ARTICLE

Lymphoma

Sphingosine-1-phosphate signalling drives an angiogenic transcriptional programme in diffuse large B cell lymphoma

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Abstract

Although the over-expression of angiogenic factors is reported in diffuse large B-cell lymphoma (DLBCL), the poor response to anti-VEGF drugs observed in clinical trials suggests that angiogenesis in these tumours might be driven by VEGF-independent pathways. We show that sphingosine kinase-1 (SPHK1), which generates the potent bioactive sphingolipid sphingosine-1-phosphate (S1P), is over-expressed in DLBCL. A meta-analysis of over 2000 cases revealed that genes correlated with SPHK1 mRNA expression in DLBCL were significantly enriched for tumour angiogenesis meta-signature genes; an effect evident in both major cell of origin (COO) and stromal subtypes. Moreover, we found that S1P induces angiogenic signalling and a gene expression programme that is present within the tumour vasculature of SPHK1-expressing DLBCL. Importantly, S1PR1 functional antagonists, including Siponimod, and the S1P neutralising antibody, Sphingomab, inhibited S1P signalling in DLBCL cells in vitro. Furthermore, Siponimod, also reduced angiogenesis and tumour growth in an S1P-producing mouse model of angiogenic DLBCL. Our data define a potential role for S1P signalling in driving an angiogenic gene expression programme in the tumour vasculature of DLBCL and suggest novel opportunities to target S1P-mediated angiogenesis in patients with DLBCL.

Introduction

Despite the use of rituximab and chemotherapy (R-CHOP), the survival of patients with diffuse large B-cell lymphoma (DLBCL) remains poor with a significant proportion of

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patients being refractory or relapsing after therapy [1, 2]. Gene expression profiling has identified two clinically distinct DLBCL subgroups, the activated B cell (ABC) and germinal centre B cell (GCB) forms, characterised by different, therapeutically tractable, molecular abnormalities. Gene expression profiling has also identified tumour microenvironment-derived molecular signatures, including the Stromal-2 signature that is reported to be enriched for angiogenesis-associated genes [3].

Although there may be therapeutic benefits in targeting angiogenesis in DLBCL, the results of clinical trials of bevacizumab, a monoclonal antibody against VEGFA, have been disappointing. Although this drug was well tolerated when delivered as a single agent in relapsed DLBCL [4] or in combination with R-CHOP in first-line treatment [5], a phase III study of RA-CHOP (MAIN trial) was stopped due to increased cardiotoxicity in the absence of any significant impact on progression-free survival [6]. One explanation for the limited clinical efficacy of bevacizumab in DLBCL is that angiogenesis in DLBCL does not depend on VEGF; a contention supported by studies showing that VEGF expression does not correlate with micro-vessel density in DLBCL [7, 8].

S1P is a bioactive sphingolipid metabolite which promotes cell growth and survival and has been shown to be a potent inducer of angiogenesis [9]. SPHK1, one of the two isoenzymes responsible for the production of S1P, is overexpressed in different cancers [10, 11]. Secreted S1P is a ligand for a family of five G-protein-coupled S1P receptors (S1PR1-5). Signalling through S1PR1 and/or S1PR3 has been shown to stimulate DNA synthesis, chemotactic motility and tube formation in endothelial cells and to induce angiogenesis in vivo [12–15]. As with other potent bioactive mediators, S1P levels are tightly regulated and controlled by the balance between its generation and its degradation by S1P lyase and S1P phosphatases [16]. Although it has been suggested that SPHK1 might play a role in haematological malignancies [17], it's role in DLBCL remains to be established [18]. Furthermore, the effects of S1P signalling on the DLBCL microenvironment, including its influence on the tumour vasculature, have not been explored.

In the present study, we have shown that the overexpression of SPHK1 correlates with an angiogenic transcriptional programme in DLBCL. We defined an endothelial cell transcriptional signature of S1P signalling and used this to show that the expression of S1P target genes in these cells was correlated with that of SPHK1 in primary DLBCL. Moreover, Siponimod, a small-molecule functional antagonist of S1PR1 [19], reversed S1P signalling and reduced angiogenesis and tumour growth in an S1P-producing mouse model of DLBCL. Our data suggest novel opportunities to target S1P-mediated angiogenesis in patients with DLBCL.

Materials and methods

Cells and tissues

Tonsils and DLBCL samples were obtained with informed consent and ethical approval (REC_RG_HBRC_12-071). DLBCL cases were reviewed by haematopathologists (ZR, YLH, UZ). Isolation of tonsillar germinal centre (GC) and blood-derived B cells was described before [20–22]. Endothelial cells (EC) were isolated from umbilical cords (HUVEC) under informed consent (REC_RG_HBRC_14-180) using collagenase treatment [23] and cultured in M199 media supplemented with 5% fetal bovine serum (FBS), 1% glutamine, 1% penicillin/streptomycin (ThermoFisher Scientific, Waltham, MA, USA) and 1% EC-growth supplement (Caltag Medsystems, Buckingham, UK) at 37 °C/5% CO_2. HT, Karpas-442, OCI-LY1, OCI-LY7, SUDHL4, SUDHL5, SUDHL6 are EBV-negative GC-DLBCL lines, Farage is an EBV-positive GC-DLBCL line. OCI-LY3 and U2932 are EBV-negative ABC-DLBCL lines. Lines were from DSMZ (Braunschweig, Germany), OCI (Ontario, Canada) or ATCC (Manassas, VA, USA) and were cultured in RPMI1640 or IMDM (OCI-LY1, OCI-LY7) media (ThermoFisher Scientific) supplemented with 10% FBS and 1% penicillin/streptomycin.

Mouse xenografts and flow cytometry

3×10^6 SUDHL6 cells were injected subcutaneously into NSG mice (Charles River Laboratories, Wilmington, MA, USA). After 17 days (when tumour volume averaged 63 mm^3) mice were randomised into two groups (each $n = 4$) and treated orally with either vehicle (0.1% DMSO in 10% 2-hydroxypropyl-β-cyclodextrin; Cayman Chemical, MI, USA) or 6 mg/kg Siponimod (Selleckchem.com, Munich, Germany) every 48 h. Mice were culled when average tumour volumes in control mice reached 400 mm^3 (28 days). Organs were weighed, minced and incubated with Liberase DL/Liberase TL and DNASEI (Roche, Basel, Switzerland) [24]. Cell suspensions were labelled with mouse CD31 and CountBright absolute counting beads (Thermofisher Scientific) and analysed by flow cytometry on LSRII and FACS diva 8 (BD, Franklin Lakes, NJ, USA). Details of the other mouse models tested are in Supplementary Materials and Methods. All mouse experiments were done according to UK Home Office guidelines.

S1P measurements

For intracellular S1P measurements, cell pellets were snap-frozen in liquid nitrogen. For secreted S1P measurements, SUDHL4 cells were cultured in serum-free RPMI (without phenol-red) supplemented with 1% tissue-culture grade

fatty acid-free BSA (Sigma-Aldrich., St Louis, MO, USA) for indicated times. Supernatants were harvested into pre-chilled HPLC grade methanol (Sigma-Aldrich) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific). S1P levels were quantified by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS; 4000 QTRAP, AB Sciex, Framingham, MA, USA) as previously described [25].

Treatment of cells

S1P (Sigma-Aldrich) was prepared as in Supplementary Materials and Methods and as before [26]. Prior to treatments, HUVEC were cultured in full media depleted of EC-growth supplement for 16 h and stimulated with 0.5 μM S1P (or control/vehicle) for 5 min to detect ERK1/2 activation or for 4 h to detect S1P transcriptional targets. For S1P inhibition, Sphingomab or isotype/control antibodies (LPath Inc., San Diego, CA, USA) were incubated with S1P (at 150 μg/ml per μM of S1P) for 1 h prior to S1P stimulation. For S1PR1 inhibition, HUVEC were treated with 100 nM S1PR1 functional antagonists Siponimod/BAF312, Ozanimod/RPC1063 or Ponesimod/ACT-128800 (Selleckchem. com) for 1 h prior to S1P stimulation.

Quantitative RT-PCR (Q-PCR)

RNA was extracted with RNeasy Mini kit including genomic DNA removal with RNase-Free DNase Set (Qiagen, Manchester, UK). cDNA was generated with qScript™ cDNA SuperMix (QuantaBio, Beverly, MA, USA). Gene transcripts were quantified with commercial gene expression assays (ThermoFisher Scientific; Supplementary Table 1a) [21]. The $2^{-\Delta\Delta CT}$ method was used to quantify target expression relative to housekeeping control. The normalized values are shown relative to the reference sample that was set to a relative quantity value of 1.

Protein analysis

Immunoblotting was by standard methodology [20]. Detection was with enhanced chemiluminescence (ECL; GE Healthcare, Chalfont St Giles, UK) on a ChemiDoc MP (Bio-Rad, Watford, UK). Immunohistochemistry (IHC) was on 4 μm FFPE sections and HUVEC grown on microscope slides, using either citrate or EDTA buffer antigen retrieval and 0.3% H_2O_2 and 5× casein blocking [26–28]. Slides were incubated either overnight at 4 °C or for 1 h at room temperature in primary antibodies in 0.05% PBS/Tween and visualised with species-specific ImmPRESS kits, followed by diaminobenzidine or ImmPACT NovaRED (Vector Laboratories, Peterborough, UK) with haematoxylin

counterstain. DLBCL were stained for BCL6, CD10 and IRF4, and defined as either GCB or non-GCB type by Hans algorithm [29]. A subset of DLBCL was also stained for SPHK1, S1PR1, CXCL12, SELE, COL1A1 and MAP1B. Mouse tumours were stained for Ki67, cPARP and CXCL12. For CXCL12, a Mouse-on-Mouse block (Vector Laboratories) was performed prior to incubation in casein. cPARP- and Ki67- positive cells were enumerated in at least 10 high power fields per tumour. Photomicrographs were acquired on a Nikon Eclipse E400 microscope with ×20 or ×40 objectives (NA 0.40 and 0.65) at room temperature and a Nikon DS-Fi-1 camera. Supplementary Table 1b lists antibodies used for immunoblotting and IHC.

RNAseq analysis

RNAseq was performed on 4 biological replicates of HUVEC treated with S1P or vehicle/control for 4 h. Quality control (RNA integrity number >7), library construction and sequencing was performed by BGI Tech Solutions (Hong Kong). RNA was hybridised to Illumina HiSeq 2000 platform and data obtained using 10 M clean reads.

Analysis of gene expression

RNAseq data were aligned to the hg19 human genome using Rsubread aligner [30] and assigned to genes with featureCounts function. Read counts normalised between samples were converted to counts-per-million (CPM) reads using the edgeR package in R [31]. RNAseq data for 32 ABC and 54 GCB DLBCL were from the controlled access area of NIH database of genotypes and phenotypes (dbGaP; accession code phs000532.v5.p2) [32]. The RNAseq data for 4 GC B cell samples were from GEO (GSE45982) [33]. Differentially expressed genes were identified using edgeR with $p < 0.05$ and read CPM >1 in at least half of the samples. Microarray data for 11 DLBCL and 10 GC B cell samples were from GEO (GSE12453) [34] and analysed with MAS5 algorithm of the Affymetrix Expression Console to generate expression levels for each probe set (GCOS Signal). The MAS5 TGT was set to 100. The series matrix expression and clinical data reported in Lenz et al. [3] were downloaded from GEO (GSE10846). Meta-analysis of 11 different DLBCL datasets was performed as before [35]. Spearman test was used to correlate gene expression between samples.

Statistics

Statistical tests are indicated in relevant sections. All experiments were performed at least in triplicate, and for B cells on at least three separate donors. Tests were considered statistically significant if $p < 0.05$.

Results

SPHK1 is over-expressed in the tumour cells of DLBCL

To explore the contribution of S1P signalling to angiogenesis in DLBCL, we examined the expression of SPHK1, an enzyme which generates S1P from sphingosine. We measured SPHK1 mRNA in published datasets, comparing global gene expression in DLBCL with that in normal GC B cells [32, 34]. SPHK1 mRNA levels were significantly higher in DLBCL compared to GC B cells (Fig. 1a) and in both ABC- and GCB- DLBCL subtypes when analysed separately (Fig. 1b).

We performed IHC using an antibody we previously showed is specific for SPHK1 [26]. We found that SPHK1 was present in normal GC B cells, and in the tumour cells of all 32 cases of DLBCL examined; including cases of both GCB and non-GCB type defined by the Hans algorithm [29] (Fig. 1c; Supplementary Table 2). The intensity of SPHK1 expression in tumour cells was variable but was generally higher than in background of non-malignant cells, including macrophages and small reactive lymphocytes (not shown). Furthermore, in contrast to the strong staining observed in tumour cells, SPHK1 was either only weakly detectable or undetectable in EC of most (27/31) evaluable cases (Supplementary Table 2; Fig. 1c).

SPHK1 mRNA and protein were also expressed in DLBCL lines (Fig. 1d). Because the catalytic activity of SPHK1 is enhanced by its phosphorylation at Ser225 we used an antibody specific for this phosphorylation site [36, 26]. Immunoblotting with this antibody revealed that SPHK1 was phosphorylated in all cell lines examined (Fig. 1d; lower panel and Supplementary Fig. 1A). S1P levels were significantly higher in all six DLBCL cell lines examined compared to normal B cells (Fig. 1e). Because S1P is a ligand for S1P receptors present on the cell surface, it was of interest to determine whether DLBCL cells such as SUDHL4, which express SPHK1 and produce S1P intracellularly, can also secrete it. We found that DLBCL cells readily export S1P outside the cells reaching a maximum level of around 1 nM within 2 min (Fig. 1f).

Finally, we stained the DLBCL cases for S1PR1. We found that S1PR1 was expressed on endothelial cells in all 32 cases and in tumour cells in 20/32 cases (Supplementary Table 2, Supplementary Fig. 1B upper panels). In keeping with this, Q-PCR revealed that some, but not all, DLBCL cell lines expressed S1PR1 (Supplementary Fig. 1B, lower panel). In primary tumours, there was no relationship between tumour cell expression of S1PR1 and disease subtype.

SPHK1 expression is associated with an angiogenic transcriptome in DLBCL

We next performed a meta-analysis of 11 primary DLBCL gene expression datasets comprising over 2000 cases of DLBCL [35]. For each dataset, genes were ordered by their variance across the patient samples and the top 80% were used to calculate the Spearman's rank correlations for their expression against that of SPHK1. The resultant p-values and correlation matrices were merged across the 11 datasets by taking the median values. A SPHK1-correlated gene set was created by taking all genes which were present in six or more datasets with a median $p < 0.05$. This identified 2236 genes positively correlated and 1658 genes negatively correlated with SPHK1 expression in DLBCL (Supplementary Table 3). We noted that genes positively correlated with SPHK1 included well known angiogenesis-associated genes, such as VEGF and VEGFR ($r = 0.28$, $p = 0.0001$; and $r = 0.19$, $p = 0.0042$, respectively). A gene ontology (GO) analysis [37] also showed a significant enrichment of angiogenesis and vasculature functions among genes positively correlated with SPHK1 (Fig. 2a). To provide further confirmation of a relationship between SPHK1 expression and angiogenesis, we utilised a published 'tumour vascular gene signature' generated from a meta-analysis of more than 1000 primary human cancers [38]. We found that genes positively correlated with SPHK1 expression in DLBCL were significantly enriched for tumour vascular signature genes (odds ratio (OR) = 7.92, $p < 0.0001$), whereas genes negatively correlated with SPHK1 were significantly depleted for tumour vascular signature genes (OR = 0.37, $p < 0.0001$; Fig. 2b).

Repeating the meta-analysis, we found that tumour vascular signature genes were significantly enriched among genes positively correlated with SPHK1 expression in GCB and ABC subtypes when analysed separately (both $p < 0.0001$; Supplementary Fig. 2). As a further check on the association between SPHK1 and angiogenesis, we used two further gene signatures of angiogenesis (the "Hallmark" angiogenic signature, M5944, and the signature for the GO term 'angiogenesis'). For both alternative angiogenic gene sets, we again found a significant overlap with genes positively correlated with SPHK1 (both $p < 0.0001$, not shown). Finally, we directly compared the mRNA expression of SPHK1 with that of classical EC markers in a further published RNAseq dataset reported by Morin et al. [32], not included in our original meta-analysis. This revealed a significant positive correlation between the expression of SPHK1 and that of CD34, CDH5, PECAM1/CD31 and VWF (Fig. 2c; Supplementary Fig. 3).

We also used the same 11 primary DLBCL datasets described above to perform a meta-analysis of genes correlated with S1PR1 in DLBCL. In keeping with the observed

Fig. 1 SPHK1 is over-expressed in primary DLBCL. **a** SPHK1 mRNA expression in the re-analysis of a published microarray dataset [34] and **b** SPHK1 mRNA expression in the re-analysis of published RNAseq data [32]. **c** IHC for SPHK1 protein expression in normal GC B cells and also in the tumour cells of representative cases of DLBCL. Arrows indicate tumour-associated EC which did not express SPHK1 (lower middle and lower right panels). GC germinal centre, MZ mantle zone. Original magnifications ×200 and ×600. **d** Upper panel: qPCR analysis for the expression of SPHK1 mRNA in DLBCL cell lines. Data are in triplicate and each is representative of three separate biological replicates. Lower panel: Immunoblotting of DLBCL cell lines for phosphorylated and total SPHK1 (pSPHK1, tSPHK1). β-tubulin is a loading control. **e** Intracellular S1P levels in primary B cells and DLBCL cell lines. Data show means (± SEM) of four biological replicates. Statistics were based on a comparison of individual cell lines with B cells#2. *Denotes $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t-test). **f** S1P levels secreted by SUDHL4 cells incubated in serum-free media for indicated times measured by mass spectrometry. Data show means (± SEM) of three biological replicates. *Denotes $p < 0.05$
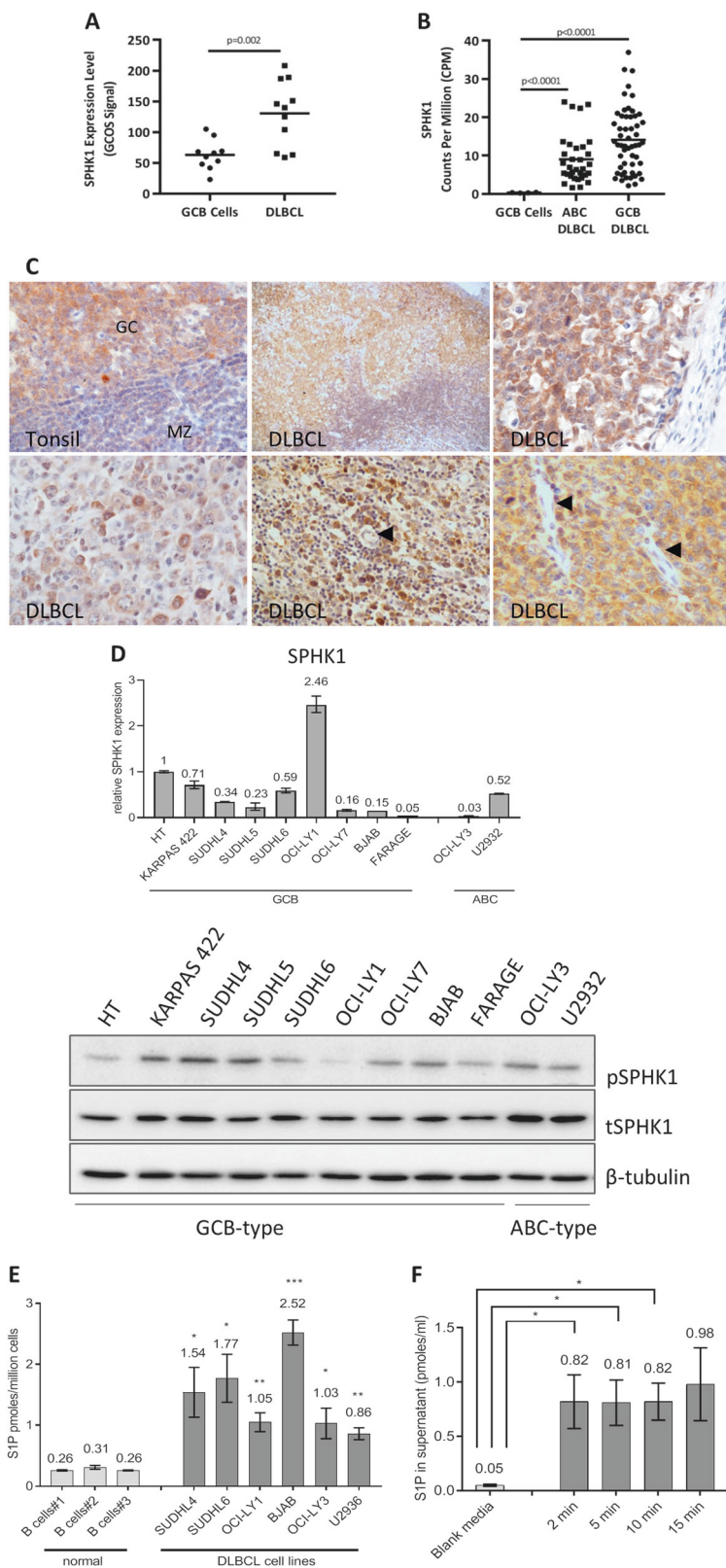
strong expression of S1PR1 in the tumour vasculature of DLBCL, we found that genes positively correlated with S1PR1 in primary tumours were also significantly enriched for tumour vascular signature genes (OR = 7.92, $p < 0.0001$; not shown). Furthermore, we also found that S1PR1 was positively correlated with CD34, (Spearman test, $r = 0.48$; p

Fig. 2 SPHK1 expression is associated with an angiogenic transcriptome in primary DLBCL. **a** Enrichment of angiogenesis-related terms (red arrows) in a GO analysis of genes positively correlated with SPHK1 expression in primary DLBCL. The top 10 significant GO categories are shown ordered by *p*-value. **b** Tumour vascular signature genes [38] were significantly enriched among genes positively correlated with SPHK1 expression in primary DLBCL (left panel), but significantly depleted among those negatively correlated with SPHK1 expression in primary DLBCL (right panel) (Fisher's exact test for both comparisons). **c** Re-analysis of gene expression from primary DLBCL [32] reveals a statistically significant positive correlation between SPHK1 expression and the expression of EC marker genes CD34, CDH5, PECAM1 and VWF ($r =$ Spearman rank correlation coefficient)
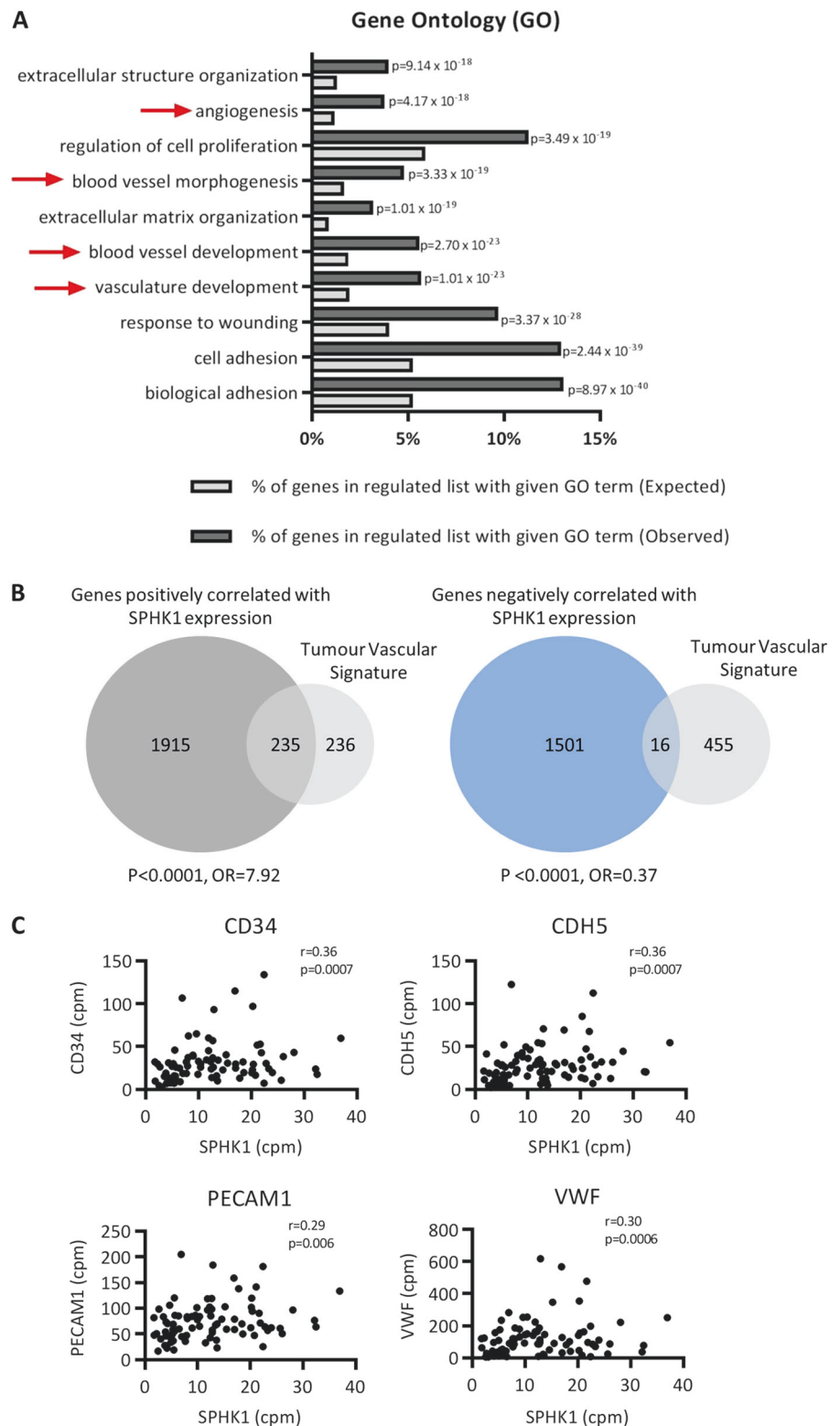
CDH5 ($r = 0.42$; $p < 0.0001$), PECAM1 ($r = 0.46$; $p < 0.0001$), and VWF ($r = 0.44$; $p < 0.0001$) in the dataset reported by Morin et al. [32].

We conclude that the expression of both SPHK1 and S1PR1 is associated with an angiogenic transcriptome in DLBCL.
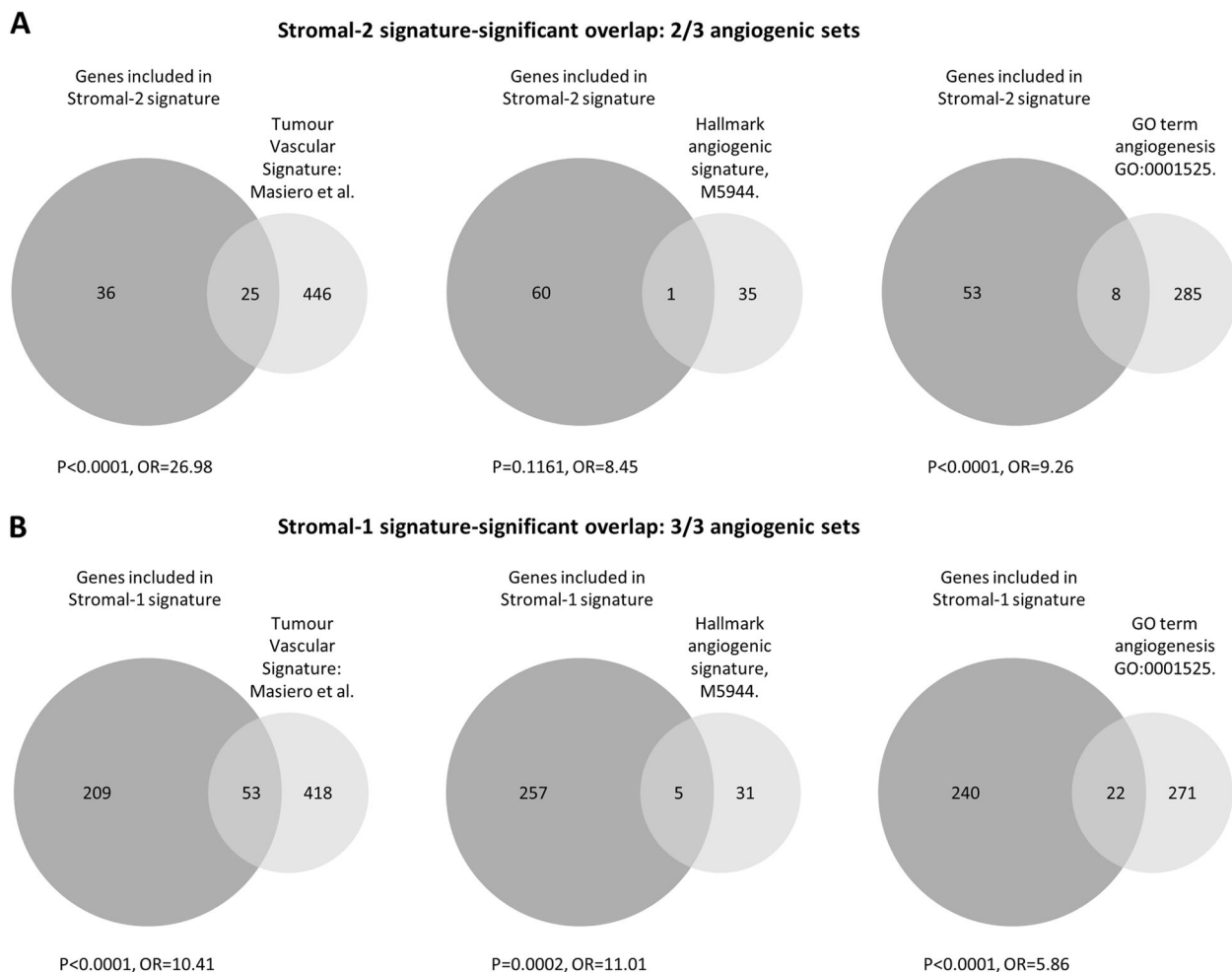
A   **Stromal-2 signature-significant overlap: 2/3 angiogenic sets**

P<0.0001, OR=26.98   P=0.1161, OR=8.45   P<0.0001, OR=9.26

B   **Stromal-1 signature-significant overlap: 3/3 angiogenic sets**

P<0.0001, OR=10.41   P=0.0002, OR=11.01   P<0.0001, OR=5.86

Fig. 3 Both Stromal-1 and Stromal-2 gene signatures are enriched for angiogenic genes. **a** Overlap between Stromal-2 genes and genes comprising the three different angiogenic gene signatures. **b** Overlap between Stromal-1 genes and the angiogenic gene signatures

SPHK1 expression is associated with angiogenesis in both Stromal-1 and Stromal-2 DLBCL subtypes

Given that angiogenesis was reported to be associated with the Stromal-2 signature in DLBCL [3], we next explored the correlation of SPHK1 expression with the expression of stromal signature genes [3]. First, we studied the extent to which Stromal-2 genes overlapped with the angiogenic gene signatures. As expected, we found a significant overlap of Stromal-2 signature genes, but only with two of the three angiogenic gene sets (Fig. 3a; upper panel). Consistent with this, we found that SPHK1 expression was positively correlated with Stromal-2 gene expression (calculated as the average expression across genes in the Stromal-2 signature [3] (Spearman correlation = 0.46 and 0.47 for CHOP- and R-CHOP-treated patients, respectively; $p < 0.0001$, not shown). However, when we repeated this analysis, this time using Stromal-1 signature genes [3], we were surprised to find that SPHK1 expression was even more highly correlated with

Stromal-1 gene expression (Spearman correlation = 0.80 and 0.77, respectively, $p < 0.0001$) and included a highly significant overlap with all three angiogenic gene sets (Fig. 3b; lower panel). In contrast, we observed that S1PR1 expression was positively correlated only with Stromal-2 gene expression (Spearman correlation = 0.34 and 0.45 for CHOP- and R-CHOP-treated patients, respectively; both $p < 0.0001$), and not with Stromal-1-gene expression (Spearman correlation $r = 0.02$, $p = 0.807$; and $r = 0.09$, $p = 0.212$, for CHOP- and R-CHOP-treated patients, respectively). We conclude that genes positively correlated with SPHK1 are enriched in both stromal signatures of DLBCL, most likely reflecting the contribution of SPHK1 to angiogenesis in both stromal subgroups.

An in vitro transcriptional signature of S1P signalling in endothelial cells

With the aim of revealing a more direct association between S1P signalling and angiogenesis in DLBCL, we next

defined a transcriptional signature of S1P signalling in EC. To do this we used human umbilical vein endothelial cells (HUVEC) which we showed express the same pattern of S1P receptors as the EC of DLBCL, marked by the high expression of S1PR1 and the absence of S1PR2 and S1PR3 in most cases (Fig. 4a). We showed that S1P treatment of HUVEC induced the robust phosphorylation of ERK1/2, a well-defined downstream target of S1P signalling in EC [12] (Fig. 4b).

We next used RNAseq to describe the global transcriptional consequences of S1P signalling in HUVEC. Treatment of HUVEC with S1P was followed by the up-regulation of 115 genes and the down-regulation of 126 genes (Supplementary Table 4). We used Q-PCR to validate a subset of the genes which were both upregulated by S1P and positively correlated with SPHK1 in our meta-analysis, including IL-8, ICAM1, SELE, PDGFA, ANGPTL4 and CXCL12 (Fig. 4c).

To confirm the specificity of these effects, we repeated these experiments in the presence of Sphingomab, a monoclonal antibody which depletes extracellular S1P [39]. Sphingomab greatly suppressed S1P-induced phosphorylation of ERK1/2 (Fig. 4d) and the upregulation of its downstream transcriptional targets (Fig. 4e). In contrast, S1P induced the phosphorylation of ERK1/2 and the up-regulation of its target genes in the presence of the isotype control antibody.

Genes upregulated by S1P signalling and correlated with SPHK1 mRNA are expressed in the tumour vasculature of DLBCL

A gene ontology analysis revealed that genes upregulated by S1P in HUVEC were significantly enriched for GO terms associated with angiogenesis, and for GO terms associated with the known effects of S1P in EC, including protection from apoptosis, leukocyte adhesion and migration (Fig. 5a). Furthermore, genes up-regulated (OR = 4.30, $p < 0.0001$), but not those downregulated (OR = 0.78, $p = 0.48$), by S1P were significantly enriched among genes positively correlated with SPHK1 in DLBCL (Fig. 5b, left panel). Similarly, genes downregulated (OR = 2.58, $p < 0.0001$), but not those up-regulated (OR = 0.73, $p = 0.48$), by S1P were significantly enriched in genes negatively correlated with SPHK1 (Fig. 5b, right panel). The enrichment of upregulated S1P targets among genes positively correlated with SPHK1 was also evident when the GCB and ABC subtypes were analysed separately (Supplementary Fig. 4).

To provide direct evidence of the expression of S1P target genes in the tumour vasculature of DLBCL we performed IHC for CXCL12 and SELE, as well as COL1A1 and MAP1B, two further genes up-regulated in S1P-treated HUVEC, positively correlated with SPHK1 expression in DLBCL, and that had available antibodies that work robustly in FFPE tissues. We found that all four markers were expressed in the EC of both subtypes of DLBCL. In contrast, tumour cells were either negative (CXCL12, COL1A1, SELE) or showed only weak expression (MAP1B) (Supplementary Table 5). In summary, these data provide evidence of an S1P-regulated transcriptional programme in the tumour vasculature of DLBCL.

S1PR1 inhibition reverses S1P signalling in vitro and reduces angiogenesis and tumour growth in a mouse model of angiogenic DLBCL

Finally, we explored the effects of inhibiting S1P signalling in a mouse model of DLBCL. We initially studied ERK1/2 phosphorylation in vitro following the stimulation of HUVEC with S1P, in the presence or absence of Siponimod, Ozanimod and Ponesimod, three functional antagonists of S1PR1 [19, 40, 41]. We found that all three drugs decreased ERK1/2 phosphorylation in HUVEC, confirming their ability to reduce the effects of S1P on downstream signalling (Fig. 6a). We next studied the impact of inhibiting S1P signalling in a mouse model of angiogenic DLBCL, focussing on Siponimod, a potent functional antagonist of S1PR1 and S1PR5 (EC50:0.39 and 0.98 nM, respectively) with > 1000-fold selectivity for S1PR1 versus S1PR2, S1PR3 and S1PR4 [42]. For these experiments we used SUDHL6 cells, not only because they produce high levels of S1P (Fig. 1e), but also because as xenografts they induce levels of angiogenesis that are almost 8-fold-higher than those found in xenografts of OCI-LY1 cells, and nearly 60-fold higher than those observed in the A20 syngeneic mouse model of DLBCL (Supplementary Fig. 5A). We treated established xenografts of SUDHL6 cells with Siponimod for up to two weeks before culling the animals and measuring mouse EC numbers in tumours by flow cytometry. We found that compared to controls, treatment of Siponimod significantly reduced the numbers of CD31-positive mouse EC in tumour tissues (Fig. 6b) as well as reducing their expression of the S1P target gene, CXCL12 (Supplementary Fig. 5B). Furthermore, Siponimod led to a significant reduction in tumour volumes in treated animals at the later time points (Fig. 6c) that was associated with increased numbers of apoptotic cells as measured by immunohistochemistry for cleaved PARP (Supplementary Fig. 5C). In contrast, we did not observe significantly reduced tumour volumes following Siponimod treatment of xenografts of the poorly angiogenic OCI-LY1 and A20 lines (Supplementary Fig. 5D, E). We conclude that the inhibition of S1P signalling can reduce angiogenesis and tumour growth in a mouse model of angiogenic DLBCL.

Fig. 4 Identification of S1P target genes in endothelial cells. **a** Representative images of IHC for S1PR1, S1PR2 and S1PR3 in primary DLBCL and HUVEC. DLBCL-associated EC (left and middle panels) were positive for S1PR1 and negative for S1PR2 and S1PR3 in most cases (black arrows). Red blood cells stained strongly for S1PR2 (red arrows) and served as an internal positive control. S1PR3-positive tumour cells (bottom left) and/or squamous epithelium (not shown) were internal positive controls for S1PR3 expression. HUVEC (right panel) were positive for S1PR1 and negative for S1PR2 and S1PR3. Original magnifications ×200 (tissues) and ×600 (cells). **b** S1P treatment of HUVEC increased ERK1/2 phosphorylation, shown here for 4 separate donors compared with vehicle only treated cells (H1-H4). β-tubulin is a loading control. The same protein lysates were used to detect phosphorylated and total ERK1/2 (pERK1/2, tERK1/2) in separate blots. **c** Q-PCR for S1P transcriptional targets in HUVEC of four donors following treatment with S1P (dark grey bars) compared with vehicle (light grey bars) normalised to 1. *Denotes $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t-test). **d** Immunoblotting for pERK1/2, tERK1/2 and β-tubulin in HUVEC representative of three donors treated with S1P in the presence of Sphingomab (SmAb) or isotype/control antibody (CmAb). The same protein lysates were used to detect pERK1/2 and tERK1/2 in separate blots. **e** Q-PCR for S1P targets following treatment of HUVEC with S1P in the presence of either SmAb or CmAb. Data shown are representative of three donors. *Denotes $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t-test)
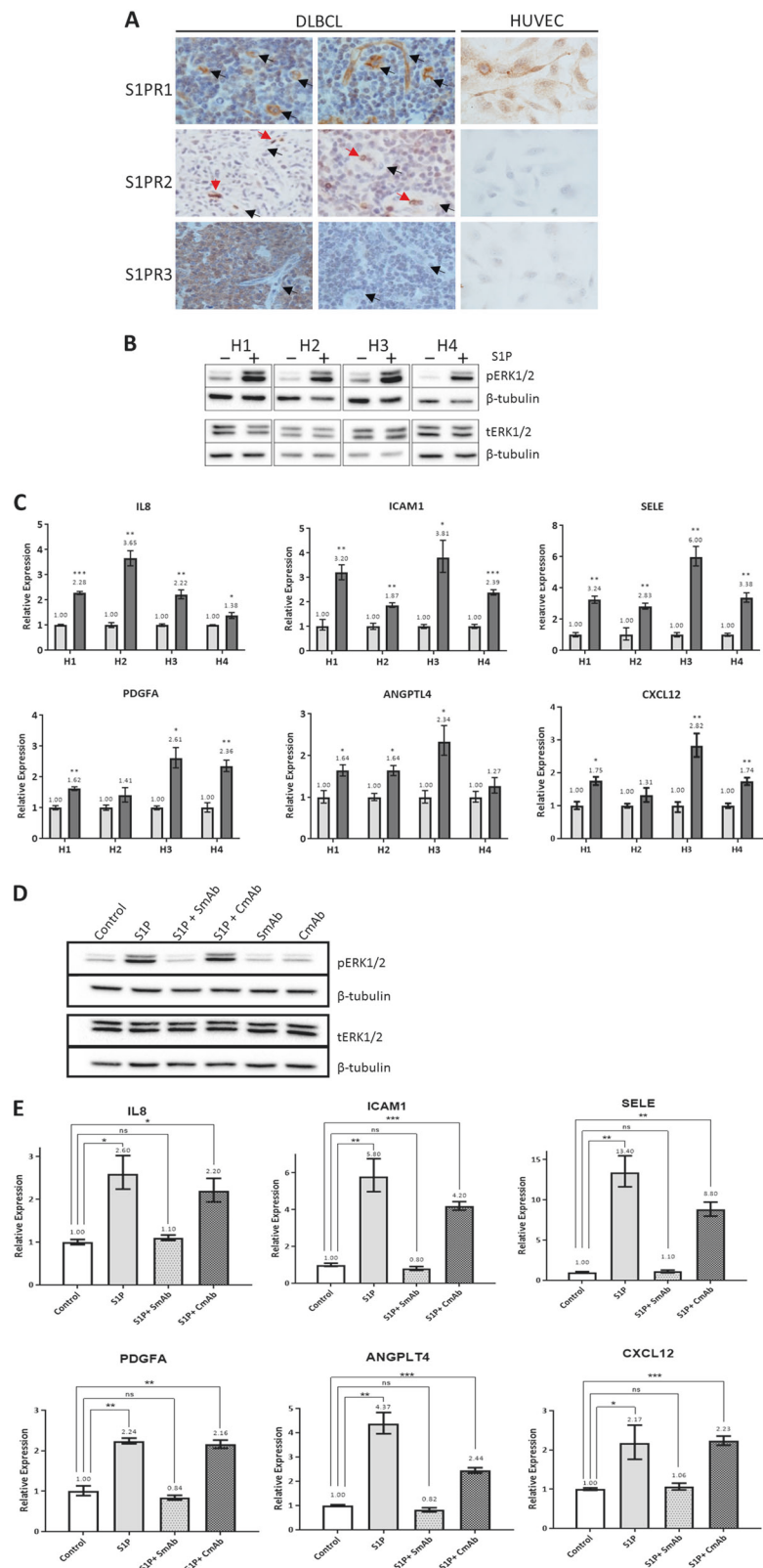
Fig. 5 Genes upregulated by S1P and correlated with SPHK1 mRNA are expressed in DLBCL tumour vasculature. **a** GO analysis of genes upregulated by S1P in HUVEC revealed a significant enrichment of GO terms associated with angiogenesis. **b** Genes upregulated (left panel), but not those downregulated (not shown) in S1P-treated HUVEC were enriched among genes positively correlated with SPHK1 expression in primary DLBCL. Genes downregulated (right panel), but not those upregulated (not shown) in S1P-treated HUVEC were enriched among genes negatively correlated with SPHK1 expression in primary DLBCL. **c** Representative examples of the expression of the S1P target genes, CXCL12, SELE, COL1A1 and MAP1B, in the tumour vasculature of DLBCL. Original magnifications ×200
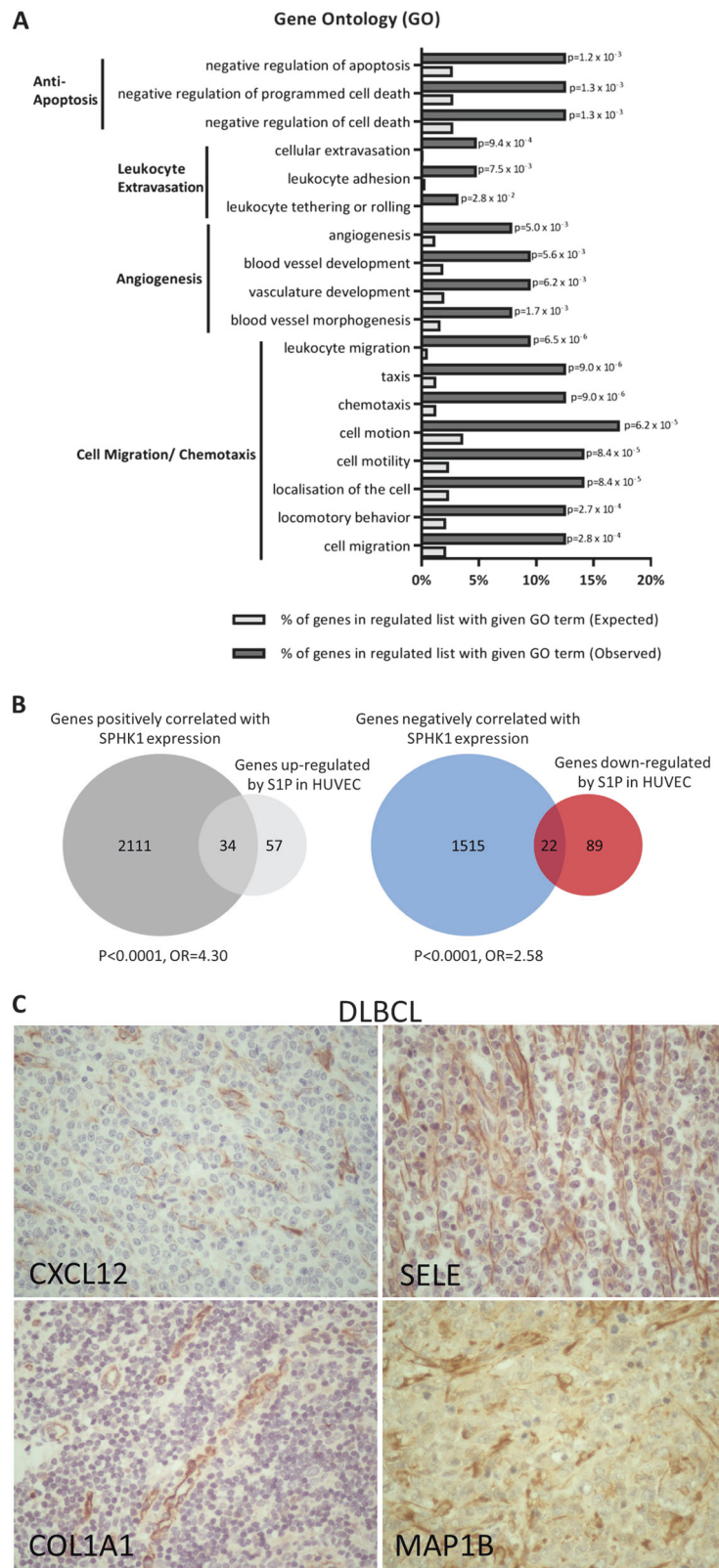
Discussion

Although previous studies have shown that targeting VEGF or its receptors can decrease tumour vascularization and reduce the growth of DLBCL-derived cell lines in vivo, a phase III trial of patients with aggressive NHL receiving R-CHOP plus bevacizumab showed cardiotoxicity without improvement in progression-free survival [6]. We speculated that DLBCL-associated angiogenesis could be driven by VEGF-independent signalling; a contention supported by other studies which reveal no correlation between lymphoma cell VEGF expression and micro-vessel density in primary DLBCL [7, 8]. Here, we have focused on the potential contribution of the potent bioactive sphingolipid metabolite, S1P. We have shown that SPHK1, the major enzyme responsible for the production of S1P, which can be secreted, is over-expressed in DLBCL. SPHK1 is strongly implicated in tumour angiogenesis in other tumours [43], and is important for the generation of S1P that drives tumour-induced hemangiogenesis and lymphangiogenesis in murine models of breast cancer [44]. The underlying mechanisms responsible for the over-expression of SPHK1 in DLBCL are not known but do not usually involve amplification and/or mutation; our review of five separate cohorts of DLBCL has revealed that the frequency of amplification and/or mutation of the SPHK1 gene is very low (<1%; not shown; TCGA Research Network: http://cancergenome.nih.gov/; [32, 45–47]).

SPHK1 can be activated by growth factors and cytokines [48–52], and its catalytic activity is enhanced by ERK1/2-mediated phosphorylation at Ser225 [36]. The pSer225 antibody used in our study showed that SPHK1 was constitutively phosphorylated at Ser225 in DLBCL cell lines. In keeping with this, we showed that S1P production was increased in DLBCL cell lines compared to normal B cells, and furthermore that this S1P was secreted into the extracellular environment.

Our meta-analysis of DLBCL revealed a strong correlation between the expression of SPHK1 and that of angiogenesis meta-signature and EC-classifier genes. One explanation for this finding could be expression of SPHK1 in tumour-associated EC. However, we observed SPHK1 expression predominantly in cancer cells, consistent with the notion that SPHK1 expression is upregulated primarily in the tumour cell population [18].

Although the meta-analysis and our in situ expression data suggested that angiogenesis in DLBCL is driven by tumour-derived SPHK1, these approaches do not directly implicate S1P. Interactions between tumour and EC mediated by S1P have been shown to be important for angiogenesis in many solid cancers, including those of the breast, prostate, liver and kidney [53–55]. To investigate the involvement of S1P in DLBCL-associated angiogenesis, we

B endothelial cells-SUDHL6 (n=4)

C Tumour volume-SUDHL6 (n=4)
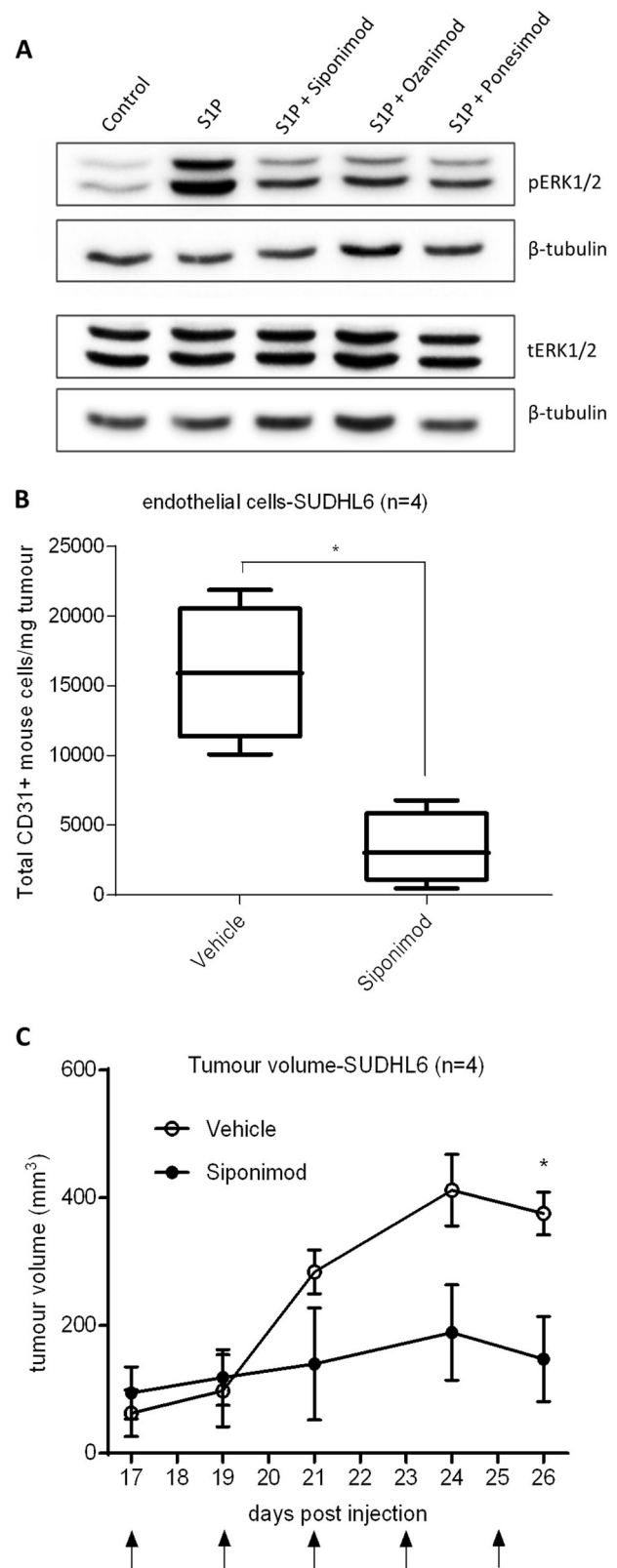
defined a transcriptional signature of S1P signalling in HUVEC [23]. Gene ontology analysis revealed that genes up-regulated in this transcriptional signature were enriched

Fig. 6 S1PR1 inhibition decreases S1P-induced signalling and reduces angiogenesis in a mouse model of DLBCL. **a** Immunoblot of HUVEC pre-treated for 1 h with 100 nM of S1PR1 functional antagonists, Siponimod, Ozanimod and Ponesimod and stimulated with S1P or control (S1P vehicle) for 5 min. S1P or control only treated cells received the equivalent dose of DMSO (drug solvent) to S1PR1 inhibitor-treated cells (0.01 μl DMSO per 1 mL of media). β-tubulin was used as a loading control. The same protein lysates were used to detect pERK1/2 and tERK1/2 in separate blots. Data are representative of three separate donors. **b** Flow cytometry analysis of numbers of CD31-positive mouse cells/per mg tumour of SUDHL6 xenografts in NSG mice treated with Siponimod compared with drug vehicle (4 mice for each group). **c** Tumour volumes in days after injection with SUDHL6 cells in Siponimod-treated versus vehicle-treated animals. Arrows indicate treatment days. *Denotes $p < 0.05$ in Student's t-test

for angiogenesis-associated GO terms, as well as for GO terms reflecting known functions of S1P [9, 12, 13, 56, 57]. We used this transcriptional signature to show that genes upregulated by S1P were enriched among genes correlated with SPHK1, an association that was also evident separately for both COO subtypes. IHC revealed that S1P target genes were expressed in the tumour vasculature of SPHK1-expressing DLBCL, with little or no tumour cell expression, suggesting that the enrichment of S1P target genes observed in SPHK1-expressing DLBCL was primarily a consequence of their expression in tumour-associated EC.

Recent studies exploring the transcriptional landscape of DLBCL have defined tumour microenvironment-derived gene signatures predicting clinical outcome [2, 3]. The 'Stromal-1' signature, associated with a favourable outcome, is characterised by expression of extracellular matrix- and macrophage-associated genes. In contrast, the 'Stromal-2' signature has an unfavourable outcome, and is reportedly enriched for angiogenesis-associated genes [3]. We formally tested the relationship between angiogenesis and these stromal signatures [3]. As expected, we found a significant enrichment of angiogenesis-associated genes within the Stromal-2 signature. However, we were surprised to find an even stronger enrichment of angiogenesis genes within the Stromal-1 signature. Both stromal signatures were enriched for genes positively correlated with SPHK1 expression, suggesting that SPHK1 contributes to angiogenesis in both stromal subtypes.

Our data suggest novel therapeutic opportunities for patients with SPHK1-expressing DLBCL insofar as the S1PR1 functional antagonists, Siponimod, Ozanimod and Ponesimod, were all able to block S1P signalling in EC. Although we observed the same effect with the S1P-specific monoclonal antibody, Sphingomab, we suggest that S1PR1 is a preferred target; S1P-blocking agents will affect signalling through all S1P receptors. Potentially unwanted side-effects of blocking S1P could include the inhibition of S1PR2 signalling which we and others have reported to be tumour suppressive in DLBCL [58, 59, 60]. Moreover, we showed that Siponimod, a highly potent functional antagonist

of S1PR1 that is effective and safe in patients with multiple sclerosis [61], can reduce angiogenesis and tumour growth of SUDHL6 xenografts which produce S1P and which we used as a mouse model of angiogenic DLBCL. Targeting tumour cell over-expression of S1PR1, which is observed in a subset of poor prognosis DLBCL, is also potentially advantageous, since S1PR1 has been shown to be important for the proliferation and survival of DLBCL [62, 63]. S1P/S1PR1 signalling also promotes the accumulation of Treg while inhibiting CD8+T cell recruitment and activation in xenograft models of breast cancer and melanoma [64]. Therefore, blocking S1PR1 might also promote anti-tumour immunity. Taken together our observations suggest that specific S1PR1 antagonists should be investigated for their therapeutic potential in DLBCL patients [40, 65, 66].

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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