DEPTOR Regulates Vascular Endothelial Cell Activation, Proinflammatory and Angiogenic Responses

Sarah Bruneau¹,², Hironao Nakayama³, Craig. B. Woda¹,², Evelyn A. Flynn¹,² and
David. M. Briscoe¹,²*

¹Transplant Research Program, Pediatric Transplant Center, Boston Children’s Hospital, Boston, MA and the Division of Nephrology, Department of Medicine, Boston Children’s Hospital, Boston MA, USA and the ²Department of Pediatrics, Harvard Medical School, Boston, MA, USA and the ³Vascular Biology Program, Department of Surgery, Boston Children’s Hospital, and Harvard Medical School, Boston, MA, USA.

*To whom correspondence should be addressed: David M. Briscoe, Boston Children’s Hospital, Transplant Research Program, Division of Nephrology, 300 Longwood Ave, Boston, MA 02115. Phone: (617) 355-6129 ; Fax: (617) 730-0130 ; E-mail: david.briscoe@childrens.harvard.edu

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Running Head: Function of DEPTOR in Endothelial Cell Responses
Key Points:

1. DEPTOR is expressed in vascular endothelial cells and serves as an endogenous inhibitor of mTORC1, ERK1/2 and STAT1 activity.

2. DEPTOR is potent to regulate endothelial cell expression of chemokines and adhesion molecules, leukocyte-endothelial adhesion and endothelial migratory responses.
Abstract:

The maintenance of normal tissue homeostasis and the prevention of chronic inflammatory disease is dependent on the active process of inflammation resolution. In endothelial cells (EC), pro-inflammation results from the activation of intracellular signaling responses and/or the inhibition of endogenous regulatory/pro-resolution signaling networks that, to date, are poorly defined. In this study, we find that DEPTOR is expressed in different microvascular EC \textit{in vitro} and \textit{in vivo} and, using a siRNA knockdown approach, we find that it is potent to regulate mTORC1, ERK1/2 and STAT1 activation in part through independent mechanisms. Moreover, using limited gene arrays, we observed that DEPTOR regulates EC activation including the mRNA expression of the T cell chemoattractant chemokines CXCL9, CXCL10, CXCL11, CX3CL1, CCL5 and CCL20 and the adhesion molecules ICAM-1 and VCAM-1 \((P<0.05)\). DEPTOR siRNA-transfected EC also bound increased numbers of PBMC \((P<0.005)\) and CD3\(^+\) T cells \((P<0.005)\) in adhesion assays \textit{in vitro}, and had increased migration and angiogenic responses in the spheroid sprouting \((P<0.01)\) and wound healing \((P<0.01)\) assays. Collectively, these findings define DEPTOR as a critical upstream regulator of EC activation responses, and suggest that it plays an important role in endogenous mechanisms of anti-inflammation and pro-resolution.
Introduction

The initiation of acute and chronic inflammation is characteristically associated with the activation of microvascular endothelial cells (EC) responding to local pro-inflammatory cytokines released from infiltrating leukocytes and/or resident macrophages\(^1,2\). The induced expression of adhesion molecules and chemokines by EC initiates the recruitment of leukocytes into inflamed tissues, and the expression of cell surface molecules such as major histocompatibility complex (MHC) molecules and costimulatory molecules by EC serves to facilitate local lymphocyte activation responses in the course of a cell-mediated immune reaction\(^1,3\). In addition, cell-mediated immune and delayed-type hypersensitivity reactions are associated with leukocyte-induced angiogenesis that fosters the development and progression of the chronic inflammatory microenvironment\(^1-3\).

Nevertheless, recent studies have also highlighted a paradigm where endogenous mechanisms of inflammation resolution function to maintain normal tissue homeostasis\(^4,5\). Increasing data indicate that these mechanisms are critical for the prevention of chronic inflammatory diseases, in as much as the efficiency and/or the speed of pro-resolution determines the outcome of an inflammatory reaction\(^4\). Pro-resolution is an active process involving the secretion of specific mediators, but is also dependent on the level of expression and/or the activity of intracellular regulatory adaptor molecules, kinases and/or select inhibitory proteins\(^6-8\).

mTOR-mediated signaling responses have been reported to function in EC activation, including the induced expression of adhesion molecules and chemokines\(^9,10\). In addition, mTOR activity is potent to facilitate EC proliferation and migration \textit{in vitro}\(^11\), is functional in tumor
angiogenesis, wound healing angiogenesis and in leukocyte-induced angiogenesis that is characteristic of chronic inflammation *in vivo*\(^{12-14}\). mTOR is an evolutionarily conserved serine/threonine kinase\(^ {15}\) that forms two distinct multiprotein complexes, composed of either mTOR, raptor and mLST8 (called mTORC1\(^ {16}\)), or mTOR, rictor, Sin1, protor and mLST8 (called mTORC2\(^ {17}\)). mTORC1 controls cell growth in part by phosphorylating S6K1 and 4E-BP1\(^ {15,18}\), key regulators of protein synthesis. mTORC2 modulates cell survival and activation in response to growth factors by phosphorylating the Akt kinase\(^ {19}\) or via activation of the protein kinase C (PKC) pathway\(^ {20}\). Signaling in response to mTOR activation is tightly regulated by several negative feedback loops as well as by distinct proteins that serve to inhibit its activity. One such regulatory protein is DEPTOR\(^ {21,22}\) that was recently reported to associate with both mTORC1 and mTORC2 and inhibit their activity in cancer cells\(^ {21,23,24}\), in myocytes\(^ {25}\) and in adipose tissue\(^ {26}\). Nevertheless, despite its potential importance as an upstream modulator/regulator of mTOR signaling, little is known about DEPTOR expression and function in normal cell types, and its effect(s) in EC-dependent mechanisms of inflammation is not known.

In these studies, we demonstrate that DEPTOR is expressed in normal human EC where it functions as a cell-intrinsic regulator of mTORC1, ERK1/2 and STAT1 signaling, and the inducible expression of adhesion molecules and chemokines. In addition, we show that it is potent to regulate leukocyte-EC adhesion as well as EC migration/angiogenesis. Our data define a paradigm where DEPTOR functions as a critical upstream regulator of EC activation responses and suggest that it plays an important role in endogenous mechanisms of inflammation resolution.
Materials and Methods

Antibodies and Reagents: See Supplementary Materials and Methods.

Cell culture: Single donor HUVEC were purchased from Clonetics (Lonza, Allendale, NJ) and cultured in complete endothelial growth medium (EGM-2, Lonza), subcultured and used between passages 3 and 6. For some experiments, we also used primary cultures of EC isolated from umbilical cords and cultured as described27. Human coronary artery EC (HCAEC), human lung and dermal microvascular EC (HMVEC) and renal tubular epithelial cells (RPTEC) were purchased from Lonza and cultured in the recommended growth medium. 786-0 cells were gifted to the laboratory (from Debabrata Mukhopadhyay, Mayo Clinic, Rochester, MN) and HEK293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Western blot analyses: Cells were lysed with RIPA buffer (Boston Bioproducts, Boston, MA) containing protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL). Proteins were separated on a SDS-polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked for 1 hour with TBS-0.1% Tween-20 containing 5% BSA, and incubated with the primary antibody overnight at 4°C. Membranes were then washed and incubated with a species-specific secondary peroxidase-linked antibody for 1 hr at room temperature, and the protein of interest was detected by chemiluminescence (Thermo Scientific, Pierce, Rockford, IL).
**Immunofluorescence microscopy:** Human neonatal foreskins and discarded cardiac tissue from patients undergoing surgery were collected as approved by the Institutional Review Board (IRB) at the Brigham and Women’s Hospital and Boston Children’s Hospital. Informed consent was obtained in accordance with the Declaration of Helsinki. A description of the immunofluorescence technique used in these studies is provided in supplementary Material Methods, as previously reported²⁸.

**Immunoprecipitation assays:** HUVEC were lysed in CHAPS-containing lysis buffer lacking NaCl and supplemented with protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL). Immunoprecipitations were performed using 500 μg of total protein and excess antibody with the Dynabeads® Protein G immunoprecipitation kit, according to the manufacturer’s instructions (Life Technologies, Invitrogen, Grand Island, NY).

**siRNA knockdown:** Small interfering RNAs (siRNAs) for DEPTOR were purchased from Qiagen (Valencia, CA) and a negative control siRNA was purchased from Invitrogen. Transfection of HUVEC with siRNA (up to 50 nM) was performed using RNAimax lipofectamine (Life Technologies, Invitrogen, Grand Island, NY), according to the manufacturer’s instructions. Following siRNA transfection, the efficiency of knockdown was assessed regularly by quantitative RT-PCR and Western blot analysis vs. controls; our siRNAs were consistently found to be approximately 90% efficient. (Supplementary Figure 1). All experiments were performed with at least 2 different siRNAs, as indicated.
**Protein arrays:** Protein arrays were performed using the Human Phospho-Kinase Array Kit (Proteome Profiler™ Array) purchased from R&D Systems (Minneapolis, MN), according to the manufacturer’s instructions.

**RNA isolation and PCR arrays:** Total mRNA was isolated from HUVEC 48hrs following control or DEPTOR siRNA transfection, using the RNeasy isolation kit (Qiagen, Valencia, CA). cDNA was generated using the qScript Supermix from Quanta Biosciences (Gaitherburg, MD), according to the manufacturer’s instructions and two commercial PCR-arrays (‘endothelial cell biology’ and ‘chemokines and cytokines’, SABiosciences/Qiagen) were used to examine endothelial activation responses. Gene expression levels were evaluated using data analyzer template provided by the manufacturer and GAPDH, β-actin, HPRT1 and ribosomal protein L13α were used as references.

**Real-time PCR:** Quantitative real-time PCR analyses were performed using the 7300 real-time PCR System and specific TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). Relative expression of each gene vs. the GAPDH control was calculated according to the 2^(-ΔΔCt) method, as previously described29.

**ELISA:** The human CXCL10/IP-10 DuoSet® ELISA kit (R&D Systems, Minneapolis, MN) was used for ELISA assays in culture supernatants, according to the manufacturer’s instructions.

**Adhesion assays:** Human peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers, consented in accordance with IRB approval at Boston Children’s Hospital.
PBMC (2.5\times10^6/well) were co-cultured with untreated or siRNA-transfected HUVEC in 6-well plates at 37°C for 60 min. Non-adherent PBMC were removed by three PBS washes and adherent cells were evaluated by microscopy or were harvested using trypsin and processed for FACS analysis (FACsCalibur, BD Biosciences, San Jose, CA) using anti-CD3, anti-CD45 and anti-CD31 fluorescent antibodies (BD Biosciences). 10,000 cells per group were analyzed using FlowJo Software (TreeStar, Ashland, OR) and the adhesion index was calculated as the ratio of the percentage of PBMC or CD3⁺ T cells within each experimental group compared to the untreated group.

For quantitative assays, CD3⁺ T cells were purified from PBMC using the human Pan T cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. Either PBMC or CD3⁺ T cells were labeled with CFSE (5 μM, Life Technologies) and were co-cultured (5 \times 10^5 per well) with confluent monolayers of untreated or siRNA-transfected HUVEC in 24-well cell culture plates at 37°C for 60 min. Non-adherent cells were removed by three PBS washes and adhesion was assessed by the measurement of fluorescence in each well using an automated plate reader (Victor, Perkin Elmer, Waltham, MA). The number of adherent cells in each well was calculated from a standard fluorescence intensity curve generated with increasing numbers of CFSE labeled PBMC or CD3⁺ T cells in control wells (Supplementary Figure 2).

For each assay, experiments were performed with two siRNAs to DEPTOR, and results were pooled for the final quantification. In addition, the adhesion of PBMC or CD3⁺ T cells to TNFα-treated HUVEC (100U/ml for 6 hrs) was evaluated in each experiment as a positive control.
Spheroid sprouting assays: HUVEC were transfected with control or DEPTOR siRNAs and cultured for 24 hours. Cells were then allowed to aggregate in hanging drops (500 cells per drop/spheroid), and after 24 hours, spheroids were embedded into a collagen type I matrix, as described30. After another 24 hours, the spheroids were fixed for 15 min with 4% paraformaldehyde, and were subsequently permeabilized for 5 min with 0.2% Triton X-100, and the F-actin cytoskeleton was stained with Alexa Fluor® 488-conjugated phalloidin (Life Technologies, Invitrogen, Grand Island, NY). Samples were examined using a Perkin Elmer UltraVIEW Vox confocal microscope (Santa Clara, CA) using a Plan Apo 20x (0.75 NA) objective. Images were acquired using the Volocity® Software (Improvision, Perkin Elmer), and the length of EC sprouts from 5 spheroids per condition was measured in 3 independent experiments, using ImageJ software.

In vitro migration assays: HUVEC were transfected with control or DEPTOR siRNAs, and after 48 hours, a linear wound was created in confluent cell monolayers by scratching with a pipette tip. After an additional 18 hours, cell migration into the wound was assessed by microscopy (10x objective) using a digital inverted microscope (AMG Evos XL Core, Fisher Scientific, Pittsburgh, PA). The degree of wound closure was measured as the percentage of the area covered by migrating cells in the initial wound, in nine wounds per test condition, using the ImageJ software. Migration assays were performed using two siRNAs to DEPTOR, and results obtained with each siRNA were pooled for quantification and statistical analyses.

Statistical analyses: Statistical analyses were performed using the Student t test and P values <0.05 were considered statistically significant.
Results

Expression of DEPTOR in vascular endothelial cells: We initially evaluated the expression of DEPTOR at the mRNA and protein levels in human umbilical vein EC (HUVEC), human coronary artery EC, human dermal and lung microvascular EC. As illustrated in Figure 1A, using quantitative real-time PCR and Western blot analysis, we found similar levels of expression of DEPTOR in each type of EC. Although DEPTOR mRNA expression was slightly higher in dermal HMVEC, as compared to primary cultures of HUVEC, this difference was not statistically significant and was not translated into increased DEPTOR protein expression by Western blot analysis (Figure 1A, lower panels). However, DEPTOR expression in HUVEC was low compared to other cell lines previously shown to express high levels of DEPTOR (Figure 1B). Furthermore, DEPTOR mRNA expression in the different EC was quite variable, suggesting that DEPTOR expression may be regulated in these cells. Indeed, previous studies have shown that DEPTOR expression is rapidly downregulated at the transcriptional and post-translational levels by mTOR itself, in response to growth signals. Consistent with this possibility, we found that stimulation of EC with TNFα (10-100 U/ml) results in a rapid decrease in DEPTOR mRNA expression (Figure 1C).

We also evaluated the pattern of expression of DEPTOR by immunofluorescence in situ in human heart (Figure 1D, upper panels) and in human skin (Figure 1D, lower panels) and found a prominent expression pattern within vascular EC. However, expression was focal and levels of expression varied among EC, again suggesting that DEPTOR is regulated in vivo.
Function of DEPTOR in the regulation of mTOR signaling in endothelial cells: DEPTOR has consistently been found to associate with both mTORC1 and mTORC2 and to inhibit their activity in different types of cancer cells\(^{21,34}\). We initially evaluated whether it also interacts with mTOR complexes in EC (Figure 2). Using immunoprecipitation assays, we observed that DEPTOR forms a complex with mTOR and raptor (mTORC1) in EC, but minimally associates with rictor (mTORC2, Figure 2A). We transfected HUVEC with increasing concentrations of two siRNAs to DEPTOR, or a control siRNA, and after 48hrs we analyzed levels of pS6K1 (T389) and pAkt (S473) by Western blot. As anticipated\(^{21,25,34}\), we found that knockdown of DEPTOR in EC led to a marked increase in the phosphorylation of S6K1 (Figure 2B), but surprisingly knockdown had little effect on the level of expression of pAkt (S473) (Figure 2B and Supplementary Figure 3A). Similar results were obtained following DEPTOR siRNA transfection of coronary artery, dermal and lung EC (Supplementary Figure 3B).

We postulated that increased mTORC1 activity and augmented phosphorylation of S6K1 in DEPTOR siRNA-transfected EC may result in an inhibition of mTORC2 activity via well-established negative feedback loops\(^{35,36}\) and may be responsible for the apparent absence of function of DEPTOR on the regulation of mTORC2 activity. Therefore, to further test if DEPTOR directly regulates mTORC2, we transfected HUVEC with DEPTOR siRNA, and we treated the cells with rapamycin (10 ng/ml for 1 hour) to target mTORC1. While rapamycin inhibited the phosphorylation of S6K1, DEPTOR knockdown again failed to increase the phosphorylation of Akt (S473) (Figure 2C). We also analyzed the levels of pSGK (S422), another target of mTORC2, in HUVEC transfected with control or two DEPTOR siRNAs. Similarly, as illustrated in Figure 2D, knockdown of DEPTOR in HUVEC had no effect on the
level of phosphorylation/activation of SGK. Thus, DEPTOR both associates with and regulates mTORC1, but it has no effect on mTORC2 activity in EC.

Function of DEPTOR in the regulation of ERK1/2 and STAT1 activity in EC: To study whether DEPTOR regulates additional signaling pathways in EC, we next performed a protein kinase array and thus compared the relative levels of phosphorylation of multiple kinases and/or their protein substrates in control siRNA- and DEPTOR siRNA-transfected HUVEC. As illustrated in Figure 3A and Supplementary Table 1, the array confirmed a regulatory effect of DEPTOR on mTORC1 activity, but there were notable additional effects on the phosphorylation/activation of ERK1/2 and STAT1. Other phosphokinases and adaptor molecules were also increased in DEPTOR siRNA-transfected EC (vs. controls), including p38α, Paxillin, PLCγ-1 and FAK, but each to a lesser extent than ERK1/2. The marked regulatory effect of DEPTOR on pERK1/2 (T202/Y204) and pSTAT1 (Y701) expression was confirmed by Western blot analysis (Figure 3B).

To determine whether DEPTOR regulates these signaling pathways through interrelated mechanisms, we transfected HUVEC with control or DEPTOR siRNAs, and treated the cells with U0126 (10 μM for 18 hrs) and/or with rapamycin (10 ng/ml for 18 hrs) or Torin1 (1 μM for 18 hrs) to inhibit ERK1/2 or mTOR activity respectively. As illustrated in Figure 3C-D, we found that DEPTOR knockdown resulted in a marked increase in the phosphorylation of S6K1, in the absence or presence of pharmacological ERK1/2 inhibition. Also, DEPTOR knockdown resulted in an increase in pERK1/2 levels in the absence or presence of rapamycin (Figure 3C), even though mTORC1-S6K1 activation was inhibited. Treatment of DEPTOR siRNA-transfected cells with Torin1, an established ATP-competitive inhibitor of both mTORC1 and
mTORC2 further confirmed that DEPTOR regulates ERK1/2 in the absence of mTOR signaling.

We also observed an increase in the levels of total STAT1, pSTAT1 (Y701) and pSTAT1 (S727) in DEPTOR siRNA-treated cells vs. controls. In addition, we found that overexpression of pSTAT1 (Y701) was unchanged in siRNA-transfected EC that were treated with rapamycin (Figure 3C) or Torin 1 (Figure 3D), and levels of expression were minimally reduced in EC treated with U0126 (Figure 3C-D). However, when DEPTOR siRNA-transfected EC were treated with both Torin1 and U0126 in combination, the increase in pSTAT1 (Y701) was almost completely inhibited, and levels of pSTAT1 (S727) as well as total STAT1 were markedly reduced. We interpret these observations to suggest that DEPTOR interacts with the mTOR and the ERK1/2 signaling pathways through independent mechanisms and that it regulates STAT1 signaling in part via crosstalk among intermediaries within both the ERK1/2 and the mTOR pathways.

Effects of DEPTOR on endothelial cell activation and pro-inflammation: We next evaluated the effect of DEPTOR on the regulation of functional EC activation responses using two commercial PCR-based arrays. Of a total of 155 genes examined, 47 genes were strongly modulated in DEPTOR siRNA-transfected vs. control siRNA-transfected HUVEC. Of these, a total of 27 genes were strongly induced (up to ~360 fold) and 20 genes were moderately downregulated following DEPTOR siRNA transfection (Supplementary Table 2). There was a most notable effect of DEPTOR knockdown on the induction of T cell chemoattractant chemokines, including CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC), CCL5 (RANTES), CX3CL1 (Fractalkine) and CCL20 (MIP3A) in the array (Supplementary Table 2) as well as in subsequent real-time
qPCR analyses (Figure 4A-B). The mRNA expression of the adhesion molecules ICAM-1 and VCAM-1 was also significantly upregulated in DEPTOR siRNA-transfected EC vs. controls (Figure 4A-B). By ELISA (Figure 4C), we also found that CXCL10 was dramatically increased in the supernatant of DEPTOR siRNA-transfected cells (mean=1415 +/- 562 pg/ml) vs. control siRNA-transfected HUVEC (mean=94 +/- 13 pg/ml, P<0.05), and was in the same range as that observed in HUVEC treated with IFNγ for 24 hours as a positive control (mean=1037 +/- 50 pg/ml). These potent effects of DEPTOR on activation responses were similar in EC derived from different vascular beds (skin, lung and coronary artery, data not shown). To test whether DEPTOR regulates EC activation through mTORC1- and/or ERK1/2-dependent signals, we also evaluated EC activation responses in DEPTOR siRNA-transfected HUVEC pre-treated for 24hrs with rapamycin (10 ng/ml) and/or U0126 (10μM). As illustrated in Supplementary Figure 4, we found that each inhibitor had different effects on the overexpression of individual chemokines and adhesion molecules, suggesting that the DEPTOR-mediated regulation of EC activation likely results from the simultaneous modulation of multiple signaling pathways.

We next questioned whether these effects of cell intrinsic DEPTOR on EC activation responses are of functional importance in pro-inflammation. We co-cultured freshly isolated human PBMC with confluent cultures of control siRNA- or DEPTOR siRNA-transfected HUVEC for 10 to 60 min at 37°C. Co-cultures of PBMC with TNFα-activated EC served as a positive control. At each time point, non-adherent PBMC were removed by washing and adherent leukocytes were assessed by microscopy. Cultures of HUVEC and adherent leukocytes were also collected and analyzed by FACS. As illustrated in Figure 5A-B, we found higher numbers of adherent PBMC in co-cultures with DEPTOR siRNA-transfected EC vs. control siRNA-transfected EC or untreated EC at all time points examined, but the effect of DEPTOR
knockdown on leukocyte adhesion was most prominent after 30-60 min co-culture \( (P<0.005) \). We also evaluated the number of \( CD3^+ \) T cells within each population of adherent PBMC, and found a significant effect \( (P<0.005) \) of DEPTOR knockdown on \( CD3^+ \) T cells-EC interactions \((Figure 5C)\).

To quantify leukocyte-EC adhesion, HUVEC were transfected with control or DEPTOR siRNAs, and co-cultured with freshly isolated and CFSE labeled human PBMC or \( CD3^+ \) T cells for 60 min. After washing, the numbers of adherent PBMC or \( CD3^+ \) T cells in each experimental condition was evaluated by measuring fluorescent intensity; quantification of cell number was determined by comparison to a standard curve \((Supplementary Figure 2)\). As illustrated in \( Figure 5D-E \), we found that DEPTOR knockdown in HUVEC resulted in a marked increase in the number of adherent PBMCs \((\sim 2 \text{ fold}, P<0.01, Figure 5D)\) and \( CD3^+ \)T cells \((\sim 2.5 \text{ fold } P<0.05, Figure 5E)\) as compared to controls. In addition, we found that the pretreatment of DEPTOR siRNA-transfected HUVEC with rapamycin (10 ng/ml, to target mTORC1) and/or U0126 (10 \( \mu \)M, to target ERK1/2) for 24hrs prior to the assay did not inhibit the adhesion of PBMC to EC \((Figure 5D)\). However, treatment of DEPTOR siRNA-transfected EC with both inhibitors, alone or in combination, decreased the binding of \( CD3^+ \) T cells to the EC monolayer, although the effect was not statistically significant \((Figure 5E)\). Of note, pre-treatment of control siRNA-transfected EC with rapamycin and/or U0126 did not decrease, and tended to increase adhesion of PBMC and \( CD3^+ \) T cells \((data not shown)\). Collectively, these findings suggest that the endogenous expression of DEPTOR in EC is of functional importance in leukocyte-EC adhesion and pro-inflammation.
Functional effects of DEPTOR on angiogenic responses: We also used two well-established in vitro models to evaluate the function of DEPTOR in angiogenic responses. In the tridimensional spheroid EC sprouting assay, DEPTOR siRNA-transfected HUVEC were seeded to form spheroids and embedded in a collagen type I matrix, as described\textsuperscript{30}. After 24hrs, total sprout length was quantified in > 5 spheroids per condition. Consistently, we found a significant increase ($P<0.01$) in total sprout length in DEPTOR siRNA-transfected EC vs. controls, as visualized and quantified by direct phase microscopy (Figure 6A, upper panels) and following staining with phalloidin (Figure 6A, lower panels). In the wound healing assay, DEPTOR siRNA- or control siRNA-transfected EC were grown to confluence and a linear wound was subsequently created in the monolayer. The migration of EC into the wound was quantified over a 12-24hrs period, as described\textsuperscript{7}. Consistent with our findings in the spheroid assay, we found that DEPTOR siRNA-transfected EC migrated into the wound at an increased rate ($P<0.01$) vs. controls (Figure 6B). In addition, to evaluate whether DEPTOR regulates EC migration through mTORC1- and/or ERK1/2-dependent signals, we treated siRNA-transfected EC with rapamycin or U0126 respectively, and we evaluated migration in the wound-healing assay. As illustrated in Figure 6B, each inhibitor alone partially attenuated the increase in migration induced by DEPTOR knockdown ($P=\text{NS}$), but treatment with both rapamycin and U0126 in combination significantly reduced the EC migration response ($P<0.05$, Figure 6B). This suggests that DEPTOR controls EC migration responses by regulating both mTORC1 and ERK1/2 activity. In contrast, EC proliferative responses (as assessed by $[^{3}\text{H}]$ Thymidine incorporation after 72hrs) and rates of apoptosis (as assessed by annexin V and propidium iodide staining) were similar in DEPTOR siRNA-transfected EC vs. controls (Supplementary Figure 5).
Discussion

Microvascular EC participate in all aspects of acute and chronic inflammation, from their initial encounter with leukocytes in the course of recruitment and transmigration, to the immune angiogenesis reaction that is characteristic of delayed-type hypersensitivity^{1-3,38}. Activation responses and pro-inflammatory intracellular signals in EC are initiated and coordinated by cytokines^{38}, growth factors^{2,28} as well as cell-cell surface receptor-mediated interactions^{38-40}. In contrast, EC-dependent signals mediating inflammation resolution are poorly defined and less well understood. In these studies, we find that a reduction in the level of expression of cell intrinsic DEPTOR results in striking effects on mTORC1-dependent signaling, ERK1/2 and STAT1 activity in EC. DEPTOR knockdown also results in EC activation, including the induced expression of several T cell chemoattractant chemokines and adhesion molecules. Also, DEPTOR-regulated EC activation is associated with leukocyte-endothelial cell adhesion and a marked increase in EC migratory responses in vitro. Our studies are suggestive that DEPTOR has potent regulatory effects within EC and that the level of DEPTOR expression is likely of importance for the maintenance of a quiescent EC phenotype.

Similar to other cell types^{21,23-25}, we find that DEPTOR expression in EC is potent to regulate mTORC1-dependent activation responses, but in these studies we also identify an important regulatory role for DEPTOR in ERK1/2 activity. Although there is a well-established intracellular crosstalk among the mTOR signaling pathway (and intermediaries) and ERK1/2 signaling^{41-43}, we find that the treatment of EC with pharmacological inhibitors of mTOR activity (either rapamycin or Torin1) fails to alter the effect of DEPTOR knockdown on the augmentation of ERK1/2 activity. Furthermore, we find that the treatment of EC with a
pharmacological inhibitor of MEK-ERK1/2 activity (U0126) fails to alter the effect of DEPTOR knockdown on mTORC1-induced signals. Thus, our findings indicate that DEPTOR is potent to selectively regulate the endogenous activity of ERK1/2 as well as mTORC1 via independent mechanisms. While it is known that DEPTOR binds to mTOR\textsuperscript{21}, this observation is consistent with a report suggesting that DEPTOR contains a putative ERK-binding site\textsuperscript{31} and that it associates with ERK1/2 in cells that have been cotransfected with a Flag-tagged DEPTOR construct and HA-tagged ERK1/2\textsuperscript{32}. However, we did not find any association between endogenous DEPTOR and ERK1/2 in our EC (data not shown). Thus, we suggest that DEPTOR may interact with another intermediary to regulate endogenous ERK1/2 signaling.

In these studies, we also identify a critical role for DEPTOR in the regulation of STAT1 expression and activity through a mechanism that is dependent in part on both mTOR and ERK1/2. Indeed, pharmacological inhibition of either pathway alone in DEPTOR siRNA-transfected EC failed to suppress phosphorylation of STAT1, but combined pharmacological inhibition (using U0126 and Torin1) was associated with a significant reduction in pSTAT1 levels. Interestingly, in contrast to Torin1, rapamycin had no effect on the phosphorylation of STAT1, suggesting that the ability of mTOR to regulate STAT1 activity is rapamycin-resistant. In addition, we found that DEPTOR regulates levels of both pSTAT1(Tyr701) and pSTAT1(Ser727). Since pSTAT1(Tyr701) is JAK dependent\textsuperscript{44}, and since pSTAT1(Ser727) is in part ERK1/2-dependent\textsuperscript{45}, this observation is consistent with the possibility that DEPTOR also has a direct effect(s) on STAT1 itself. Indeed, in general, DEPTOR knockdown resulted in an increase in total STAT1 levels within EC, indicating that it may also function via a direct STAT1-dependent autoregulation loop.
In our studies, knockdown of DEPTOR in EC resulted in the overexpression of several pro-inflammatory molecules that are both STAT1-dependent (e.g., IP-10/CXCL10) and STAT1-independent (e.g., VCAM-1) and there was an associated increase in the adhesion of leukocytes to DEPTOR siRNA-transfected EC. Also, we found that the treatment of DEPTOR siRNA-transfected EC with rapamycin (to inhibit mTORC1) or U0126 (to inhibit ERK1/2), alone or in combination, had variable effects on the overexpression of individual chemokines and adhesion molecules and leukocyte-EC adhesion. These findings suggest that the effects of DEPTOR on the regulation of EC activation responses is likely related to its broad functions as a cell intrinsic inhibitor of several intracellular signaling pathways. In addition, knockdown of DEPTOR resulted in enhanced EC migration and angiogenesis responses that are well established to be associated with mTOR and ERK1/2 signaling; consistently, we found that the treatment of DEPTOR siRNA-transfected EC with the combination of rapamycin and U0126 dramatically decreased the induced EC migratory response. Some DEPTOR-regulated chemokines (e.g. IP-10/CXCL-10) have well-established anti-angiogenic effects, further suggesting that these pro-migratory effects of DEPTOR knockdown are associated with cell intrinsic signaling. Collectively, these data are suggestive that the potent regulatory effects of DEPTOR on mTORC1 and ERK1/2 in EC translate into its ability to sustain EC quiescence, anti-inflammation and/or inflammation resolution mechanisms.

DEPTOR expression has been shown to be downregulated by mTOR-mediated signals at the transcriptional and post-translational levels. mTOR signals result in the phosphorylation of DEPTOR, which leads in turn to its binding to the F box protein βTrCP and its subsequent ubiquitination and degradation. We have found that cytokine-mediated stimulation of EC (with TNFα) results in a significant decrease in DEPTOR mRNA expression after 6 hours. Thus,
following cytokine-mediated activation of EC in association with inflammation, amplification loops may sustain a reduced expression of DEPTOR, such that regulatory responses are inhibited. Therefore, we speculate that agents known to sustain DEPTOR activity\textsuperscript{46}, and/or agents that target physiological DEPTOR degradation\textsuperscript{47-50} will have anti-inflammatory and anti-angiogenic properties.

Collectively, these studies define DEPTOR as a key endogenous regulator of EC activation and EC-dependent pro-inflammatory responses. Our findings are consistent with a new paradigm whereby the maintenance of anti-inflammation and/or inflammation resolution involves the expression and activity of endogenous regulators of EC activation. The development of therapeutics that target DEPTOR degradation will have implications for the prevention and treatment of chronic inflammation and angiogenesis-dependent diseases.
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Authorship contributions

S.B. participated in research design, performed the studies, analyzed and interpreted the data and wrote the manuscript; H.N., C.B.W. and E.A.F assisted with experiments; D.M.B. conceived and designed the study, analyzed and interpreted the data and edited the manuscript.

Conflict of Interest

This work was funded in part through an Advancing Research in Transplantation Science Grant (ARTS) from Pfizer (to DMB).
References:


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Figure Legends

Figure 1. DEPTOR expression in human vascular endothelial cells. Panel A, DEPTOR expression was analyzed at the mRNA level by qPCR and at the protein level by Western blot analysis in primary cultures of human umbilical vein endothelial cells (HUVEC), and commercially available HUVEC, human coronary artery endothelial cells (HCAEC), as well as in dermal and lung human microvascular endothelial cells (HMVEC). Illustrated is the mean fold change in mRNA expression ± 1SEM from 3 independent experiments. Panel B, qPCR and Western blot analysis of DEPTOR expression in HUVEC as compared to renal proximal tubule epithelial cells (RPTEC), renal cancer cells 786-0, and human embryonic kidney HEK293 cells. Representative of 3 experiments. Panel C, HUVEC were treated with TNFa (10U/ml or 100U/ml) for 6hrs, and DEPTOR mRNA expression was subsequently analyzed by qPCR. The bar graph represents the mean fold change in DEPTOR mRNA expression (± 1SEM) in five independent experiments (***P<0.001). Panel D, Human cardiac tissue (atrium) and skin were evaluated by double immunofluorescence for the expression of DEPTOR or endothelial-specific CD31, as indicated. Illustrated are representative confocal images where DEPTOR (red) was found to co-localize with endothelial cells (green) in each tissue. The yellow staining in the merged image is representative of co-localization.

Figure 2. DEPTOR associates with mTORC1 and inhibits its activity in endothelial cells, but it minimally interacts with mTORC2. Panel A, DEPTOR, mTOR, Raptor or Rictor were immunoprecipitated (IP) from HUVEC and HEK293 cells, and Western blot (WB) analysis was subsequently performed with anti-DEPTOR, -mTOR, -raptor and -rictor. As illustrated,
DEPTOR associates with mTOR and raptor, but minimally associates with rictor in HUVEC. Panel B, HUVEC were transfected with a control or two DEPTOR siRNAs (#1 and #2) and the expression of pS6K1 (T389), S6K1, pAkt (S473), and Akt was evaluated by Western blot analysis. Panel C, control or DEPTOR siRNA (50 nM)-transfected EC were cultured for 48 hours and treated with rapamycin (10 ng/ml) for the last 1 hour of culture. Cell lysates were analyzed by Western blot for the expression of pS6K1 (T389), S6K1, pAkt (S473), and Akt. Panel D, The expression of pSGK (S422), SGK, pS6K1 (T389), S6K1, pAkt (S473) and Akt was analyzed by Western blot in HUVEC transfected with a control or two DEPTOR siRNAs (50 nM). All the data presented are representative of at least 3 independent experiments.

**Figure 3. DEPTOR inhibits ERK1/2 and STAT1 activity in endothelial cells.** HUVEC were transfected with control or DEPTOR siRNAs (#1 and #2), and after 48 hours, a phosphokinase protein array was performed on cell lysates to analyze the relative expression of 46 individual phosphokinase proteins. Panel A illustrates a representative blot (of n=2) showing the levels of phosphorylation of individual kinases and their protein substrates in control and in two DEPTOR siRNA-transfected EC (siRNA#1 and siRNA#2). Transfection with either siRNA resulted in similar findings by phosphokinase array. In Panel B, the expression of pS6K1 (T389), pAkt (S473), pERK1/2 (T202/Y204), pSTAT1 (Y701) and GAPDH was examined in control or DEPTOR siRNAs-transfected HUVEC using Western blot analysis. Representative results of >3 independent experiments are shown. Panels C and D, HUVEC were transfected with control or DEPTOR siRNAs, cultured for 48 hours, and treated with (Panel C) rapamycin (10 ng/ml), or (Panel D) Torin1 (1 μM) and/or U0126 (10 μM) for the last 18 hours of cell culture. Cells lysates were analyzed by Western blot analysis for the expression of pS6K1 (T389), total S6K1, pAkt
(S473), total Akt, pERK1/2 (T202/Y204), total ERK1/2, pSTAT1 (Y201 and S727), total STAT1 and GAPDH, as illustrated. Representative blots from 3 independent experiments are shown.

**Figure 4. DEPTOR regulates the expression of proinflammatory chemokines and adhesion molecules in endothelial cells.** mRNA array analysis (Supplemental Table 2) identified 27 genes that were induced in expression in DEPTOR siRNA-transfected EC vs. controls. Of these, eight represented established EC activation response genes, members of the chemokine and adhesion molecule families. In Panel A, qPCR was performed on control or two DEPTOR siRNA-transfected EC after 48hrs to validate the function of DEPTOR on the expression of the 8 genes, CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC), CX3CL1 (Fractalkine), CCL5 (RANTES) and CCL20 (MIP-3A) and the adhesion molecules VCAM-1 and ICAM-1. Graphs represent the mean fold change in mRNA expression (± 1SEM) in three independent experiments. Panel B summarizes the average fold induction (high to low) in mRNA expression of each gene, as indicated, in DEPTOR siRNA-transfected EC vs. controls. In Panel C, HUVEC were transfected with control or with two DEPTOR siRNAs, and the concentration of IP-10 protein was evaluated by ELISA in culture supernatants after 96 hours. Control siRNA-transfected HUVEC treated with IFNγ (1000 U/ml) for 24 hours were used as positive control. The bar graphs illustrate the mean IP-10 concentration (± 1SEM) from 3 experiments (*P<0.05, **P<0.01, ***P<0.001).

**Figure 5. Function of DEPTOR in leukocyte-endothelial interactions in vitro.** Panels A-C, Confluent cultures of untransfected, control siRNA- and DEPTOR siRNA-transfected HUVEC
were co-cultured with freshly isolated human PBMC at 37°C for 10 min to 60 min. Subsequently, the cultures were washed 3 times, and HUVEC and the number of adherent PBMC were evaluated by microscopy or by FACS analysis. **Panel A** shows representative photomicrographs of HUVEC transfected with control or DEPTOR siRNAs and cultured with human PBMC for 10, 30 and 60 mins. Cells cultured with TNFα-treated EC (100U/ml for 6 hrs) are illustrated as a positive control. Microscopy was carried out (10x objective) using a digital inverted microscope (AMG Evos XL Core, Fisher Scientific). **Panel B** shows representative FACS dot plots of the patterns of FSC (Forward Scatter) and SSC (Side Scatter) for 10,000 cells per group, illustrating the percentage of adherent PBMC to each group of HUVEC after 60 min. The expression of CD45 within the PBMC gate (lower left panel, open histogram), as well as CD45 and CD31 within the HUVEC gate (lower middle and right panels, open histogram) are shown vs. isotype antibody as a control (shaded histogram). The bar graph illustrates the mean adhesion index of PBMC (± 1SEM) to EC, from 5 independent experiments. **Panel C** shows representative FACS plots of CD3+ T cells within each PBMC gate shown in Panel B. The bar graph shows the mean adhesion index of CD3+ T cells (± 1SEM) to EC from 5 independent experiments. **Panels D-E**, PBMC and freshly isolated CD3+ T cells were labeled with CFSE (5 μM) prior to co-culture in adhesion assays with untransfected, control siRNA or two DEPTOR siRNA-transfected HUVEC. EC were cultured in the absence or presence of rapamycin (labeled R, 10 ng/ml) and/or U0126 (labeled U, 10 μM) for 24 hrs prior to each assay. After 60 min of co-culture, non-adherent cells were removed by washing and adherent leukocytes were evaluated by measurement of fluorescence in each well. The number of adherent leukocytes was calculated based on a standard curve, as described in Methods. The bar graphs show the mean number (± 1SEM) of adherent PBMC (**Panel D**) and CD3+ T cells (**Panel E**) from 5 independent
experiments. Data from EC transfected with DEPTOR siRNA#1 or #2 were pooled for analysis in the bar graphs. *P<0.05, **P<0.005, ***P<0.001.

**Figure 6. Function of DEPTOR in angiogenic responses in vitro.** Panel A, spheroids derived from either control or DEPTOR siRNA-transfected HUVEC were embedded in a collagen I matrix, cultured for 24 hours, and stained with Alexa Fluor® 488-conjugated phalloidin. A representative image of each group is shown without (upper panels) and with (lower panels) staining. The bar graph shows quantitative analysis of the mean total sprout length (± 1SEM) performed on at least five spheroids per experimental group in 3 independent experiments (***P<0.01). Panel B, HUVEC were transfected with control or with two DEPTOR siRNAs and cultured for 48 hours until confluent. Subsequently, linear scratch/wounds were created in the monolayers with a pipet tip, and the migration of cells into the wound was measured after 16 hrs in the absence or presence of rapamycin (10 ng/ml) and/or U0126 (10 μM). Illustrated are representative photomicrographs of wounds at 0 hr and after 16 hrs; dotted lines highlight the linear scratch/wound for each group of cells (representative of 3 experiments). The bar graph shows the mean percentage wound closure in pooled DEPTOR siRNA-transfected cells vs. controls (± 1SEM; *P<0.05 vs. untreated control siRNA-transfected EC; #P<0.05 vs. untreated DEPTOR siRNA-transfected EC).
Figure 1

A

B

C

D

anti-CD31

anti-DEPTOR

DAPI

Mmerge

Heart

Skin
Figure 2

A

IP: mTOR  IP: DEPTOR  IP: Raptor  IP: Rictor

mTOR  HUVEC  HEK293  HUVEC  HEK293  HUVEC  HEK293  HUVEC  HEK293
Raptor  HUVEC  HEK293  HUVEC  HEK293  HUVEC  HEK293  HUVEC  HEK293
Rictor  HUVEC  HEK293  HUVEC  HEK293  HUVEC  HEK293  HUVEC  HEK293
DEPTOR  HUVEC  HEK293  HUVEC  HEK293  HUVEC  HEK293  HUVEC  HEK293

WB:

B

siRNA: pS6K1 (T389)  S6K1  pAkt (S473)  Akt

siRNA:

12.5 nM  25 nM  50 nM

C

Rapamycin:

-  +

siRNA: pS6K1 (T389)  S6K1  pAkt (S473)  Akt

D

siRNA: pSGK (S422)  SGK  pS6K1 (T389)  S6K1  pAkt (S473)  Akt

siRNA:

Control  DEPTOR #1  DEPTOR #2  Control  DEPTOR #1  DEPTOR #2

Control  DEPTOR #1  DEPTOR #2
Figure 3

A

Control siRNA transfected

DEPTOR siRNA transfected

pERK1/2 (T202/Y204)
pS6K1 (T389)
pAkt (S473)
pSTAT1 (Y701)

B

siRNA

Control
DEPTOR #1
DEPTOR #2

pS6K1 (T389)
pAkt (S473)
pERK1/2 (T202/Y204)
pSTAT1 (Y701)
GAPDH

C

Rapamycin:
U0126:

- - + - +
- - - - -
- + - + +
- - + + +

siRNA:

Control
DEPTOR #1
DEPTOR #1
DEPTOR #1
DEPTOR #1

pS6K1 (T389)
Total S6K1
pAkt (S473)
Total Akt
pERK1/2 (T202/Y204)
Total ERK1/2
pSTAT1 (Y701)
pSTAT1 (S727)
Total STAT1
GAPDH

D

Torin1:
U0126:

- - + - +
- - - - -
- + - + +
- - + + +

siRNA:

Control
DEPTOR #1
DEPTOR #1
DEPTOR #1
DEPTOR #1

pS6K1 (T389)
Total S6K1
pAkt (S473)
Total Akt
pERK1/2 (T202/Y204)
Total ERK1/2
pSTAT1 (Y701)
pSTAT1 (S727)
Total STAT1
GAPDH
Figure 4

A

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>Average fold Induction</th>
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<tbody>
<tr>
<td>CCL5</td>
<td>Chemokine (C-C motif) ligand 5 (RANTES)</td>
<td>1194</td>
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<tr>
<td>CXCL10</td>
<td>Chemokine (C-X-C motif) ligand 10 (IP-10)</td>
<td>801</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>Chemokine (C-X3-C motif) ligand 1 (Fractalkine)</td>
<td>335</td>
</tr>
<tr>
<td>CXCL11</td>
<td>Chemokine (C-X-C motif) ligand 11 (I-TAC)</td>
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<td>CXCL9</td>
<td>Chemokine (C-X-C motif) ligand 9 (MIG)</td>
<td>18</td>
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<tr>
<td>VCAM1</td>
<td>Vascular cell adhesion molecule 1</td>
<td>15</td>
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<tr>
<td>CCL20</td>
<td>Chemokine (C-C motif) ligand 20 (MIP-3A)</td>
<td>7</td>
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<tr>
<td>ICAM1</td>
<td>Intercellular adhesion molecule 1</td>
<td>3</td>
</tr>
</tbody>
</table>

B

C

IP-10 concentration (pg/ml)

IFNγ 1000 U/ml:

- -

siRNA:

Control

DEPTOR #1

DEPTOR #2
Figure 6

A

Control siRNA

DEPTOR siRNA

Phase contrast

F-actin staining

B

0hr

16hrs

Control siRNA

DEPTOR siRNA

16hrs

DEPTOR siRNA + Rapamycin

DEPTOR siRNA + U0126

DEPTOR siRNA + Rapamycin + U0126

Total sprout length (fold induction)

Wound closure (%)

siRNA: Control, DEPTOR #1, DEPTOR #2

Rapamycin: - + - +

U0126: - - + +

n=3

n=3
DEPTOR regulates vascular endothelial cell activation, proinflammatory and angiogenic responses

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