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18-430.R2 **Poulsom Edited UK English** **ORIGINAL ARTICLE**

Regulation of S1PR2 by the EBV oncogene LMP1 in aggressive ABC subtype diffuse large B cell lymphoma

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ABSTRACT

The Epstein-Barr virus (EBV) is found almost exclusively in the activated B cell (ABC) subtype of diffuse large B cell lymphoma (DLBCL), yet its contribution to this tumour remains poorly understood. We have focussed on the EBV-encoded latent membrane protein-1 (LMP1), a constitutively activated CD40 homologue expressed in almost all EBV-positive DLBCL and which can disrupt germinal centre (GC) formation and drive lymphomagenesis in mice. Comparison of the transcriptional changes that follow LMP1 expression with those that follow transient CD40 signalling in human GC B cells enabled us to define pathogenic targets of LMP1 aberrantly expressed in ABC-DLBCL. These included the down-regulation of S1PR2, a sphingosine-1-phosphate (S1P) receptor that is transcriptionally down-regulated in ABC-DLBCL, and when genetically ablated leads to DLBCL in mice. Consistent with this we found that LMP1-expressing primary ABC-DLBCL were significantly more likely to lack S1PR2 expression than were LMP1-negative tumours. Furthermore, we showed that the down-regulation of S1PR2 by LMP1 drives a signalling loop leading to constitutive activation of the phosphatidylinositol-3-kinase (PI3-K) pathway. Finally, core LMP1-PI3-K targets were enriched for lymphoma-related transcription factors and genes associated with shorter overall survival in patients with ABC-DLBCL. Our data identify a novel function for LMP1 in aggressive DLBCL.

KEY WORDS: S1P, S1PR2, EBV, LMP1, CD40 and DLBCL

INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is molecularly and clinically heterogeneous even within the activated B-cell (ABC) and germinal center B-cell (GCB) subtypes [1, 2]. Around one-third of patients have refractory disease or will relapse, and outcome generally is poor for these patients [3]. DLBCL is dependent upon de-regulated cellular signalling, including PI3-K/AKT and NF-κB pathways. Different cell-of-origin (COO) subtypes are addicted to different branches of the PI3-K signalling pathway; AKT signalling is critical for the survival of PTEN-deficient GC-DLBCL, whereas PI3-Kα/δ induces activation of pro-survival NF-κB signalling in ABC-DLBCL [4].

EBV-positive DLBCL accounts for around 10% of cases and is almost exclusively of ABC subtype. Although frequently observed against a background of immune impairment, EBV-positive DLBCL also arises without a prior history of immune suppression; particularly in older people, where senescence of the EBV-specific immune response is suspected [5]. However, EBV-positive DLBCL occurs also in apparently immunocompetent younger patients [6, 7]. Patients with EBV-positive DLBCL have an inferior clinical course and shorter overall survival compared with patients with EBV-negative DLBCL; an effect that is particularly

consistent among elderly patients even after adjustment for the adverse effects of age [8–10].

The EBV-encoded LMP1 is a homologue of the cellular CD40 receptor and is expressed in most EBV-positive cases of DLBCL [7]. However, unlike CD40, the spontaneous oligomerisation of LMP1's transmembrane domains delivers a constitutive signal. LMP1 is essential for the *in vitro* transformation of B cells by EBV and can block GC formation and induce B cell lymphomas in transgenic mice [11, 12]. It is the constitutive nature of the LMP1 signal that is transforming. Thus, the B cell-specific expression of a constitutively active CD40 receptor, comprising the transmembrane domains of LMP1 and the cytoplasmic tail of CD40 leads to lymphoma development in mice, whereas a chimera of the transmembrane domains of CD40 and the cytoplasmic region of LMP1 is not transforming [13, 14]. While LMP1 constitutively activates both the PI3-K/AKT and the NF-κB pathways, how LMP1 contributes to the pathogenesis of ABC-DLBCL has yet to be established [15–17].

Here we identified genes regulated by LMP1, but not by a transient CD40 signal, in primary GC B cells. Putative pathogenic targets of LMP1 included S1PR2, a sphingosine-1-phosphate (S1P) receptor that has been shown to be transcriptionally silenced in ABC-DLBCL and when genetically ablated to lead to DLBCL-like tumours in transgenic mice [18-23]. We found that LMP1-mediated loss of S1PR2 expression initiates a signalling loop leading to the constitutive activation of the PI3-K pathway. Moreover, core LMP1-regulated PI3-K target genes were enriched for lymphoma-related transcription factors and genes associated with

shorter overall survival in patients with ABC-DLBCL. Our data identify novel functions for LMP1 in driving aberrant cell signalling and gene expression in aggressive ABC-DLBCL.

MATERIALS AND METHODS

Cells and tissues

Tonsils and tumour samples were obtained with informed consent and ethical approval (REC_RG_HBRC_12-071, REC reference 15/NW/0079; IRAS project ID:171283). Primary GC B cells were isolated from tonsils as described [24, 25]. HT [26], SUDHL4 [26], SUDHL6 [26], BJAB [27], OCI-Ly1 [28] are EBV-negative GCB-DLBCL lines, Farage [29] is an EBV-positive GCB-DLBCL line. OCI-Ly3 [28], OCI-Ly18 [28] and U2932 [30] are EBV-negative ABC-DLBCL lines. DG75 [31] is an EBV-negative Burkitt lymphoma line. L591 [32] is an EBV-positive Hodgkin lymphoma line. Cell lines were purchased from DSMZ (Braunschweig, Germany), OCI (Toronto,ON, Canada) or ATCC (Manassas, VA, USA). Cells were cultured at 37 °C/5% CO_2 in RPMI1640 or IMDM (for OCI-Ly1, OCI-Ly3, OCI-Ly18), supplemented with 10% fetal bovine serum (FCS) and 1% penicillin-streptomycin stock (ThermoFisher Scientific, Waltham, MA, USA).

Cell transfections and treatments

GC B cells were nucleofected (Lonza, Slough, UK), cultivated for 16 h and enriched as described previously [24, 25]. SUDHL4, DG75, OCI-ly1 and U2932 cells were nucleofected. BJAB cells were electroporated (Bio-Rad Laboratories, Hemel Hempstead, UK) and enriched as described [33, 34]. Cells were harvested for RNA extraction, protein extraction or immunofluorescence 24 h after transfection. Dual luciferase reporter gene assays (Promega Corporation, Southampton, UK) using pGLS1PR2luc promoter were performed as described

[24]. Cloning of the S1PR2 promoter fragment and all plasmid details are presented in supplementary material, Supplementary materials and methods.

For CD40 stimulation, cell lines and GC B cells from 9 donors were seeded at $2x10^6$/ml and cultivated in the presence or absence/control of 200 ng/ml CD40L (Autogen Bioclear, Calne, UK) for indicated times. For PI3-K/AKT inhibition, 5 μM Ly294002 (Pan-PI3-K inhibitor), CAL-101 (p110-δ inhibitor), MK-2206 (Pan-AKT inhibitor) or DMSO were added after transfection and cells cultivated for 24–48 h.

Quantitative RT-qPCR

RNA was isolated using RNeasy® Mini/Micro Kit (QIAGEN, Crawley, UK) and amplified as described [24, 25]. CDNA was synthetized using M-MLV Reverse Transcriptase kit (Invitrogen, ThermoFisher Scientific). Relative mRNA levels were determined in triplicate using real-time fluorimetric measurement of cDNA and comparative C_T method. RT-qPCR assays are detailed in supplementary material, Table S1A.

Global gene expression analysis

Tonsillar GC B cells of 9 individuals were cultivated in the presence or absence of CD40L for 3 h. Extracted RNA was labelled and hybridized to Affymetrix Human Genome U133A 2.0+ Arrays. Scanned images of microarray chips were analysed with GeneChip Operating Software and Expression Console with target signal set to 100. Probe level quantile normalisation and robust multi-array analysis on the raw CEL files were performed with affy

package of the Bioconductor project (http://www.bioconductor.org) [35, 36]. Differentially expressed probe sets were identified using limma with false discovery rate adjusted p value <0.05 [37]. The primary data are available in GEO (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE96709). Gene ontology (GO) analysis was performed using the DAVID Gene Functional Classification Tool (http://david.abcc.ncifcrf.gov).

Analysis of publicly available datasets

Probe level quantile normalisation was performed as above on datasets GSE10846 [38], GSE12453 [39], GSE94610 [40] and GSE10821 [24]. Differentially expressed probe sets were identified using limma (p value <0.05, fold-change >1.5) [37]. RNA-seq data from human DLBCL were downloaded in April 2016 from the controlled access area of the NIH database of genotypes and phenotypes (dbGap; http://www.ncbi.nlm.nih.gov/gap) using accession number phs000532 [41, 42]. RNA-seq data for four GC B cell samples were from GSE45982: GSM1129344, GSM1129345, GSM1129346 and GSM1129347 [43]. RNA-seq data were aligned to hg19 human genome using Rsubread aligner and assigned to individual genes with featureCounts function [44]. Read counts were normalized between samples and converted to counts-per-million (CPM) reads for each gene by edgeR package [45]. Differentially expressed genes were identified using edgeR with criteria of CPM>1 in at least four samples, p<0.05, fold-change>2.0. To identify genes differentially expressed in DLBCL, we compared gene expression in primary DLBCL with that in normal GC B cells. Kaplan-Meier analyses with samples split by median expression value were performed using the

"survival" package in R. P-values were calculated using the log-rank test. A list of shared NF-κB and PI3-K targets in ABC-DLBCL were taken from Kloo *et al* [40].

Protein analysis and* in situ *hybridization

Protein expression was analysed by immunohistochemistry (IHC), immunoblotting and flow cytometry [34]. IHC was carried out using citrate antigen retrieval and H_2O_2 and 5x casein or background reducing reagent (S3022; Dako, Ely, UK) blocking before adding primary antibodies in PBS (listed in supplementary material, Table S1B) and visualisation using diaminobenzidine (Vector Laboratories, Peterborough, UK) with haematoxylin counterstain. An Opal™ 3-Plex Kit was used for multiplex immunofluorescence (IF) (PerkinElmer, Seer Green, UK; NEL791001KT) [46]. Antibody stripping by microwaving in pH 6 citrate buffer was used between steps. Omission of individual antibodies was used to assess adequate stripping. Slides were scanned using a Vectra 3.0.3 System for detection of cyanine 5, cyanine 3, DAPI, texas red and fluorescein. Multispectral images were analysed using InForm Automated Image Analysis software version 2.2.1. Images were acquired using an Olympus BX-51WI microscope (Olympus, Tokyo, Japan) with 10x, 20x and 40x objective lenses. Comparisons of marker expression were performed using Pearson's Chi-squared test with Yates' continuity correction. EBV status was assessed by EBER *in situ* hybridisation [47].

Statistics

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

RESULTS

Identification of putative pathogenic LMP1 targets in DLBCL

To identify pathogenic targets of LMP1, we compared the transcriptional effects of this viral oncogene with those of its physiological homologue, CD40, in primary human GC B cells. To identify CD40 targets, tonsillar GC B cells were stimulated with 200 ng/ml CD40L for 3 h, a time-point optimized to give maximal regulation of known CD40 targets in preliminary experiments (not shown) [24, 25, 48]. To reduce the effects of inter-donor variation, stimulation of the CD40 receptor was performed on 9 separate donors and the mean expression of each gene across all donors calculated. To define LMP1 targets, we re-analysed our previous microarray dataset describing the transcriptional effects of LMP1 in GC B cells [24]. Of the 767 genes up-regulated and 1435 genes down-regulated by CD40 (supplementary material, Table S2A,B), 224 and 429 genes were also up- and down-regulated, by LMP1, a statistically significant overlap that confirms the partially overlapping functions of LMP1 and CD40 in B cells (Figure 1A; $p<0.0001$). Although aberrantly regulated CD40 targets might also contribute to the pathogenesis of DLBCL, our primary interest was in those LMP1 targets not regulated by CD40 (supplementary material, Table S2C,D). We found that genes regulated by LMP1, but not CD40, were significantly enriched in the set of genes concordantly differentially-expressed when primary DLBCL were compared to normal GC B cells in our re-analysis of published gene expression dataset (Figure 1A; $p<0.0001$; supplementary material, Table S2E–H) [39]. We also observed a significant enrichment of LMP1 targets among genes shown to be differentially expressed when the ABC- (Figure 1B;

p<0.0001) and EBV-positive (not shown; p<0.0001) subtypes of DLBCL were compared separately to normal GC B cells (supplementary material, Table S3A–D) [41-43]. RT-qPCR confirmed the regulation of selected genes; notably, they included sphingosine kinase 1 (*SPHK1*), which encodes the enzyme responsible for sphingosine-1-phosphate (S1P) production (Figure 1C).

LMP1, but not CD40, down-regulates expression of sphingosine-1-phosphate receptor 2 (S1PR2)

LMP1 target genes included *S1PR2*, which encodes an S1P receptor that has been shown to be transcriptionally down-regulated in ABC-subtype DLBCL, and which, when genetically ablated, induces DLBCL in mice [18-20, 23]. RT-qPCR confirmed that *S1PR2* expression was down-regulated by LMP1, but not CD40, in GC B cells (Figure 2A). We next studied the regulation of S1PR2 by LMP1 in SUDHL4 and OCI-Ly1 GCB-DLBCL cell lines which have high *S1PR2* expression (supplementary material, Figure S1). LMP1 expression, but not CD40 stimulation, significantly decreased *S1PR2* mRNA expression in both lines (Figure 2B). To rule out the possibility that CD40 might regulate S1PR2 at the later time points, we performed CD40 stimulation also for 6 h and 24 h, but observed no significant change in *S1PR2* levels (Figure 2B). Furthermore, we confirmed that the CD40 signalling pathway was intact in the cell lines used, since we could induce expression of CD40 targets, including ICAM1 (supplementary material, Figure S2A), which we have previously shown is also an LMP1 target and which was significantly induced following the stimulation of GC B cells by

CD40 [24]. As a further a control we measured S1PR2 expression following the addition of CD40L to mock electroporated cells. We did not observe any decrease in S1PR2 levels, in contrast to the levels of ICAM1 which were increased, as expected (data not shown). To show that the S1PR2 downregulation is mediated by the constitutive signal induced by the membrane part of LMP1, we used an expression vector that contains the cytoplasmic signalling domain of CD40 fused to the transmembrane domain of LMP1 [14]. This fusion protein provides a constitutive CD40 signal to the cell. We found that this constitutive CD40 signal leads, like LMP1, to the downregulation of *S1PR2* in B cell lymphoma lines (supplementary material, Figure S2B). Next, we used multiplex IF to investigate the impact of LMP1 on S1PR2 protein levels in SUDHL4 cells which show high levels also of S1PR2 protein (not shown). SUDHL4 cells ectopically expressing LMP1 were significantly more likely to lack S1PR2 protein than un-transfected LMP1-negative cells in the same population (Figure 2C). As a control, we showed that LMP1 significantly increased ICAM1 expression in SUDHL4 cells (Figure 2C). To show that the down-regulation of S1PR2 protein by LMP1 was not an artefact associated with transfection, we stained Farage and L591 which are EBV-positive lymphoma cell lines that endogenously express LMP1. Compared with their LMP1-negative counterparts, significantly more LMP1-positive Farage cells and L591 cells were S1PR2-negative (Figure 2C, lower right panels). These effects appear to be predominantly mediated at the level of the *S1PR2* promoter, since we showed that LMP1 significantly reduced *S1PR2* promoter activity in the BJAB and DG75 lines which show robust effects in

luciferase assays (Figure 2D). We conclude that LMP1, but not its physiological homologue CD40, down-regulates S1PR2 expression in B cells.

Down-regulation of S1PR2 in LMP1-expressing primary DLBCL

Next, we studied the expression of *S1PR2* mRNA in primary DLBCL. Re-analysis of two large expression datasets confirmed the down-regulation of S1PR2 in DLBCL compared to normal GC B cells, and showed that this was particularly pronounced in ABC-DLBCL (Figure 3A) [38, 41-43]. To study the relationship between the expression of S1PR2 and LMP1 in primary tumours we performed IHC using an antibody that we previously showed is specific for S1PR2 [46]. In contrast to GC B cells which expressed S1PR2, 114/167 DLBCL showed no tumour cell S1PR2 expression (Figure 3B). We stained all cases for BCL6, CD10 and IRF4 and defined each as either GCB or non-GCB type using the Hans algorithm [49]. 14 cases, all non-GCB type, were EBER-positive, 10 of these were LMP1-positive. EBER-positive and LMP1-positive non-GC cases were significantly more likely to be S1PR2-negative compared with EBER-negative and LMP1-negative non-GCB cases ($p<0.05$ for both comparisons; supplementary material, Table S4). Moreover, a meta-analysis of eleven different DLBCL gene expression datasets [50] (supplementary material, Table S5) revealed an inverse relationship between genes regulated by S1PR2 and LMP1. Thus, genes negatively correlated (OR=3.4; $p<0.0001$), but not those positively correlated (OR=0.69; $p=0.43$), with S1PR2 were significantly enriched among genes up-regulated by LMP1 in primary DLBCL. Likewise, genes positively correlated with S1PR2 were significantly enriched (OR=10.79;

p<0.0001), and those negatively correlated with S1PR2 significantly depleted (OR=0.42; p<0.001), from those down-regulated by LMP1.

LMP1-mediated down-regulation of S1PR2 promotes constitutive PI3-K signalling in DLBCL

Having shown that S1PR2 expression is frequently lost in LMP1-positive ABC-DLBCL, we next explored the mechanism responsible for this effect, focussing on PI3-K signalling. Consistent with previous reports, we found that LMP1 increased the phosphorylation of the PI3-K downstream target, AKT, in the B cell lines (Figure 4A) [15]. To study if PI3-K/AKT was responsible for S1PR2 down-regulation, we transfected SUDHL4 cells with a plasmid expressing a MYC-tagged constitutively active *AKT1* gene (MYC-caAKT1) and measured S1PR2 protein levels by multiplex IF. Induction of pAkt in SUDHL4 cells following MYC-caAKT1 transfection significantly decreased the numbers of cells expressing S1PR2 (p<0.0001; Figure 4B). To determine if the down-regulation of S1PR2 by PI3-K/AKT was at the promoter level, we repeated transfections, also using a plasmid expressing a constitutively active p110 (PI3-K catalytic subunit) and performed *S1PR2* promoter assays. The ectopic expression of LMP1, MYC-caAKT1 and p110 significantly inhibited *S1PR2* promoter activity (Figure 4C). Conversely, the inhibition of PI3-K significantly increased *S1PR2* promoter activity in LMP1-transfected, and in control-transfected, DLBCL cell lines (Figure 4D). To show a relationship between S1PR2 expression and PI3-K signalling also in primary DLBCL, we re-analysed published data on the transcriptional effects of the PI3-K inhibitor, 15e on the ABC cell line, HBL1 [40]. We found that genes regulated by LMP1 and

correlated with S1PR2 in DLBCL were significantly enriched for genes concordantly regulated by PI3-K in HBL1 cells ($p<0.0001$; supplementary material, Table S6A,B).

S1PR2 itself can also inhibit AKT activity in mouse B cells [18]. We investigated if this was also the case in DLBCL. Re-expressing S1PR2 in U2932 cells, an ABC line with low endogenous *S1PR2* levels, reduced AKT phosphorylation (Figure 4E). Gene editing of both alleles of *S1PR2* in SUDHL4 cells (supplementary material, Figure S3A,B), which have wild-type *S1PR2*, *GNA13*, *P2YR8* and *ARHGEF1* genes and therefore intact S1PR2 signalling [41, 42] also increased AKT phosphorylation (Figure 4F). Therefore, loss of S1PR2 expression can itself further increase PI3-K activity. Thus, our data reveal a signalling loop which acts to sustain PI3-K signalling in DLBCL (Figure 4G).

LMP1-regulated PI3-K targets are enriched for lymphoma-associated transcription factors and associated with shorter survival in ABC-DLBCL

Integration of the different gene expression datasets enabled us to identify a core set of 41 genes concordantly regulated by LMP1 and PI3-K signalling and significantly and inversely correlated with S1PR2 expression in DLBCL ($p<0.0001$; Figure 5A). These included *S1PR2* itself, as expected. GO analysis revealed that this core set was significantly enriched for transcription factors (Figure 5B), including *ATF3*, *BATF* and *BATF3* which were up-regulated, and *BCL6*, *BPTF*, *KLF12*, and *MEF2C* which were down-regulated, by LMP1. RT-qPCR confirmed the down-regulation of *BCL6*, *BPTF*, *KLF12* and *MEF2C* by LMP1 (Figure 5C) and by

AKT1 (supplementary material, Figure S4A). *BCL6*, *KLF12*, and *MEF2C* were also up-regulated following the treatment of control or LMP1-transfected cells with the pan-PI3-K Ly294002, the p100-δ-specific CAL-101 and the pan-AKT MK-2206 inhibitors (supplementary material, Figure S4B).

Re-analysis of published global gene expression data confirmed that the transcription factors in our core set were also concordantly differentially expressed in ABC-DLBCL (supplementary material, Figure S5A,B). Using IHC and commercially available antibodies suitable for FFPE tissues, we confirmed the increased expression of BATF and BATF3, and the decreased expression of two transcription factors; MEF2C and KLF12, not previously described before in ABC-DLBCL (Figure 5D).

Finally, we explored the overlap of LMP1-PI3-K targets with genes associated with clinical outcome in DLBCL. Re-analysing the dataset reported by Lenz *et al* [38], but confining our analysis to only those patients with ABC-DLBCL treated with R-CHOP, the current standard-of-care treatment, we found that genes down-regulated by LMP1-PI3-K, including *MEF2C* and *KLF12*, were significantly enriched among genes for which lower expression was associated with poorer overall survival (p=0.0011; Figure 5E).

We conclude that LMP1 and PI3-K regulate a core set of B cell-associated transcription factors, including several not previously described in ABC-DLBCL that are associated with shorter overall survival in patients with ABC-DLBCL.

DISCUSSION

We have compared the transcriptional effects of the EBV-encoded LMP1, with that of its physiological homologue, CD40, in normal human GC B cells to identify the oncogenic effects of LMP1 relevant to the pathogenesis of DLBCL. We used GC B cells because they are the cell type readily isolatable from tonsils which are the nearest equivalent to the stage of B cell differentiation from which ABC-type DLBCL is believed to derive. We found that LMP1, but not CD40, down-regulated the expression of S1PR2, a G-protein-coupled receptor that binds the bioactive sphingolipid metabolite S1P, and which signals through its ability to couple to the small G proteins, Gα12 and Gα13 [51]. Although we used soluble CD40L, which is considered to provide a relatively weak CD40 signal compared to the membrane bound trimeric forms of CD40L, Basso *et al*, reported the effects of trimeric CD40L (NIH3T3mCD40L cells) on B cells and also did not observe any downregulation of S1PR2 even after 24 h of stimulation [52].

Our IHC studies also revealed for the first time the loss of S1PR2 protein expression in DLBCL. Moreover, LMP1-expressing ABC-DLBCL were significantly more likely to lack tumour cell expression of S1PR2 than were LMP1-negative cases suggesting that LMP1 is important for the down-regulation of S1PR2 in EBV-positive primary DLBCL. S1PR2 is critical for the maintenance of normal GC integrity. For example, in mice, S1PR2 deficiency leads to the increased survival and migration of GC B cells in response to antigen stimulation, resulting in enlarged and less well-defined GC [18]. Thus, the down-regulation of S1PR2 by LMP1 might

also explain the previous observation that unlike CD40, LMP1 disrupts normal GC formation in transgenic mice [12].

Several lines of evidence show that S1PR2 is a tumour suppressor in B cells. For example, S1PR2 deficient mice have an increased propensity to develop lymphomas that resemble human DLBCL [20]. Furthermore, ectopic expression of S1PR2 has been shown to induce apoptosis in DLBCL cell lines; an effect that was equally efficient in both GCB and ABC-derived lines [22]. S1PR2 can also suppress the outgrowth of xenografts of DLBCL cells in immuno-deficient mice, and its loss accelerates MYC-driven lymphoma development in mice [22].

Our data support the existence of an alternative pathway to inactivate S1PR2 in DLBCL. Thus, in GCB-DLBCL, S1PR2 signalling is silenced primarily by mutations in *S1PR2* itself, or by mutations or methylation of other genes involved in S1PR2 signalling, including *GNA13*, *ARHGEF1* and *P2RY8* [18–21, 41]. In contrast, in ABC-DLBCL, S1PR2 inactivation appears to occur primarily as a consequence of transcriptional down-regulation. In some cases this is mediated by over-expression of the FOXP1 transcription factor [22]. Our data reveal that LMP1 is another upstream repressor of S1PR2 transcription important for the EBV-positive forms of ABC-DLBCL. The regulation of S1PR2 by LMP1 may be independent of FOXP1 since it has been shown that *FOXP1* mRNA levels are lower in EBV-positive compared with EBV-negative DLBCL [53]. In keeping with this we observed either no change or only a modest

up-regulation of *FOXP1* mRNA following LMP1 expression in different B cell lymphoma cell lines (supplementary material, Figure S6).

We showed that the transcriptional down-regulation of *S1PR2* or its knock out by CRISPR/Cas9 increased PI3-K signalling in DLBCL. Moreover, we showed that the transcription of *S1PR2* itself can be suppressed by the activation of either PI3-K or AKT. Thus, our data support a model in which the transcriptional down-regulation of *S1PR2* is sufficient to activate the PI3-K/AKT pathway, in turn initiating a signalling loop leading to further loss of S1PR2. Our data also suggest that in EBV-positive cases of DLBCL, this signalling loop is likely to be driven by the EBV oncoprotein, LMP1. Our data do not agree with the findings of Flori *et al* [22] who showed no effect of the ectopic expression of S1PR2 on AKT phosphorylation in DLBCL cell lines, including U2932, used in our study. We observed robust decreases in AKT phosphorylation in U2932 cells transfected with S1PR2, but not at the later time points (>48 h) when there were substantially reduced numbers of S1PR2-transfected compared to vector-transfected cells (not shown); presumably reflecting the well-described negative effect of S1PR2 on cell survival.

Dependency on PI3-K signalling for survival is apparent in both COO subtypes, but is driven by different molecular abnormalities [54, 55]. Thus, PTEN loss is an important mechanism of PI3-K/AKT activation in some GCB-DLBCL. In contrast, ABC-DLBCL cells are not dependent upon AKT signalling for survival, but require the PI3-K mediated induction of NF-κB activation [40, 56]. In keeping with this we observed that the core set of LMP1-PI3-K

regulated genes significantly overlapped with NF-κB target genes that were previously shown to be dependent upon PI3-K signalling in ABC-DLBCL (OR=38.16; p<0.0001, not shown) [40]. Our experiments also revealed that LMP1 can down-regulate S1PR2 in the absence of a functional PI3-K pathway, suggesting that NF-κB might also directly as well as indirectly impact on the down-regulation of S1PR2 expression in ABC-DLBCL. In support of this, the *S1PR2* promoter contains a number of NF-κB binging sites (supplementary material, Figure S3B).

In keeping with a previous report [22], we found that low S1PR2 expression was associated with significantly reduced overall survival in patients with DLBCL (not shown). However, when we refined this analysis we found that this effect was no longer apparent when patients were stratified by COO subtype (not shown), presumably because, as we showed here, the mRNA levels of *S1PR2* are substantially lower in ABC-DLBCL, compared to GCB-DLBCL. In contrast, while S1PR2 alone had no impact on prognosis in the different COO subtypes when analysed separately, we found that among ABC patients receiving R-CHOP therapy, the LMP1-driven gene expression signature associated with the concomitant loss of *S1PR2* and the activation of PI3-K signalling, was enriched among genes associated with poor survival. These observations are consistent with reports that PI3-K/AKT signalling confers inferior survival in ABC-DLBCL [57] and could explain the reported poorer outcomes for patients with EBV-positive DLBCL [5, 6, 8-10].

Several of the transcription factors present in this core signature have to our knowledge not previously been reported before in DLBCL. They include BPTF, a c-MYC interactor that is required for c-MYC chromatin recruitment and transcriptional activity [58], as well as KLF12 and MEF2C, which we found to be associated with poor prognosis. KFL12, a member of the Kruppel-like family of transcription factors, is a putative tumour suppressor gene that regulates anoikis by promoting cell cycle transition through S phase [59]. MEF2C is necessary for GC formation [60], and may like its relative MEF2B which is mutated in DLBCL, also regulate BCL6 transcriptional activity [61]. Furthermore, we found that low expression of KLF12 was associated with poorer overall survival in both RCHOP- and CHOP-treated DLBCL patients, whereas low expression of MEF2C was associated with shorter overall survival only in RCHOP patients suggesting a possible interaction with rituximab (not shown).

Pharmacological inhibition of PI3-K signalling can suppress lymphoma growth and survival in pre-clinical tumour models of DLBCL [4, 40, 55]. For example, the PI3-Kα/δ inhibitor AZD8835 has potency in ABC-DLBCL models, whereas the AKT inhibitor AZD5363 induces apoptosis in PTEN-deficient DLBCL [4]. Our data suggest that targeting S1P or its receptors could provide an alternative approach to suppress aberrant PI3-K signalling in ABC-DLBCL patients with deficient S1PR2 expression.

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Author contributions statement

MV, KV, EN, CW, DKu, GT, PGM designed research; MV, KV, MI, EN, SM, LL, WS, AS, TO, MA, SF, AD, ZR performed research/analysed data; RH, RT, MC, DK, WW, PGM performed statistical analysis; MV, KV and PGM wrote the manuscript. All authors approved the manuscript.

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*Cited only in supplementary material.

FIGURE LEGENDS

Figure 1. Identification of putative pathogenic LMP1 targets in DLBCL. (A) Comparison of the transcriptional targets of LMP1 and CD40 in primary human GC B cells revealed a statistically significant overlap ($p<0.0001$). Moreover, LMP1 target genes were significantly enriched in genes concordantly differentially expressed when primary DLBCL was compared to normal GC B cells ($p<0.0001$). Shown here is data from Brune et al. In this dataset DLBCL was not split by COO subtype [39]. (B) LMP1 target genes were also significantly enriched in genes differentially expressed when ABC-DLBCL was compared to normal GC B cells ($p<0.0001$). This analysis used data from Morin *et al* (DLBCL) [41, 42] and Beguelin *et al* (GC B cells) [43]. (C) RT-qPCR was used to confirm the regulation of selected genes by LMP1, but not by CD40. Data are in triplicate and each is representative of the results of three separate donors. TNFAIP3, a known LMP1 and CD40 target, is used as a positive control. Values are presented as $2^{-\Delta\Delta CT}$ and relative to a GC B cell control. *denotes $p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's *t*-test).

Figure 2. LMP1, but not its physiological homologue CD40, down-regulates the expression of S1PR2 in B cells. (A) RT-qPCR analysis of *S1PR2* expression in LMP1-transfected and CD40-stimulated GC B cells. Data presented are triplicates and each is representative of the results of three separate donors. Values are presented as $2^{-\Delta\Delta CT}$ relative to GC B controls. **denotes $p<0.01$ (Student's *t*-test). (B) *S1PR2* expression measured by RT-qPCR in LMP1-transfected or CD40-stimulated DLBCL cell lines, SUDHL4 and OCI-Ly1. Data presented are triplicates and

each is representative of the results of three separate biological replicates. Statistics are presented as $2^{-\Delta\Delta CT}$ relative to controls. *denotes p<0.05 (Student's t test). ns=non-significant, ns^ indicates no significant down-regulation. (C) Multiplex IF for LMP1 (green) and either S1PR2 or ICAM1 (red) in SUDHL4 cells transfected with LMP1. LMP1-positive cells (white arrows) were significantly more likely to be S1PR2-negative and ICAM1-positive than LMP1-negative cells. Lower panels show the proportions of SUDHL4 cells positive for S1PR2 or ICAM1 by LMP1 status and data from Farage and L591 cells and are representative of three separate biological replicates. Original magnification x600. (D) *S1PR2* mRNA (top panel) and promoter activity (bottom panel) in LMP1-transfected DG75 and BJAB cells. Data are representative of three biological replicates. *denotes p<0.05, ***p<0.001, and ****p<0.0001 (Student's *t*-test).

Figure 3. Down-regulation of S1PR2 in LMP1-expressing primary DLBCL. (A) Left panel: Significantly lower *S1PR2* expression in ABC- and GCB-DLBCL compared with normal GC B cells in a re-analysis of existing RNAseq datasets (p<0.0001 and p=0.002 respectively) [41–43]. Right panel: Significantly lower *S1PR2* expression in ABC-DLBCL compared with GCB-DLBCL (p<0.0001) [38]. (B) Top left panel: Normal GC B cells express S1PR2 protein. Top middle panel: a case of testicular DLBCL that is negative for S1PR2. Tumour cells are shown by the black arrow and positive staining for S1PR2 in normal seminiferous tubules by the white arrow. Top right panel: a case of DLBCL that is S1PR2-positive. Bottom left panel: co-staining for LMP1 (red) and S1PR2 (green) in a representative case of EBV-positive DLBCL showing that the LMP1-positive tumour cells are S1PR2-negative (white arrows). A blood

vessel is stained for S1PR2. Bottom right panel; a case of LMP1-negative DLBCL expressing S1PR2 stained for CD20 (red) and S1PR2 (green). Original magnifications x200 and x600.

Figure 4. LMP1-mediated down-regulation of S1PR2 promotes constitutive PI3-K/AKT signalling. (A) LMP1 increased the phosphorylation of AKT in BJAB and SUDHL4 cells. SUDHL4 lysates were from the same experiment in which we had shown that LMP1 down-regulates S1PR2 expression (Figure 2B; left panel). Levels of each protein are shown in LMP1-transfected cells compared with those transfected with empty vector (control). (B) SUDHL4 cells were transfected with a plasmid expressing a MYC-tagged constitutively active AKT1 and the cells stained for the MYC tag, pAKT and S1PR2 by multiplex IF. Cells transfected with the AKT1 plasmid (white arrows) and expressing the MYC-tag (green) were significantly more likely to be pAKT-positive (yellow) and to lack expression of S1PR2 (red) than were un-transfected cells in the same population ($p<0.0001$). Original magnification x600. (C) *S1PR2* promoter activity in BJAB and DG75 cells transfected either with LMP1, a constitutively activated AKT1, or a constitutively activated p110. (D) Inhibition of PI3-K signalling by LY294002 increased *S1PR2* promoter activity in LMP1-transfected and in control-transfected B cell lymphomas. (E) Ectopic expression of S1PR2 in U2932 cells reduced the levels of phosphorylated AKT. (F) Compared with wild-type controls (designated LA2, HB2, HB4), CRISPR/cas9-mediated knockout of *S1PR2* in three clones of SUDHL4 cells (designated HA1, HA2, HC3) resulted in increased levels of phosphorylated AKT. For all RT-qPCR, S1PR2 promoter assays and immunoblotting experiments, representative examples of three separate biological replicates are shown. *denotes $p<0.05$, ***$p<0.001$, and

****p<0.0001 (Student's *t*-test). G) Diagrammatic representation of our proposed signalling loop driven by LMP1, in which S1PR2 down-regulation activates PI3-K/AKT signalling, in turn leading to the further down-regulation of S1PR2 expression.

Figure 5. LMP1-regulated PI3-K targets are enriched for lymphoma-associated transcription factors and associated with shorter overall survival in ABC-DLBCL. (A) Identification of a subset of 41 transcriptional targets of LMP1 that were also differentially expressed following treatment with a PI3-K inhibitor and anti-correlated with *S1PR2* in DLBCL, including S1PR2 itself. (B) Gene set enrichment analysis of these genes revealed significant enrichment of GO terms associated with transcriptional regulation (asterisks). Grey bars show the observed percentage, and black bars the expected percentage of genes in a GO category. Transcription factors among the 41 target genes are shown in boxes in A). (C) RT-qPCR shows that the down-regulation of S1PR2 by LMP1 in B cell lymphoma cell lines was accompanied by the down-regulation of, *BCL6*, *BPTF*, *KLF12*, and *MEF2C*. Shown are data in three cell lines; each is representative of three separate biological replicates. *denotes p<0.05, **p<0.01, ***p<0.001 (Student's *t*-test). (D) Representative examples of BATF, BATF3, MEF2C and KLF12 staining in normal lymphoid tissues and primary ABC-DLBCL (Original magnifications x200 and x600), and right panel, H-scores of these cases confirming the up-regulation of BATF and BATF3 and the down-regulation of KLF12 and MEF2C proteins in the tumour cells of ABC-DLBCL classified according to the Hans algorithm. (E) Kaplan–Meier curves showing that the down-regulation of *MEF2C* and *KLF12* was associated with

significantly reduced overall survival in patients with ABC type DLBCL receiving R-CHOP therapy.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods YES

Supplementary figure legends NO, because legends are embedded in the single Suppl Fig PPTX file

Figure S1. Expression of *S1PR2* in DLBCL cell lines

Figure S2A. Expression of ICAM1 after stimulation of different B cell lymphoma cell lines with CD40L

Figure S2B. Downregulation of S1PR2 after the expression of a constitutively active CD40

Figure S3A. DNA and protein sequences of three independent CRISPR knockout clones (HA1, HA2 and HC3)

Figure S3B. Predicted transcription factor binding sites in the *S1PR2* promoter

Figure S4A. Regulation of lymphoma-associated transcription factors by PI3-K/AKT signalling

Figure S4B. Regulation of lymphoma-associated transcription factors by PI3-K/AKT signalling

Figure S5A. Differential expression of LMP1-regulated transcription factors in primary DLBCL (data from Morin *et al* [41, 42] and Beguelin *et al* [43])

Figure S5B. Differential expression of LMP1-regulated transcription factors in primary DLBCL (data from Lenz *et al* [38])

Figure S6. Expression of *FOXP1* after transfection of different B cell lymphoma cell lines with LMP1

Table S1A-B. RT-qPCR assays and antibodies used

Table S2E–H. Genes regulated by LMP1 and differentially expressed in DLBCL

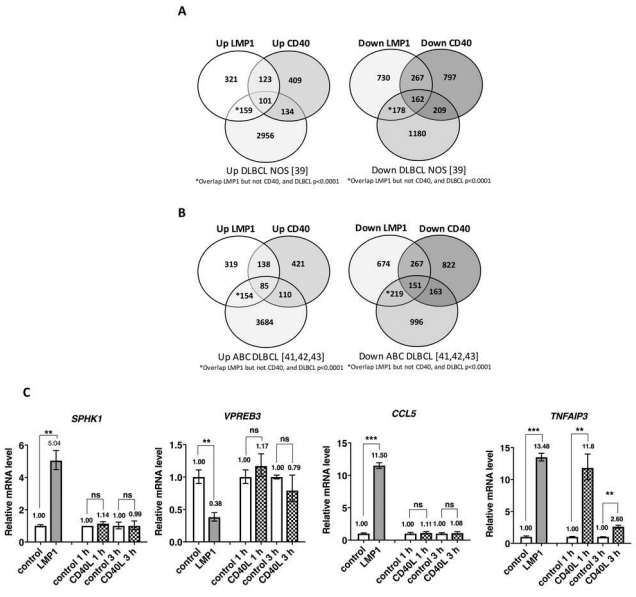
Table S3A–D. Genes differentially expressed in DLBCL

Table S3E,F. Counts for SUDHL4 cells stained with LMP1 and either S1PR2 or ICAM1

Table S4. Counts for S1PR2, LMP1 and EBER positivity in non-GC cases

Table S5. Genes correlated with *S1PR2* in DLBCL (all significant genes)

Table S6A,B. Genes differentially expressed in HBL1 cells after treatment with 15e



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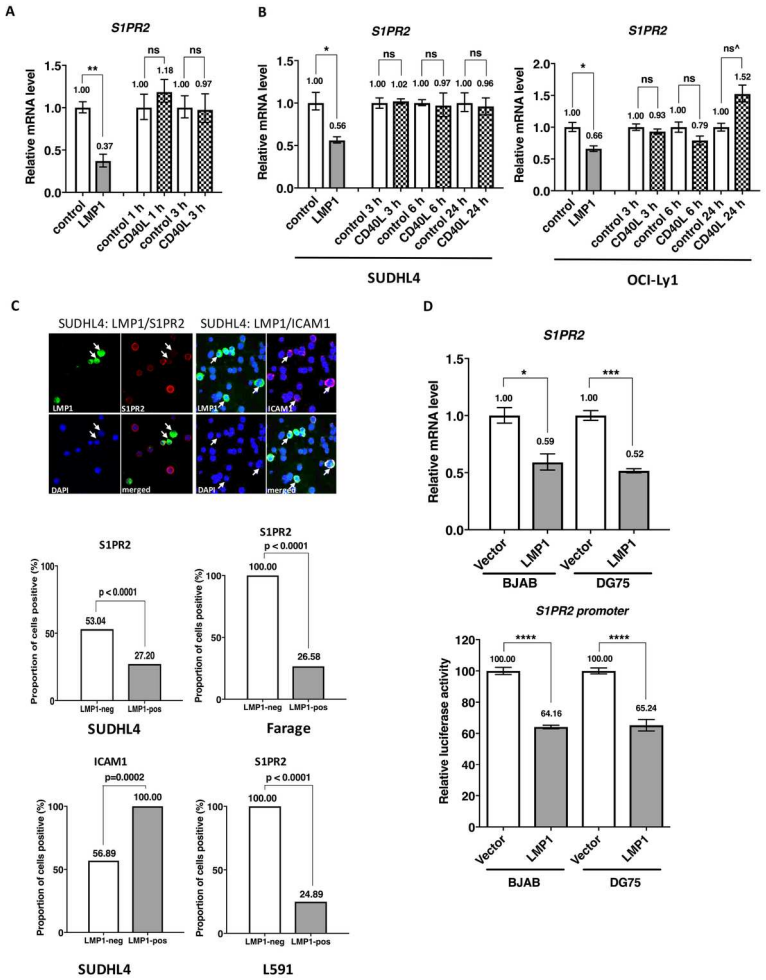

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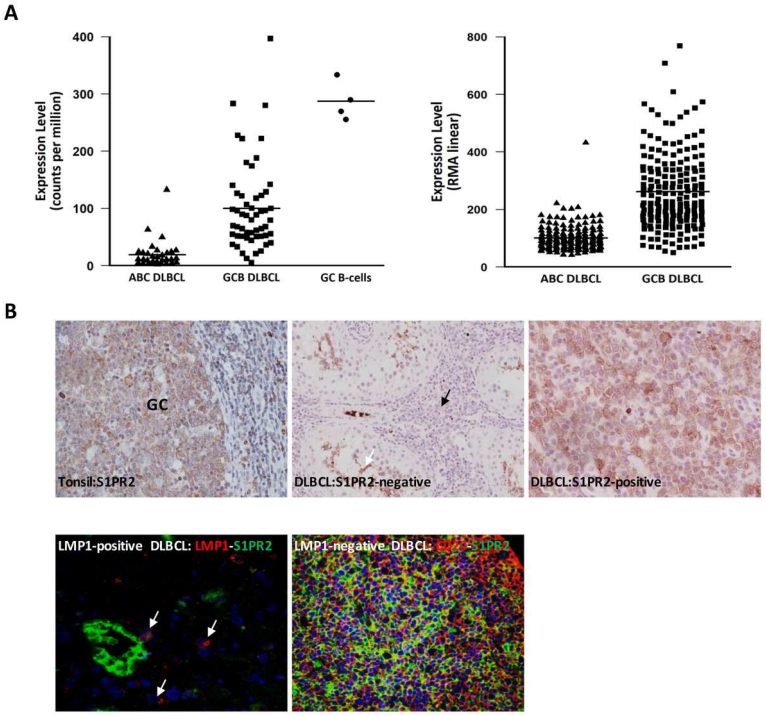

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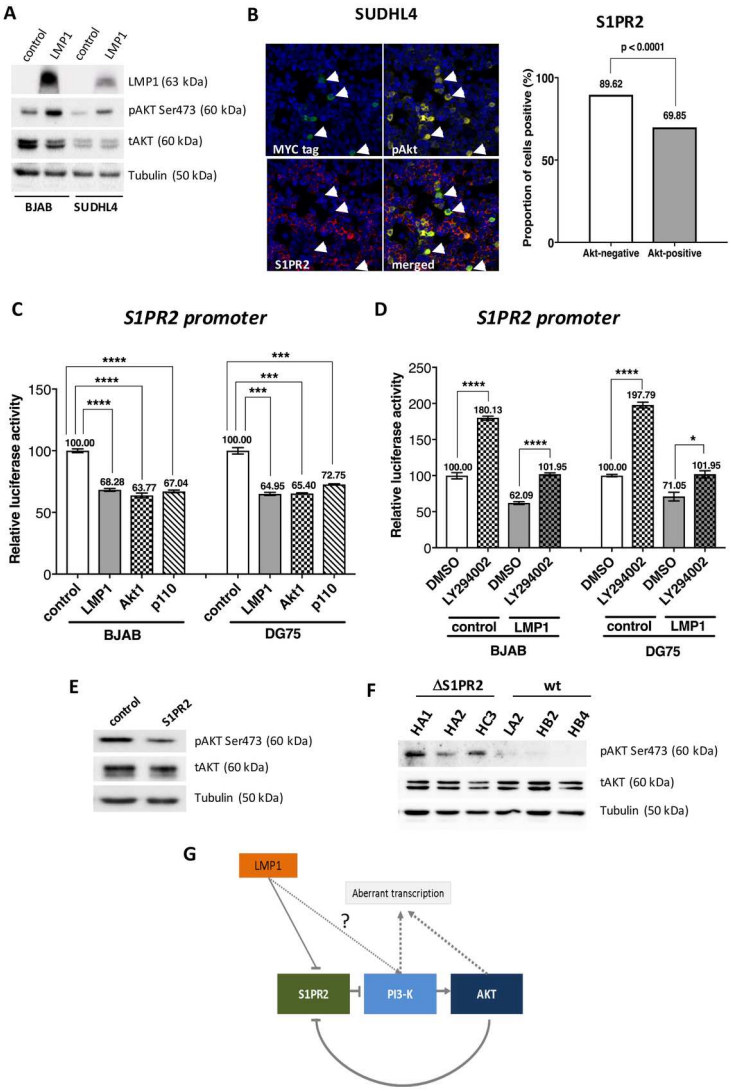

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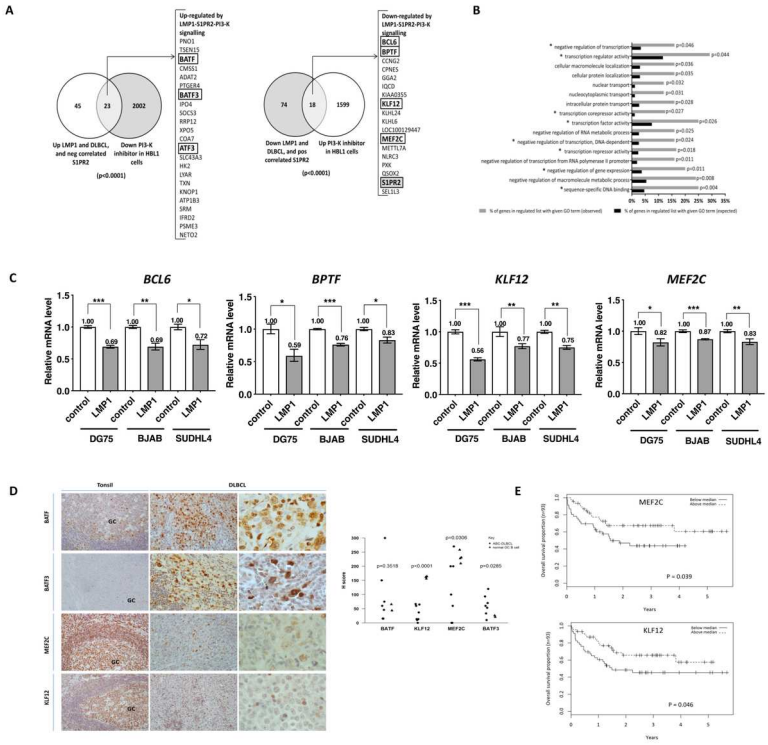

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