing KDR) will respond to local overproduction of VEGF within allografts leading to chemoattraction. It will be interesting in the future to determine if KDR expression on circulating or intragraft T cells predicts and/or correlates with graft failure.

Also, although beyond the scope of our studies, our data also provide insight into how the local overproduction of VEGF by tumors may result in T regulatory cell recruitment that may serve to inhibit a destructive immune response. Indeed, it has been reported that VEGFR-expressing T regs are present within tumors. We suggest that, in part, the absence of antigen-specific sensitization in patients with cancer skews the biological effect of tumor derived VEGF to be selective for the recruitment of circulating VEGFR-expressing T regulatory cells. Perhaps, in the future, therapies that augment the generation of VEGFR-expressing tumor-specific cytotoxic T cells, with potential to migrate in response to VEGF, will enable T cell-dependent targeting of tumors.
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Authorship Contributions and Conflicts of Interest

Monika Edelbauer (Children's Hospital Boston): Performed research, interpreted data, helped to design research, contributed to technical and analytic tools, Contributed to writing of the manuscript. Conflicts of Interest Disclosure: None.

Dipak Datta (Children's Hospital Boston): Performed research, contributed to analytic tools and the collection and interpretation of data. Contributed to writing of the manuscript. Conflicts of Interest Disclosure: None.

Ingrid Vos (Children's Hospital Boston): Performed research, contributed to analytic tools and the collection and interpretation of data, Contributed to writing of the manuscript. Conflicts of Interest Disclosure: None.

Aninda Basu (Children's Hospital Boston): Performed research, interpreted data. Conflicts of Interest Disclosure: None.

Maria Stack (Children's Hospital Boston): Performed research, interpreted data. Conflicts of Interest Disclosure: None.

Marlies Reinders (Children's Hospital Boston): Performed research. Conflicts of Interest Disclosure: None.

Masayuki Sho (Children's Hospital Boston): Performed research. Conflicts of Interest Disclosure: None.

Katiana Calzadilla (Children's Hospital Boston): contributed to use of vital research reagents and use of analytical technical tools. Conflicts of Interest Disclosure: None.

Peter Ganz (Brigham and Women's Hospital): contributed vital reagents and in the interpretation of data. Conflicts of Interest Disclosure: None.
David M. Briscoe (Division of Nephrology): Designed research, Supervised and contributed to technical and analytic tools, supervised and counseled on data generation, interpreted data, Wrote the manuscript. Conflicts of Interest Disclosure: Research Grant Support: RoFAR, ROTRF and Investigator originated basic research proposals from Wyeth and Astellas. Consultant services provided to: Life Cycle Pharma, Wyeth Pharma
REFERENCES


FIGURE LEGENDS:

**Figure 1:** *Expression of KDR on mitogen-activated CD4+ and CD8+ T cells:* CD4+ and CD8+ T cells were purified from human blood by positive selection, and were stimulated with anti-CD3/anti-CD28 (as outlined in Methods) for 72 hours or as indicated. The expression of KDR was evaluated by FACS (*Panel A*), by real-time PCR (*Panel B*), by Western blot analysis (*Panel C*) and by Confocal Microscopy (*Panel D*). In *Panel A*, the difference (delta, C) in mean fluorescence staining (experimental minus isotype control) is shown within each FACS plot. *Panel B*, the relative fold induction in KDR mRNA expression in 6 and 12 hour mitogen-activated CD4+ and CD8+ T cells is shown. *Panel C*, illustrates a representative Western Blot of KDR in unactivated (lane 1) and 12 hour mitogen-activated T cells (lane 2). *Panel D*, representative confocal microscopy images of 72 hr anti-CD3/anti-CD28-activated CD4+ T cells stained for CD3 and KDR. Approx. 20% of all activated T cells in our cultures express KDR (as illustrated) and the remainder of the T cells in the cultures have non-detectable or low levels of expression (not shown). All experiments are representative of n ≥ 5 per condition (*Panels A-C*) and n=3 (*Panel C*).

**Figure 2:** *Expression of KDR on T cells within human cardiac and renal allografts:* *Panel A*, Representative photomicrographs illustrating the expression of KDR within human cardiac allografts. *Upper Panels*, immunohistochemical staining of KDR (rose brown color) in three human cardiac allografts with few (left upper panel), moderate (middle upper panel) and diffuse (right upper panel) infiltrates. *Middle and Lower Panels*, immunofluorescence staining of CD3 and KDR in a representative cardiac allograft at x400 mag (Middle Panels) and x 800 mag
(Lower Panels). Merged images illustrate coexpression of CD3 and KDR (yellow color, arrows). **Panel B**, confocal microscopy of a representative cardiac allograft with evidence of rejection illustrating co-expression of CD3 with KDR. **Panel C**, scatter graphs showing the percent of CD3⁺ stained T cells that coexpress KDR in cardiac and renal biopsies (black dots represent individual biopsies). The line shows the mean percent expression in all biopsies examined.

**Figure 3:** Effect of anti-VEGF on the transmigration of human CD4⁺ and CD8⁺ T cells across endothelial cells. Endothelial cells were cultured to confluency on FluroBlok™ 3μm pore membranes in the upper chambers of transwells. Subsequently, CFSE-labeled T cells (5x10⁵ cells) were added into the upper chamber and migration into the lower chamber was monitored real-time by the assessment of increasing fluorescence. The number of transmigrated cells in each lower chamber was determined using a standard curve, and the percentage of transmigrated cells at each time point was calculated. Transmigration assays were performed in the presence of control IgG (black dots) or anti-human VEGF (Genentech, 1μg/ml, open squares). **Panels A-B**, representative experiment illustrating transmigration across unactivated endothelial cells. No difference between anti-VEGF vs. control IgG treatment is evident. **Panels C-D**, representative experiment illustrating transmigration across 6 hour TNFα-activated endothelial cells. **Panels E-F** the mean percent inhibition of transmigration by anti-VEGF across unactivated (white bars) or TNFα-activated endothelial cells (black bars, n=5 per experimental group). Anti-VEGF significantly inhibits transmigration across activated endothelial cell at times >2hrs. Asterix-* represents P< 0.01 vs. Control IgG.
Figure 4: Effect of anti-KDR on the transendothelial migration of human CD4+ and CD8+ T cells. Endothelial cells were cultured to confluency on 3μm pore membranes in FluroBlok™ transwells. CFSE-labeled CD4+ or CD8+ cells (5x10^5 cells) were added into the upper chamber and migration into the lower chamber was monitored real-time as described in Methods. Panel A illustrates the mean percent inhibition of transmigration of CD4+ or CD8+ cells (as indicated) by anti-KDR (R&D Systems) vs. control IgG across unactivated (white bars) or TNFα-activated EC (black bars, n=5 experiments, Asterix-* represents P< 0.01 vs control IgG). Panel B illustrates a representative experiment showing patterns of inhibition of CD4+ or CD8+ T cell transmigration by anti-KDR across TNFα-activated EC (Control IgG, solid dots; anti-human KDR, open squares). Panel C, Western blot analysis of control siRNA and KDR siRNA transfected endothelial cells. Upper and middle blots illustrate different exposure times of the same blot. Panel D, representative experiments illustrating transmigration of CD4+ T cells across non-transfected, control siRNA transfected endothelial cells and KDR siRNA transfected endothelial cells. Panels E-F, representative experiments illustrating the transmigration of CD4+ T cells across control siRNA transfected EC (E) or KDR siRNA transfected EC (F) in the absence (solid dots) or presence (open squares) of anti-KDR (2μg/ml). Panels G-H, illustrate the mean percent inhibition (n=3 experiments) of transmigration of CD4+ T cells (G) and CD8+ T cells (H) across control siRNA transfected EC compared to: non-transfected EC (black bars) or KDR siRNA transfected EC (grey bars) in the absence or presence of anti-KDR as indicated. Also shown is the inhibitory effect of anti-KDR on T cell transmigration across control siRNA transfected EC (white bars, as in Panel E) or KDR siRNA transfected EC (hatched bars, as in Panel F). The asterix-* represents P< 0.01 vs control IgG.
**Figure 5:** *Effect of anti-KDR on the transendothelial migration of mitogen-activated human* CD4⁺ and CD8⁺ T cells. Endothelial cells were cultured to confluency on FluroBlock™ 3μm pore transwells. Subsequently, 72hr mitogen-activated (anti-CD3/CD28) CD4⁺ or CD8⁺ T cells were CFSE labeled and added into the upper chamber of transwells and migration into the lower chamber was monitored real-time. **Panel A,** mitogen-activated T cells were pretreated with control IgG or anti-KDR for 3 hours and were washed in culture medium prior to the migration assay. Representative experiments showing transmigration patterns across untreated or TNFα-activated endothelial cells are illustrated. The bar graphs represent mean percent inhibition of transmigration by anti-KDR pretreatment across unactivated EC (white bars) or TNFα-activated EC (black bars) in 5 experiments at each time point. The double asterix-** represents *P* < 0.05 comparing anti-KDR pretreated cells vs. cells pretreated with control IgG. **Panel B,** transmigration of mitogen-activated CD4⁺ or CD8⁺ T cells across unactivated EC or TNFα-activated EC in the presence of control IgG (solid dots) or anti-KDR (open squares) for the entire period of the assay. The bar graphs illustrate mean percent inhibition of transmigration by anti-KDR across unactivated EC (white bars) or TNFα-activated EC (black bars) in n=5 experiments. The asterix-* represents *P* < 0.01 comparing the effect of anti-KDR vs. control IgG. **Panel C,** unactivated (hatched bars) or 72 hr mitogen-activated CD4⁺ T cells (black bars) were placed in the upper chamber of a microchemotaxis Boyden chamber and migration into the lower chamber was assessed after 4hrs, as described in Methods. The chemotaxis response to VEGF or IP-10, as a positive control, is illustrated. As indicated, the T cells were pretreated with SU5416, a pharmacological KDR signaling inhibitor, prior to and during the chemotaxis assay. The illustrated experiment is representative of at least three performed in triplicate wells. *P* values were calculated using the Student t test (* *P* < 0.01).
**Figure 6:** *Effect of endothelial cells on inducible KDR expression by human CD4⁺ and CD8⁺ T cells.* Untreated or TNF-α-activated endothelial cells (100U/ml for 6 hours) were incubated with purified populations of either CD4⁺ or CD8⁺ T cells (1x 10⁵ cells) in a ratio of 1:1 for increasing times up to 6 hrs, as indicated. Following coculture, FACS was performed for the evaluation of T cell expression of KDR. *Panel A,* representative dot plots following culture of either CD4⁺ (upper panels) or CD8⁺ T cells (lower panels) with endothelial cells. *Panel B,* representative FACS histograms illustrating expression of KDR on TNF-α-activated endothelial cell before and after coculture with CD4⁺ or CD8⁺ T cells. *Panel C,* representative FACS histograms illustrating the temporal induction of KDR expression on CD4⁺ T cells (upper panels) or CD8⁺ T cells (lower panels) following coculture with TNF-α-activated endothelial cells. *Panel D,* confocal microscopy illustrating colocalization of CD3 and KDR on CD4⁺ T cells (upper panels) and CD8⁺ T cells (lower panels) after coculture with TNF-α-activated endothelial cells. Panels A, B and C are representative of n=5 per experimental group. Panel D is representative of n=2, with identical results.

**Figure 7:** *The effect of anti-VEGF on KDR-expressing lymphocyte recruitment in vivo.* SCID mice with human skin transplants were humanized by adoptive transfer of human PBMC intraperitoneally. At the time of humanization, and every other day, the mice received human IgG or humanized anti-human VEGF (5mg/kg in 100μl saline). After 14 days, the skin grafts were harvested and were analyzed by H&E staining and by immunofluorescence. Illustrated in the left-hand panels are representative photomicrografts of skin grafts harvested from mice treated with control IgG. The photomicrografts illustrated in the right panels are representative of
skins harvested from mice treated with anti-VEGF. Expression of CD3 is illustrated in green, KDR in red, and the merged images (lower panels, yellow color) illustrate co-expression of KDR with CD3 on T cell infiltrates. Magnification of all micrographs, X400. Boxed inserts, magnification X 800. Representative of n=4 mice in each group.
Figure 3

A) Unactivated EC

B) Unactivated EC

C) TNFα-activated EC

D) TNFα-activated EC

E) % inhibition by anti-VEGF

F) % inhibition by anti-VEGF

CD4+ T Cells

CD8+ T Cells

Time (hrs)

Time (hrs)

Time (hrs)
Figure 5

A

Activated CD4+ T Cells

Activated CD8+ T Cells

B

Activated CD4+ T Cells

Activated CD8+ T Cells

C

Transmigrated T cells (Number/Well)

VEGF (ng/ml)
SU5416 (µM)
IP-10 (ng)

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Figure 7
Effect of vascular endothelial growth factor and its receptor KDR on the transendothelial migration and local trafficking of human T cells in vitro and in vivo

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