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# Proangiogenic Function of T Cells in Corneal Transplantation

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## Abstract

**Background—**Corneal neovascularization increases the risk of T cell mediated allograft rejection. Here, we investigate whether T cells promote angiogenesis in transplantation.

**Methods—**Conventional effector T cells were collected from draining lymph nodes (DLNs) of allogeneic or syngeneic corneal transplanted BALB/c mice. T cells were either cocultured with vascular endothelial cells (VECs) to assess VEC proliferation or used in a Mixed Lymphocyte Reaction assay. mRNA expression of vascular endothelial growth factor (VEGF)-A, -C, and VEGF receptor 2 (VEGF-R2) in VECs was assessed by real-time PCR. VEGF-A protein expression was determined by ELISA. Flow cytometry was used to analyze VEGF-R2 expression in corneal $CD31^+$ cells, and VEGF-A and IFN$\gamma$ expression in corneal $CD4^+$ T cells.

**Results—**Allogeneic T cells from high-risk (HR) grafted mice induced more VEC proliferation than those from syngeneic transplant recipients (p=0.03). VEGF-A mRNA and protein expression were higher in T cells from DLNs (p=0.03 and p=0.04, respectively) and cornea (protein; p=0.04) of HR compared to low-risk (LR) grafted hosts. VEGF-A, VEGF-C and VEGF-R2 mRNA expression were increased in VECs when cocultured with T cells from HR transplants compared to LR transplants and naïve mice. In addition, IFN$\gamma$ blockade in T cell/VEC coculture increased VEC proliferation and VEGF-A protein expression, whereas blocking VEGF-A significantly reduced VEC proliferation (p=0.04).

**Conclusions—**Allogeneic T cells from corneal transplant hosts promote VEC proliferation, probably via VEGF-A signaling, while IFN$\gamma$ shows an antiangiogenic effect. Our data suggest that T cells are critical mediators of angiogenesis in transplantation.

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**Author contributions:**

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## Introduction

Corneal transplantation is the most common form of human solid tissue transplantation,[1,2] with over 100,000 cases reported annually worldwide.[3] Corneal allo-transplantation does not ordinarily require systemic or permanent immunosuppression or human leukocyte antigen (HLA) tissue matching,[1,3,4] but allograft rejection causing corneal graft failure continues to be an obstacle to transplant success.[5–7] When performed in nonvascularized and uninflamed host beds, termed low-risk (LR) transplantation, graft survival rates are over 90% under topical corticosteroid therapy. In contrast, graft rejection rates dramatically increase to near 50% when transplants are placed into inflamed and vascularized host beds, termed high-risk (HR) transplants, despite maximal immune suppressive therapy.[1,3,4] These outcomes are worse than grafts of kidney, heart, or liver.[5–7]

Host bed vascularity is a principal risk factor for allograft rejection because blood vessels are critical for delivery of immune effector cells to the graft site,[8] particularly T helper 1 (Th1) cells, the principal mediators of graft rejection in corneal transplantation[9]. The normal cornea is devoid of blood and lymphatic vessels and actively maintains a state of ‘angiogenic privilege’. In LR transplantation, transient vascular engorgement or vascular sprouting in the limbus is quickly extinguished. In contrast, grafting onto HR vascularized and inflamed host beds often leads to increased angiogenesis which further increases the risk of graft rejection.[10] Numerous studies have demonstrated that the innate immune system contributes to angiogenesis in corneal transplantation, particularly through the actions of macrophages.[11–14] In addition, several studies have outlined the effect of T cells in inducing tumor-related angiogenesis.[15] However, in transplantation, while the function of blood vessels in facilitating T cell-mediated immunity has been appreciated, very little is known whether T cells themselves can promote or regulate angiogenesis.[9] Here, we hypothesized that T cells derived from inflamed HR transplant hosts disrupt angiogenic privilege through increased expression of proangiogenic factors. The vascular endothelial growth factor (VEGF) family controls angiogenesis and targeting VEGF-A in low- and high-risk corneal transplantation has been shown to reduce angiogenesis and improve graft survival.[10] In this study, we investigated the proangiogenic effect of T cells on vascular endothelial cell proliferation, and show a direct effect of $CD4^+$ conventional T cells (conv T cells) on VEC proliferation through increased VEGF expression.

## Materials and Methods

### Animals

Male C57BL/6 (donors) and BALB/c (hosts and naïve) mice 6–8 weeks of age were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed in the Schepens Eye Research Institute animal vivarium and treated according to the guidelines set forth by the Association for Research in Vision and Ophthalmology (ARVO). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee.

### Corneal transplantation

Syngeneic (BALB/c to BALB/c) and allogeneic (C57BL/6 to BALB/c) orthotopic corneal transplantation was performed as described previously.[16] Briefly, in low-risk transplantation, 2 mm diameter donor corneal buttons from C57BL/6 mice were affixed to 1.5 mm diameter avascular and uninflamed BALB/c host beds via 8 interrupted 11-0 nylon sutures. Inflamed and vascularized high-risk host beds were created by placing 3 intrastromal sutures 14 days before transplantation in BALB/c mice as described previously.[16] After surgery, host eyelids were closed for 3 days via tarsorrhaphy and interrupted corneal sutures were removed 7 days following surgery. Corneal allografts were evaluated by slit lamp microscopy and graft clarity was scored according to a well-established 0–5+ scale, with scores of 2+ considered rejected [17]. To exclude grafts undergoing primary failure, only those grafts with scores under 1 at 14 days after transplantation were used for experimentation.

### T cell sorting

Ipsilateral draining lymph nodes (DLNs) were harvested from HR and LR mice. Single cell suspensions were created by homogenizing lymphoid tissue in 70 μm cell strainers. $CD4^+CD25^-$ T cells were magnetically sorted using a mouse T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and stimulated for 12 hours at 37° C and 5% $CO_2$ with purified anti-mouse CD3ε antibody (1 μg/ml, Biolegend, San Diego, CA) in complete Dulbecco Modified Eagle medium (DMEM).

### Cell culture

A mouse endothelial cell line, MILE SVEN-1 (MS-1), (a kind gift from Dr. Patricia D'Amore, Boston, MA, and originally purchased at ATCC, Manassas, USA),[18] was used in our experiments. We evaluated these cells for their surface expression of VEGF-R2 by flow cytometry to assure their endothelial cell characteristics before performing experiments. Cells were cultured in DMEM (Lonza, Basel, Swiss) with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA). At day 0, $1\times10^4$ MS-1 endothelial cells (ECs) were seeded in the lower compartment of a 96 PET transwell system (6.5 mm insert size, 1 μm pore size, Corning). At day 1, when the MS-1 cells covered ~30% of the lower wells, $1\times10^5$ $CD4^+CD25^-$ anti-CD3ε stimulated T cells were placed in the upper compartment of the 96 PET transwell system and cocultured in serum free DMEM medium with endothelial cells. After 24–30 hours of coculture, endothelial cell proliferation was assessed using the bromodoxyuridine (BrdU) incorporation assay (Millipore, Billerica, MA) and a spectrophotometer microplate reader (Brand, city, state). MS-1 cells cultured in DMEM only served as a negative control, and MS-1 cells treated with 20 ng/mL recombinant VEGF-A (Biolegend, San Diego, CA) were used as a positive control.

### Mixed Lymphocyte Reaction (MLR)

Purified allogeneic T cells ($1\times10^5$) were isolated from syngeneic and allogeneic HR and LR graft recipients and MACS-sorted for CD90.2 (Miltenyi Biotec), according to the manufacturer's instructions. Isolated T cells were cocultured with BALB/c APCs ($1\times10^5$) and alloantigens from C57BL/6 spleen (cell lysate was generated by sonicating spleen cells from a naïve C57BL/6 mouse and underwent a freeze/thaw cycle) for 72 hrs in 96-well,

round-bottom plates. BrdU reagent (Sigma-Aldrich) was added to each well, 16 hrs before collecting the cells. The proliferation of T cells was measured by using the BrdU incorporation assay kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions.

### Flow cytometry

At day 14 following corneal transplantation, the ipsilateral cervical lymphoid tissue was collected from allogeneic and syngeneic HR and LR transplant recipients. Single cell suspensions were prepared by homogenizing lymphoid tissue in 70 μm cell strainers. Grafts and host corneal beds were collected 21 days posttransplantation and digested with DNase (0.2 mg/ml, Roche, Basel, Switzerland) and Collagenase (0.4 mg/ml, Roche, Basel, Switzerland) to make single cell suspensions and were incubated for 4h at 37° C with 0.1% Golgistop (BD Biosciences, USA) to prevent cellular release of VEGF-A. All cell suspensions were incubated with an Fc-blocking agent (R&D Systems, Minneapolis, MN) before surface antibody staining. Corneal cells were stained with BV421 anti-CD4 (Biolegend, San Diego, CA) and Alexa Fluor488 anti-VEGF-A (Bioss, Woburn, MA) antibodies. To determine VEGFR2 expression on endothelial cells in the cornea, we stained single cell suspensions with APC-conjugated anti-VEGFR2 (R&D Systems) and PE-conjugated anti-CD31 (eBiosciences). Lymph node cells were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, St Louis, state, USA) and ionomycin (Sigma Aldrich) in the presence of Golgi-stop (BD Biosciences, USA) for 5 hours at 37° C and 5% $CO_2$, and then stained with FITC-conjugated anti-CD4 antibody (Biolegend). Cells were fixed and permeabilized overnight, and stained with APC-conjugated anti-IFN$\gamma$ antibody (Biolegend). MS-1 VECs were analyzed after coculture with CD4 T cells and stained with APC conjugated anti-VEGF-R2 (R&D Systems, Minneapolis, MN). Data were analyzed using a BD LSR II flow cytometer (BD Biosciences) and Summit v4.3 software (DAKO corporation, Carpinteria, CA).

### RNA isolation, Reverse transcription (RT) PCR, and quantitative Real-Time PCR

Anti-CD3ε stimulated conventional T cells and VECs were collected and their RNA was isolated using the RNeasy micro kit (Qiagen, Valencia, CA). The RNA was then reverse-transcribed using the Quantitec reverse transcription kit (Qiagen) with a Taqman Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was performed using a Taq Man-Mastermix and preformulated primers (Life Technologies) for murine IFNγ (Mm01168134_m1), VEGF-A (Mm01281449_m1), VEGF-C (Mm00437310_m1), VEGF-R2 (Mm01222421_m1), bFGF (Mm01285715_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm99999915_gl). Comparative threshold (CT) values were measured using a LightCycler 480 II System (Roche, Indianapolis, IN) and target CT values were normalized to the CT value of GAPDH, which served as an endogenous control. The fold change of mRNA levels relative to control groups was then calculated.

### Enzyme-linked immunosorbent assay (ELISA)

VEGF-A protein expression was measured in the supernatants of cocultured conventional T cells and VECs using an ELISA assay according to manufacturer's instructions (Mouse VEGF-A Platinum ELISA kit, eBioscience).

### In vitro IFNγ stimulation

Proliferation of MS-1 VECs stimulated with 20ng/ml VEGF-A and treated with different doses of recombinant IFNγ (1, 100, 500 ng/ml) (Prepotech, Rock Hill, NJ) or 10ng/mL of recombinant IL-1β (Biolegend) (as a positive control) was assessed using the BrdU incorporation assay (Millipore).

### In vitro neutralization

A neutralizing anti-IFNγ antibody (1mg/mL; LEAF™ purified anti-mouse IFNγ antibody, Biolegend) or an anti-VEGF-A antibody (Biolegend, San Diego, CA) and their respective isotype control antibodies (anti-IFNγ: LEAF™ purified armenian Hamster IgG isotype ctrl antibody, Biolegend; anti-VEGF-A: LEAF™ purified rat IgG2a, κ isotype Ctrl, Biolegend, San Diego, CA) were used for in vitro blockade of IFNγ or VEGF-A, respectively, in our culture system with T cells and VECs. After 24 hours of culture VECs proliferation was assessed using the BrdU incorporation assay (Millipore). After anti-IFNγ treatment we also assessed VEGF-A expression by ELISA.

### Statistical analysis

Statistical differences were evaluated using Prism 5.0 software (Prism 5.0 software, GraphPad Software, Inc., La Jolla, CA, USA). Student's T test was used to analyze differences between 2 groups. Data were expressed as the mean ± SEM of at least 2 independent experiments. A p value of $<0.05$ was considered significant (*= $P<0.05$; **=$P<0.01$; ***=$P<0.001$).

## Results

### Alloprimed T cells from transplanted mice induce vascular endothelial cell proliferation

We evaluated the effect of allogeneic and syngeneic T cells isolated from naïve, HR and LR transplanted mice on inducing VEC proliferation in vitro. We compared the proangiogenic effect of conv T cells collected from HR vs. LR transplant hosts using a coculture system with a vascular endothelial cell line (MS1). We found that allogeneic but not syngeneic conventional T cells from HR transplanted mice induced more VECs proliferation than those from LR grafted and naïve mice (HR allogeneic vs. syngeneic: $p=0.03$; Figure 1A). To verify that this effect depends on the alloreactivity of T cells, we performed MLR with T cells from syngeneic and allogeneic transplanted mice. Only allogeneic T cells were able to promote T cell proliferation but not syngeneic T cells (HR allogeneic vs. syngeneic: $p=0.008$; Figure 1B).

### Alloprimed T cells release pro-angiogenic VEGF-A

We next found that VEGF-A mRNA expression was increased in conv T cells recovered from HR recipients compared to T cells collected from LR recipients and naïve mice (HR 3.4 ± 0.2l vs. LR 1.7 ± 0.43, p=0.03; HR vs. naïve 1.09 ± 0.23, p=0.003; Figure 2A). We additionally confirmed that this increase also occurs at the protein level, as VEGF-A expression was increased in the supernatant of cultured T cells from HR but not LR graft recipients or naïve mice (HR 18±1.7 vs. LR 9.8±2.0 p=0.04; HR vs. naïve 9±0.9, p=0.01; Figure 2B). Analyzing VEGF-A expression in transplanted corneas showed a significant increase in VEGF-A expression in CD4 T cells isolated from HR and LR transplanted grafts (p=0.04; Figure 2C and D).

We also assessed bFGF mRNA expression in T cells, IL-1β concentration in the supernatant of cultured T cells (using ELISA), and IL-17 expression in T cells (using flow cytometry) from HR and LR recipients and found no difference between both groups (SDC Figure 1).

### VEGF-A, VEGF-C, and VEGF-R2 are increased in vascular endothelial cells

We next analyzed the mRNA expression of VEGF-A, VEGF-C, and VEGF-R2 in VECs after culturing them with T cells sorted from HR and LR grafted mice. T cells from HR and LR recipient mice induced VEGF-A, VEGF-C, and VEGF-R2 expression in VECs compared to VECs cultured in medium alone (VEGF-A: HR 6.3±1.13 vs. medium 1.1 ± 0.2, p=0.047; LR 2.47±0.05 vs. medium p=0.047; VEGF-C: HR 67.96±23.35 vs. medium 1.8±1.5, p=0.048; LR 12.74±6.57 vs. medium p=0.18; VEGF-R2: HR 3.3±0.73 vs. medium 0.88±0.03, p=0.01; LR 1.36±0.09 vs. medium, p=0.0006; Figure 3A–C). VEGF-A, VEGF-C, and VEGF-R2 mRNA expression in VECs was highest in VECs co-cultured with HR T cells compared to LR cells (VEGF-A, HR 6.3±1.13 vs. LR 2.47±0.5 p=0.008; VEGF-C: HR 67.9 ± 23.3, LR 12.7 ± 6.5, p= 0.08; VEGFR-2: HR 3.3±0.73; LR 1.3±0.09, p=0.049). Frequencies of VEGF-R2$^{hi}$ VECs were similar after culture with T cells from HR vs. LR grafted hosts (data not shown). When we analyzed VEGFR2 protein expression levels on CD31+ endothelial cells in the cornea posttransplantation, we found a statistically significant increase in VEGFR2 expression in endothelial cells (CD31+) in HR compared to LR transplanted corneas (p<0.0001; Figure 3D and E).

### HR transplantation promotes Th1 cells and IFNγ expression in the lymph nodes

Th1 cells are the principal mediators of acute corneal graft rejection; thus, we assessed their frequencies in draining lymph nodes and their expression of IFNγ before and 14 days posttransplantation. The frequencies of CD4$^{+}$IFNγ$^{+}$ T cells were increased in HR compared to LR transplanted mice (p=0.008; Figure 4A). Moreover, the expression level (MFI) of IFNγ in conventional T cells was increased in HR compared to LR transplant recipients and naïve mice (p=0.03; Figure 4B).

### Vascular endothelial cell proliferation is increased with IFNγ blockade and decreased with VEGF blockade

To investigate the anti-proliferative effect of IFNγ on VECs we treated them with different doses of IFNγ and added IL-1β as a positive control. Addition of IL-1β significantly upregulated VEC proliferation as shown before (p=0.003; Figure 4C).[19] High IFNγ

concentrations significantly reduced VEC proliferation compared to low IFNγ concentrations (p=0.0006; Figure 4C). Next, we cultured VECs with T cells from HR vs. LR grafted mice with or without blocking IFNγ. We found that VEC proliferation further increased when we blocked IFNγ (naïve 1±0.04 naïve + αIFNγ 2.3±0.2, p= 0.038; LR 2.7±0.46, LR + αIFNγ 4.4±0.33 p=0.04; HR 3.8±0.26, HR+αIFNγ 4.8± 0.1 p=0.03; Figure 4D). Next, we measured VEGF-A protein expression in our coculture of conventional T cells and VECs and found a significant increase in VEGF-A in LR and HR groups treated with anti-IFNγ antibody compared to untreated groups (naïve: 12.17±1.7 vs. 9.066±0.09, p=0.2; LR: 25.32±4.1 vs. 9.861±2.093, p=0.03; HR: 32.22±2.6 vs. 17.99±1.7, p=0.01; Figure 4E). Finally, we blocked VEGF-A signaling in our T and VEC coculture and found that VEC proliferation was inhibited after treatment with anti-VEGF-A (p=0.04; Figure 4F).

## Discussion

Angiogenesis represents a key process in transplant rejection in the liver,[20] kidney,[21,22] lung,[23] and cornea [24] and serves as a key risk factor for allorejection.[6] Although the contribution of innate immunity to angiogenesis has been well established,[3,5,7,11,25,26] little is known about the role of T cell-mediated adaptive immunity in regulating the development of neovessels. A recent study has reported that a decrease in anti-angiogenic signals in the cornea is mediated by graft-infiltrating allospecific T cells through reduction of corneal endostatin, a broad inhibitor of angiogenic factors.[27] In the current study, we report that alloreactive T cells from transplanted mice directly promote the proliferation of VECs in vitro, and that these T cells express proangiogenic VEGF-A, the main mediator of VEC proliferation and migration. Further, our data suggest that alloprimed T cells also promote angiogenesis by inducing VEC expression of VEGF-A and C.

Transplantation into an inflamed, or “high risk” host bed increases the chance of graft rejection.[28] Corneal neovascularization induced after chemical burns and posttrauma is primarily mediated by the innate immune system, and this angiogenic response facilitates healing and regeneration, and later spontaneously regresses.[29,30] When corneal transplantation is performed onto an inflamed bed, this early phase of neovascularization is followed by a later and more invasive neovascularization which promotes allograft rejection. Since alloreactive T cells produce VEGF-A and significantly increase VEC proliferation, we hypothesize that this second graft-threatening phase of neovascularization is mediated, at least in part by alloreactive T cells.

Here, we show that performing transplantation in an inflamed host bed leads to increased VEGF-A secretion by lymphoid (Figure 2B) and corneal T cells (Figure 2C and D), and these “high-risk” T cells promote VEC proliferation and enhanced VEGF signaling in a coculture system. VECs cocultured with alloreactive T cells, especially from HR graft recipients show increased mRNA VEGF-R2 expression compared to VECs cocultured with T cells from LR transplant recipients (Figure 3C). However, flow cytometry analysis shows only a modest increase in the frequencies of VEGFR2+ VECs cultured with T cells from HR transplants (data not shown). Analyzing VEGFR2 expression in corneal endothelial cells after transplantation shows significantly increased VEGFR2 expression in HR vs. LR transplanted corneas (Figure 3D and E). In addition, blockade of VEGF-A in our culture

system reduces VEC proliferation to a level seen in VECs cultured with syngeneic T cells (Figure 4F), suggesting a crucial role for VEGF-A in T-cell mediated angiogenesis. Autocrine VEGF-A secretion and signaling via VEGF-R2 on VECs is crucial for their vascular homeostasis and survival.[3233] It has also been shown that endothelial cells upregulate VEGF-A production under stress, such as hypoxia or inflammation. These findings suggest that an autocrine pathway may amplify the paracrine effects of VEGF-A in inducing angiogenesis.[34] Thus, in the aggregate, our data suggest that alloprimed T cells from HR grafted hosts can mediate neovascularization through increased VEGF-A expression, which is a critical factor in the mechanisms of allograft rejection; indeed, increased graft survival has been reported after in vivo neutralization of VEGF.[10,19,35]

Angiogenesis is a complex process requiring numerous interactions between pro and anti-angiogenic signals. While most scientists agree that VEGF-A is the dominant member of the family, other members expressed by T cells may also promote angiogenesis. For example, fibroblast growth factor (FGF) was first investigated for its role in angiogenesis in the 1980s,[36] and FGF-2 (also named bFGF) has been shown to have a proangiogenic role in the corneal stroma of mice.[37, 38] In addition, in a previous study, we have shown that IL-1 induces expression of VEGF-A in corneal stromal fibroblasts, and thus promotes angiogenesis in the cornea.[39] Finally, it has been reported that IL-17 can promote migration and tube formation in an in vitro model of age related macular degeneration, as well as in cancer-related angiogenesis.[40,41] However, FGF-2, IL-1, and IL-17 expression was not increased in T cells isolated from HR vs. LR transplanted recipients (SDC Figure 1).

Allogeneic T cells mediate acute alloimmune response by releasing IFNγ.[5,42–45] In this study, we show that T cells from HR hosts, despite their increased production of VEGF-A and proangiogenic role, express significantly higher levels of IFNγ compared to T cells from LR transplants (Figure 4A and B). IFNγ is known to stimulate proliferation of $CD4^+$ T cells via Signal Transducer and Activator of Transcription 1 (STAT1) in an autocrine and paracrine way, sustaining the immune response against alloantigens.[46,47] IFN-γ further exerts proinflammatory effects via upregulating IFNγ Induced Protein 10 (IP-10), which attracts Th1 cells, and thus initiates and amplifies the host alloresponse that may lead to acute rejection. Besides its proinflammatory function, however, previous studies have shown that IFN-γ blockade can inhibit neovascularization in the cornea.[48] IFNγ can suppress angiogenesis via several mechanisms: (i) It can suppress VEC proliferation and migration by activating the anti-angiogenic factor, IP-10, in VECs.[48–52] (i) IFNγ can indirectly suppress angiogenesis by suppressing the STAT3 pathway in T cells, which induces VEGF-A release by immune cells.[49,53–55] (iii) IFNγ can also downregulate the expression of metalloproteinases, which are required for the breakdown of the extracellular matrix to allow new capillaries to sprout, and thus may affect angiogenesis. (iv) It has been shown that IFNγ inhibits VEGF-A secretion[49] and endothelial cell proliferation in human cell lines.[59] Here, we demonstrate that IFNγ reduces VEC proliferation in a dose-dependent manner. High amounts of IFNγ reduce VEC proliferation by 30% compared to low doses of IFNγ (Figure 4C). Blocking IFNγ in the T cell/VEC coculture promotes VEC proliferation and enhances VEGF-A expression in vitro (Figure 4D and E). Thus, T cells and their expressed cytokines may be critical regulators of angiogenesis in transplantation. We speculate that there is a balance between T cell-secreted proangiogenic factors, principally VEGF-A, and anti-

angiogenic mediators, specifically IFNγ. Thus, T cells and their expressed cytokines may be critical regulators of angiogenesis in transplantation. Our data suggest that in the context of transplantation, performed in the inflamed microenvironment of the high-risk host bed, the net result of these 2 opposing T cell-derived mechanisms is proangiogenic (Figure 5). Thus, in this context the antiangiogenic and proinflammatory functions of IFN-γ are not mutually exclusive.

In conclusion, our study provides new insights into the role of adaptive immunity and transplantation-associated angiogenesis. We show for the first time that alloreactive host T cells promote angiogenesis after transplantation, largely through expression of VEGF-A. Accordingly, we propose that the angiogenic process following corneal transplantation develops in 2 phases: an early phase related to the postoperative healing process mediated by innate immune cells, as described elsewhere,[56–58] and a late phase mediated by alloreactive T cells responsible for graft rejection.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
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| **DLN** | Draining lymph node |
| **HR** | High-risk |
| **IFNγ** | Interferon gamma |
| **LR** | Low-risk |
| **VEC** | Vascular endothelial cells |
| **VEGF** | Vascular endothelial growth factor |

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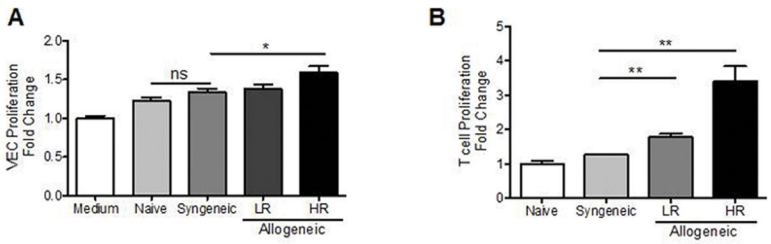


**Figure 1. Alloprimed T cells from transplanted mice induce vascular endothelial cell proliferation**

(A) Vascular endothelial cells (VECs) were cultured alone (medium), or with allogeneic or syngeneic T cells collected from naïve, low-risk (LR) or high-risk (HR) transplanted mice. After 24 hours the proliferation of VECs was assessed using the BrdU incorporation assay. (B) Purified, allogeneic or syngeneic T cells were cocultured with APCs for 72 hrs. BrdU reagent was added to each well 16 hrs before collecting the cells, and T cell proliferation was measured. Each experiment has been performed at least 2 times, with 4 mice per group per experiment.

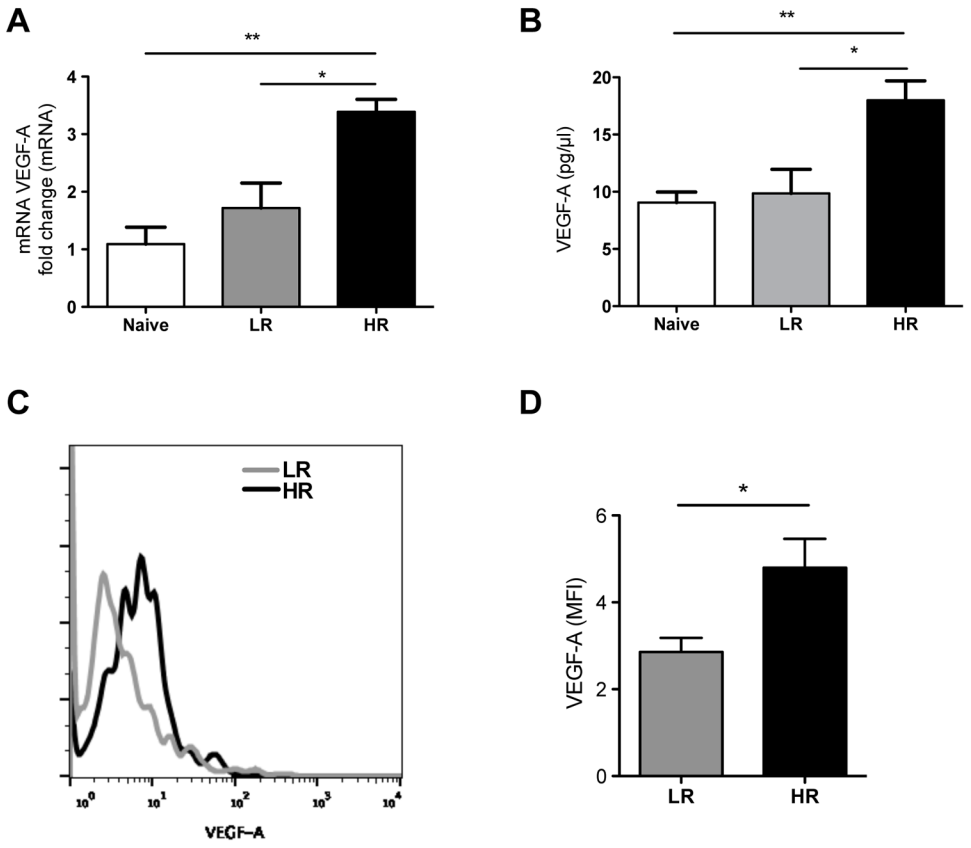


**Figure 2. Alloprimed T cells from high-risk mice release increased proangiogenic VEGF-A**

(A) T cells from naïve, low-risk (LR), high-risk (HR) transplanted mice were stimulated with an anti-CD3 antibody for 12 hours. VEGF-A mRNA expression of T cells was assessed using Real-Time PCR and (B) VEGF-A protein concentration was detected in the culture supernatant using ELISA. Each experiment has been performed at least 3 times, with 10 mice per group per experiment. (C and D) Protein expression (Median Fluorescence Intensity, MFI) of VEGF-A in corneal CD4 T cells isolated from HR and LR graft recipients 21 days posttransplantation was analyzed by flow cytometry (p=0.04; n=4/group).

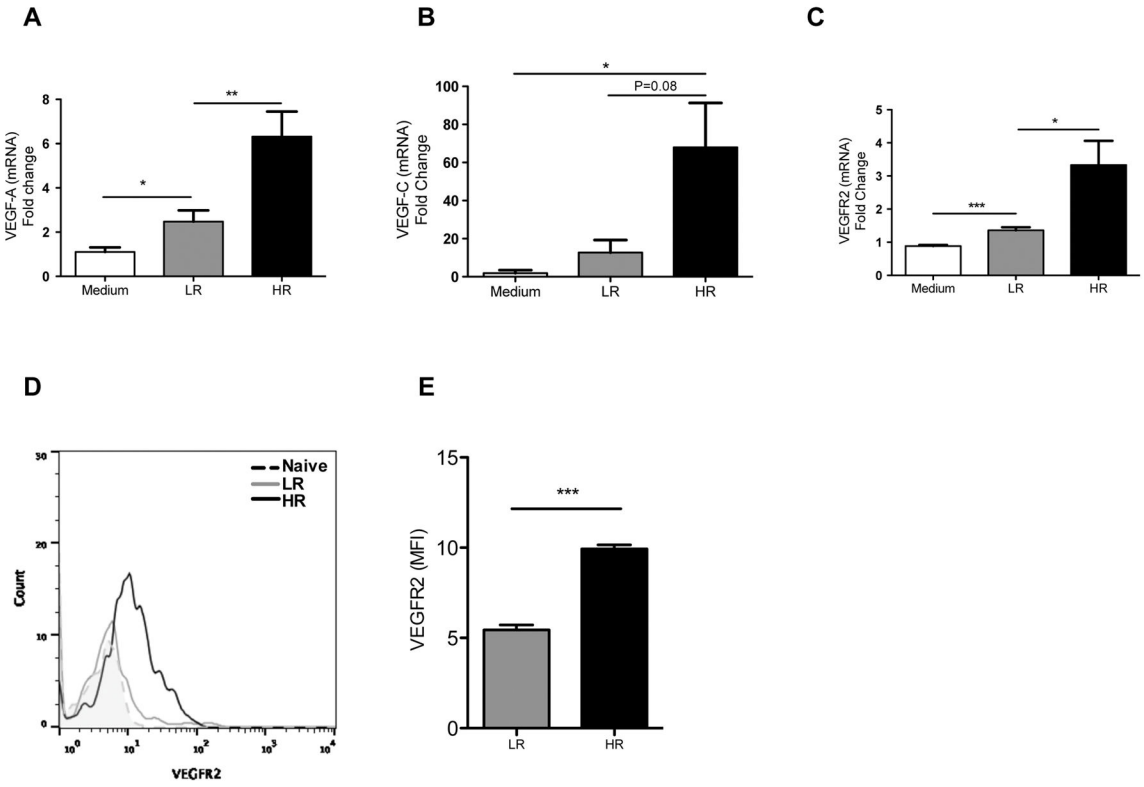


**Figure 3. VEGF-A, VEGF-C, and VEGF-R2 are increased in vascular endothelial cells**

Vascular endothelial cells (VECs) were cultured alone (medium), or with T cells collected from low-risk (LR) or high-risk (HR) transplanted mice. mRNA expression of (A) VEGF-A, (B) VEGF-C, and (C) VEGF-Receptor 2 (VEGF-R2) was determined in VECs 24 hours after coculture. Each Real-Time analysis has been performed at least 3 times, with 10 mice per group per experiment. (D and E) Flow cytometry analysis showing VEGF-R2 expression by CD31+ endothelial cells in the cornea of HR and LR transplanted mice 21 days postsurgery ($p<0.0001$; n=4/group).

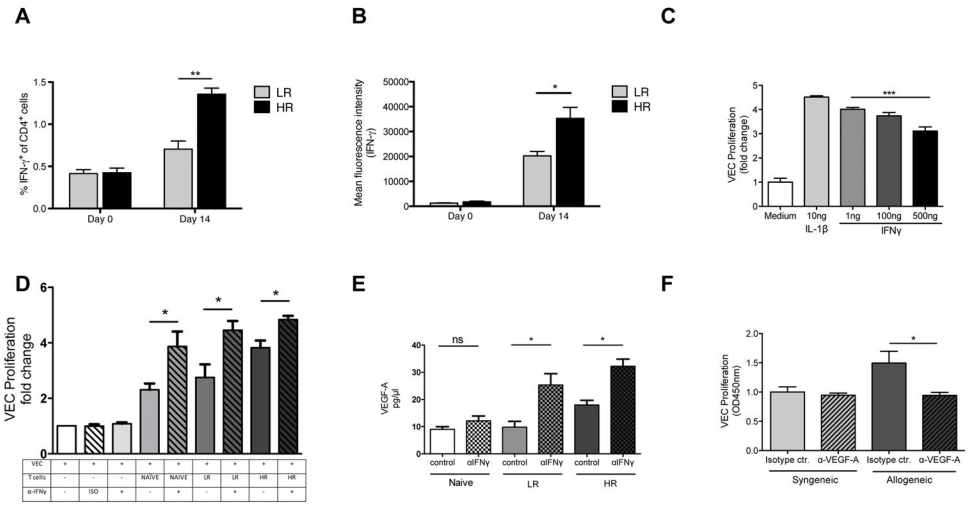


**Figure 4. Blocking IFNγ increases vascular endothelial cell proliferation**

(A and B) Before surgery (day 0) and 14 days after low-risk (LR) and high-risk (HR) corneal transplantation, draining lymph nodes were isolated and analyzed for (A) frequencies of IFNγ-producing T cells and (B) protein expression (Mean Fluorescence Intensity, MFI) of IFNγ in CD4 T cells. (C) Vascular endothelial cells (VECs) were cultured in DMEM and stimulated with VEGF-A (20ng/mL). Different concentrations of IFNγ (1, 100, 500 ng/mL) and IL-1β (10ng/mL) were added for 24 hours. VEC proliferation was assessed using the BrdU incorporation assay. (D) VECs were cultured in DMEM only, or with T cells from naïve, LR, or HR mice and treated with an anti-IFNγ or isotype control antibody as indicated. 24 hours later VEC proliferation was assessed using the BrdU incorporation assay. (E) VEGF-A protein expression was assessed in the supernatant after culture using ELISA. (F) Syngeneic and allogeneic T cells were cocultured with VECs and treated with anti-VEGF-A or isotype control for 24 hours. VEC proliferation was assessed using the BrdU incorporation assay. Each experiment has been performed at least 3 times, with 10 mice per group per experiment.

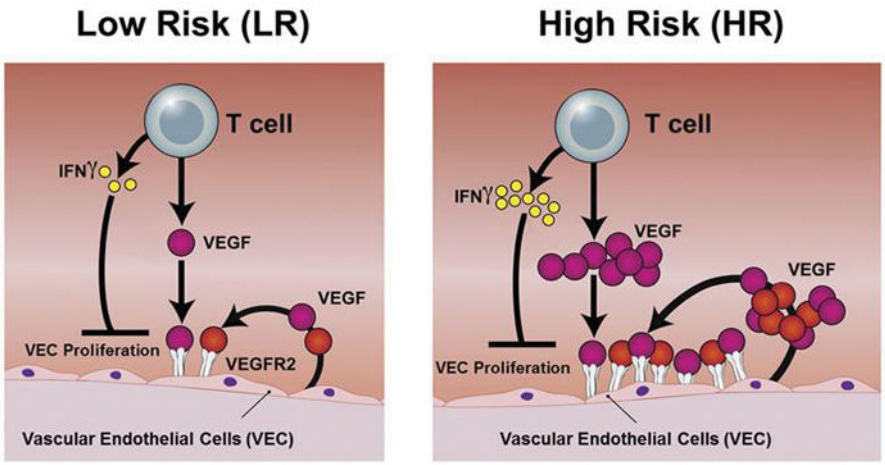


**Figure 5. Schematic diagram of T cell mediated angiogenesis**

In our vitro model, T cells promote angiogenesis in 3 ways: 1) Conventional T cells induce VEC proliferation by releasing the angioigenic factor VEGF-A. T cells collected from HR recipients (right) are more proangiogenic than T cells obtained from LR grafted mice (left); 2) VEC autocrine secretion of VEGF-A and VEGF-C promote VEC proliferation; and 3) VECs upregulate expression of VEGF-R2, which is the receptor for VEGF-A and VEGF-C. The release of these ligands and increase in expression of VEGF-R are more pronounced in the HR setting than LR setting. Our data also show an antiangiogenic effect of IFNγ; however, the balance between T cell-induced VEGF-mediated proangiogenic, and IFNγ anti-angiogenic, mechanisms appears to lie in favor of the proangiogenic effects.