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G-protein coupled receptor kinase-2 regulates the migration of chronic lymphocytic leukaemia cells to sphingosine-1 phosphate in vitro and their trafficking in vivo

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Disease progression and drug resistance in patients with chronic lymphocytic leukaemia (CLL) depend on signals from the tumour microenvironment in lymphoid sites. GRK2 inhibits the egress of normal B cells from lymphoid tissues by inducing the downregulation of the S1P-receptor 1 (S1PR1). In this study we investigated the role of GRK2 in the context of CLL using in vitro and in vivo murine models, and also primary samples from CLL patients. We found that pharmacological inhibition of GRK2 enhanced the migration of leukemic cells from CLL patients towards S1P and impaired the S1P-induced downregulation of S1PR1. Likewise, CRISPR/Cas9-mediated GRK2 deletion in a murine leukemic cell line derived from the Eμ-TCL1 mouse model of CLL also increased migratory capacity toward S1P in vitro. Furthermore, when injected into mice, GRK2-deficient murine leukemic cells exhibited an altered in vivo localization, with a higher presence in the blood and spleen compared to the bone marrow. Within the spleen, these cells displayed reduced localization to the follicles compared to control murine leukemic cells. Deletion of GRK2 on murine leukemic cells did not affect their in vitro proliferation, but notably, conferred a growth disadvantage in vivo. These findings underscore GRK2 as a critical regulator of the localization of CLL cells in vivo and suggest its potential as a therapeutic target to disrupt survival niches in CLL.

Chronic lymphocytic leukaemia (CLL) is an incurable disease characterized by the accumulation of clonal B cells in blood and lymphoid organs[1]. Proliferation of leukemic cells mainly occurs within lymphoid tissues driven by the stimulation of the B cell receptor (BCR) and by signals provided by T cells, stromal cells and macrophages[2–4]. These signals also induce the resistance of CLL cells to therapeutic agents[5–10], supporting the idea that the tumour microenvironment within lymphoid tissues contributes to disease progression and relapse. Therefore, mobilization of tissue-resident CLL cells into the circulation could be exploited as a strategy to minimize the reservoir of tumour cells within growth and survival niches.

The egress of lymphocytes from lymphoid tissues into circulation depends on the expression of the sphingosine-1 phosphate (S1P)-receptor-1 (S1PR1) in lymphocytes and the high concentrations of its ligand, S1P, in the blood and lymph[11]. The G-protein coupled receptor kinase-2 (GRK2) is responsible for the down-regulation of S1PR1 in the S1P-rich circulatory fluids which allows the lymphocyte to overcome the S1P-mediated retention in the blood and to enter into the lymphoid organs guided by chemokines[11–13]. Migration towards the high concentration of S1P in the vascular compartment also mediates the exit of B cells from lymphoid tissues to circulation. The key role of GRK2 for lymphocyte homing was confirmed in mouse models, where the deletion

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of GRK2 on B cells lead to an increased response to S1P and thus a higher presence of B cells in peripheral blood over bone marrow, lymph nodes and spleen follicles[12,14].

We have previously reported that signals present in the tumour microenvironment reduce S1PR1 expression in CLL cells[15]. Others have reported a reduced expression of S1PR1 in CLL cells from patients with poor prognosis (unmutated IGHV) or lymphadenopathies[16,17], suggesting that this low S1PR1-expression might favour leukemic cells growth by prolonging their residency in the pro-survival niche within lymphoid organs. Here we aim to evaluate the role of GRK2 in modulating the S1P response in CLL, focusing on its role as a regulator of leukemic cell homing and potential therapeutic target.

Methods and materials

CLL patients and healthy donor samples

Peripheral blood (PB) samples were obtained from CLL patients and age-matched healthy donors. The study was approved by the local Ethics Committee from the National Academy of Medicine, Buenos Aires, Argentina, according to institutional guidelines (Protocol approval code 18/24/CEIANM). Peripheral blood samples were obtained after signed informed consent. The research was performed in accordance with the Declaration of Helsinki. International ethical guidelines were strictly followed. CLL was diagnosed according to standard clinical and laboratory criteria. At the time of analysis, all patients were free from clinically relevant infectious complications and were treatment naïve or had not received treatment for ≥ 3 months before sampling. Clinical characteristics of CLL patients included in this study are depicted in Supplementary Table 1.

Mouse model

Eμ-TCL1 transgenic mouse model of CLL was kindly provided by Dr. Croce and Dr. Chiorazzi to the animal facility of the IMEX-CONICET-Academia Nacional de Medicina, Buenos Aires, Argentina. C57BL/6 mice from the IMEX-CONICET were purchased from Jackson Laboratory (ME, USA) and C57BL/6 from the ICGEB were purchased from ENVIGO Laboratories (IN, USA). Mice were maintained in specific pathogen-free (SPF) conditions at both animal facilities and were housed in standard polypropylene transparent cages under environmentally controlled conditions (temperature, 24 ± 2 °C; humidity, 50% ± 10%) with a 12 h light:12 h dark cycle. All animal procedures performed in Argentina were approved by the Institutional Animal Care and Use Committee at IMEX-CONICET-Academia Nacional de Medicina (protocol number 106/2022) in accordance to the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee, 2011). All animal procedures carried out in Italy were performed in accordance with Italian legislative decree 26/2014 and European directive 2010/63/EU and were conducted under a protocol approved by the Italian Ministry of Health (no. 218/2022-PR). Recommendation from the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020) were strictly followed. Study was reported in accordance with ARRIVE guidelines.

Cell purification and DNA isolation

Normal B cells from healthy donors and leukemic B cells from CLL patients were purified by negative selection with the B cell Microbead isolation kit (purity > 95%) or B-CLL Microbead isolation kit (purity > 98%), respectively (both from Miltenyi, Germany). Murine B cells were purified with the MojoSort™ Mouse CD19 Nanobeads (Biolegend, CA, USA) or the EasySep Mouse CD19 Positive Selection Kit II (Stem Cell Technologies, Canada) (purity > 95%). DNA was extracted with the KAPA Mouse Genotyping Kit (KAPA Biosystems, MA, USA).

CRISPR/Cas9-editing of murine cells

TCL1-355 TKO cells were edited using the CRISPR/Cas9 Alt-R system (Integrated DNA Technologies [IDT]), as previously described[18]. Predesigned CRISPR RNA (cr-RNA) for targeting the GRK2 was used. Briefly, Cas9 ribonucleoprotein (RNP) complexes were generated combining 1.5 μM cr-RNA with 1.5 μM ATTO 550-labeled trans-activating cr-RNA, 0.75 μM recombinant Cas9 protein, and 1.5 μM Alt-R Cas9 electroporation enhancer in 5 μL of nuclease-free duplex buffer (IDT). Complexes were then electroporated into 6×10^6 leukemic cells resuspended in 95 μL of mouse B-cell nucleofector solution (Lonza, Switzerland) using the Z-001 program in the Amaxa Nucleofector II device. Control cells were nucleofected with complexes without cr-RNA. ATTO 550-positive cells were analysed by flow cytometry 3 h after transfection to evaluate transfection efficiency. Editing efficiency analysis was performed on an aliquot of the nucleofected cells collected 48 h after transfection. Editing efficiency was evaluated by amplicon capillary electrophoresis of polymerase chain reaction (PCR) fragments spanning the region of genomic DNA around the targeted site[19]. Mutant allele frequency (MAF) was calculated as previously described[18]. Western blot analysis was performed to confirm lack of GRK2 expression in edited cells. cr-RNA and PCR primer sequences are provided in Supplementary Material and Methods.

In vivo localization assay and Immunofluorescence

GRK2-WT and -KO cells were labelled with CFSE and CellTrace™ Violet (CTV) respectively. Then, cells were mixed in a 1:1 ratio and 60×10^6 cells/mouse were transferred intravenously through the tail vein into 2–3 month-old female C57BL/6 mice. After 20 h, PB, spleen (SP) and bone marrow (BM) were collected and processed. Cell suspensions were stained with fixable live/dead kit and anti-CD19 mAb and acquired in a BD FacsCelesta. The ratio of GRK2-WT (CFSE+) / GRK2-KO (CTV+) cells was evaluated.

In order to evaluate cell localization within the SP, GRK2-WT and -KO cells were stained with CellTrace™ Yellow (CTY) and CTV, respectively. Cells were mixed in a 1:1 ratio, mice were injected as previously described for in vivo homing assay and after 20 h the SP was isolated and divided in two parts. One half was used for flow cytometry analysis in a BD FacsSymphony A5 SE flow cytometer, whereas the other half was processed for

immunostaining. Briefly, the latter SP section was fixed for 5 h in fixative solution (1% PFA, 0.075 M L-Lysine, 0.1 M Sodium phosphate, 0.01 M $NaIO_4$), washed 3 times in PBS and incubated in 30% sucrose for 16 h. The sucrose solution was then removed and the tissue was dried and imbedded in CRYOPLAST® (Biopack, Argentina). Crysostatic sections of 15-µm thickness were used for immunofluorescence staining with CD3 and CD169 to determinate follicle regions. Sections were mounted using Aqua-Poly-Mount[2]. Stained sections were microscopically analysed using a FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with the oil immersion objectives 20X (AN 0.75) and 4X (AN 0.16). As previously described[3], CD169 + macrophages were used to delimit the spleen follicles, and then, GRK2-WT (CTY+) and GRK2-KO (CTV+) cells were counted in each region (inside follicles/white pulp and outside follicles/red pulp) using the Fiji, ImageJ software. For each spleen section, a total of 6 to 10 different images 20X were quantified. The number of cells counted in each region was then normalized to the total area of the analysed region.

In vivo tumour growth

A mix of GRK2-WT and GRK2-KO cells (3×10^7 cells) were injected intraperitoneally into 2–3 month old C57BL/6 female mice. Two independent experiments were performed with KO: WT ratios of 95:5 and 60:40. Leukaemia expansion was evaluated by WBC counts and CD5/CD19 flow cytometry analysis. After 9 to 15 days, mice were euthanized by inhalation of 5% isoflurane and peritoneal cavity (PC) lavage, SP and PB were obtained and processed. B cells from the PC, SP and PB were purified with positive selection with anti-CD19 kits as previously detailed.

Statistical analysis

Statistical significance was determined using the nonparametric tests: Mann-Whitney test, One sample Wilcoxon test, Wilcoxon matched-pairs signed rank test, Friedman test followed by Dunn's multiple comparison test. In all cases, $p < 0.05$ was considered statistically significant. Data were analysed using GraphPad Prism software version 8.4.2.

Additional information is provided in the Supplementary Materials and Methods.

Results

Leukemic cells from CLL patients express GRK2 at similar levels as B cells from healthy donors

GRK2 expression was reported to be altered in different tumour contexts with some cases showing over-expression[20–22] and others a decreased expression[23,24] compared with non-transformed cells. As a first step, we analysed GRK2 expression in public gene expression datasets comparing mRNA levels in leukemic cells from CLL patients and circulating B cells from age-matched healthy donors[25,26]. As shown in Fig. 1A and Supplementary Fig. 1 there was no differences in GRK2 expression between both groups. We obtained similar results when we evaluated protein levels of GRK2 on purified leukemic cells from CLL patients and B cells from age-matched healthy donors by western blot (Fig. 1B and Supplementary Fig. 1A and D). We also analysed the expression of GRK2 in CLL cells from patients with mutated (M) or unmutated (UM) IGHV genes at the mRNA level in published gene expression data sets[27,28] and at the protein level in our samples, and found no differences in GRK2 expression between the two IGHV mutational subsets (Fig. 1C-D and Supplementary Fig. 1B and C).

In vitro treatment of leukemic cells with a GRK2 inhibitor increases migration towards S1P

We next investigated the effect of CMPD101, a GRK2 inhibitor commonly used to target GRK2 in cultured cells[21,29,30], on the migratory response of leukemic cells from CLL patients towards S1P. First, given that GRK2 was reported to modulate cell viability in other tumour models[21,31], we evaluated if the treatment with CMPD101 affects leukemic cell viability or drug-induced apoptosis. As shown in Fig. 1E-F, CMPD101 at doses previously used for in vitro inhibition of GRK2 (3–30 µM)[21,29,30] did not affect the viability of the leukemic cells, either alone or in combination with the Bcl-2 antagonist venetoclax.

We then evaluated whether CMPD101 will affect the migration towards S1P of ex vivo CMPD101-treated CLL cells using a transwell migration assay. As shown in Fig. 2A, the presence of CMPD101 significantly increased the migration of the leukemic cells towards S1P, increased S1PR1 expression (Supplementary Fig. 2A) and also impaired the S1P-induced down-regulation of S1PR1 (Fig. 2B). On the other hand, migration towards CXCL13 and CXCL12 was not significantly affected by CMPD101 treatment (Supplementary Fig. 2B). We also evaluated the effect of CMPD101 on the S1P response of leukemic cells from the murine Eµ-TCL1 model of CLL. Consistent with the findings with leukemic cells from CLL patients, we observed that the presence of CMPD101 increases the in vitro migratory response to S1P of murine leukemic cells isolated from both PB and lymphoid tissues (Fig. 2C-E).

GRK2 deletion in the TCL1-355 TKO cell line

To further investigate the involvement of GRK2 in the S1P-response of leukemic cells, we generated a GRK2-deficient murine cell line by CRISPR/Cas9 gene editing of the TCL1-355 TKO cell line. This cell line has an auto-reactive BCR that binds to the apoptosis-associated autoantigen phosphatidylcholine (PtC) and was generated by introducing loss-of-function mutations in CDKN2A, CDKN2B and TP53 in CLL cells that derive from the Eµ-TCL1 mouse model (TCL1-355 cells)[18]. This genetically modified leukaemia cell line has the ability to spontaneously proliferate in vitro which makes it suitable for stable gene editing[18,32]. GRK2 targeting was done by nucleofecting ribonucleoparticle (RNP) complexes containing recombinant Cas9 and the guideRNA. We confirmed GRK2 editing at DNA level by amplicon capillary electrophoresis of the CRISPR/Cas9 targeted region with a 97% editing efficiency, consistent with an almost complete absence of GRK2 protein as assessed by western blot (Fig. 3A-C). As shown in Fig. 3D, GRK2-WT (wild type) and GRK2-KO (knock-out) leukemic

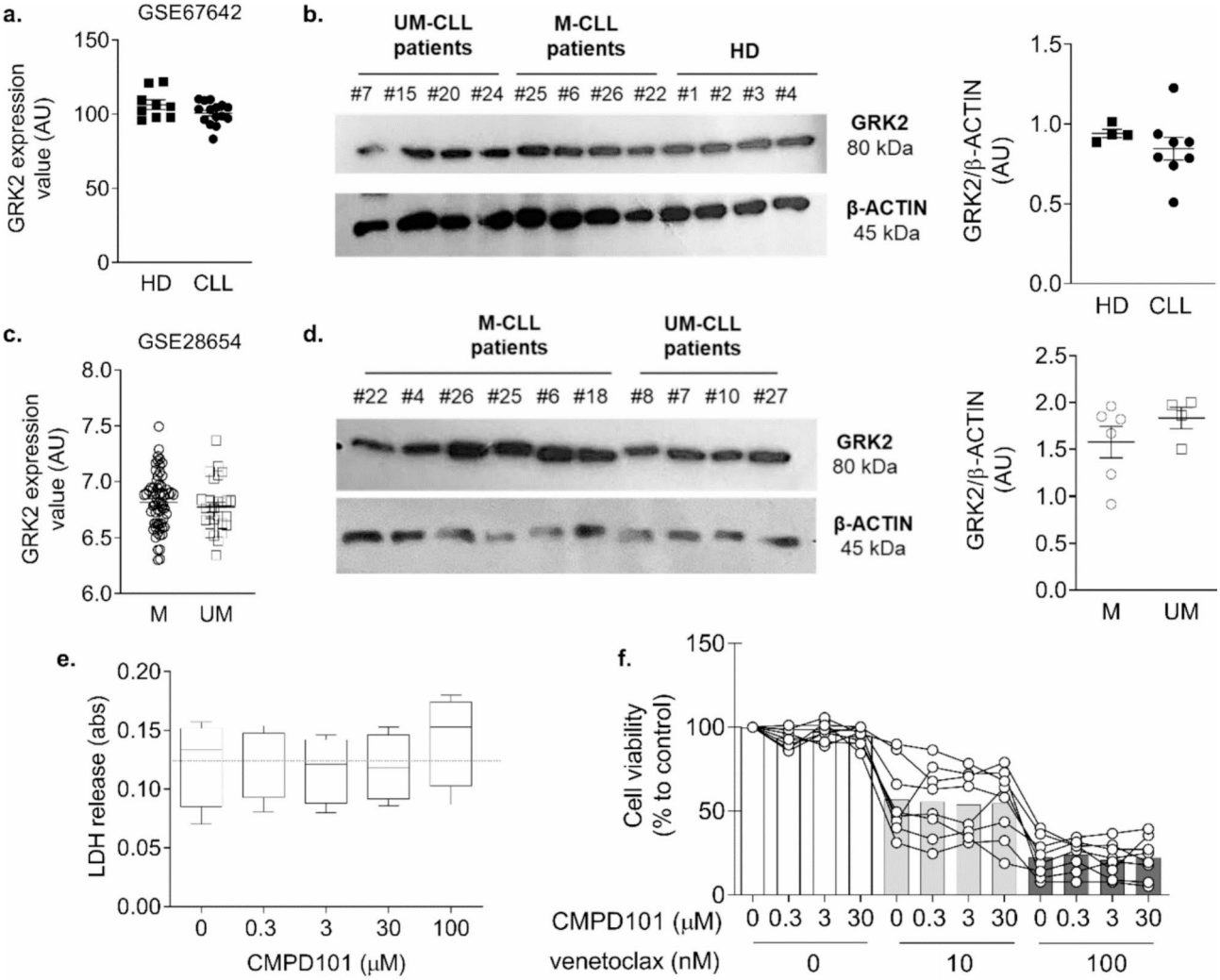


Fig. 1. GRK2 mRNA and protein levels are similar between B cells from healthy donors (HD) and CLL patients. **(a-d)** GRK2 mRNA and protein expression were analysed using different GEO datasets (GSE67642/GSE28654) **(a and c)** and western blot on purified leukemic B cells from CLL patients or B cells from age-matched HD **(b)** and **(d)**. β-actin was used as loading control. UM: unmutated IGHV. M: mutated IGHV. B cells from a total of 20 CLL patients and 10 healthy donors were analysed in different western blots. Representative blots are shown here and in Supplementary Figures. Mean ± SEM of the ratio GRK2/β-actin is shown. A.U.: arbitrary units quantified using the ImageJ software (NIH Image). Images of full length blots are shown in Supplementary Figs. 3–5. **(e-f)** PBMC from CLL patients were cultured in presence of different concentrations of the GRK2 inhibitor, CMPD101, or vehicle (DMSO) for 24 h. Then, cell viability was analysed by LDH assay ($n = 4$) **(e).** Venetoclax was added to cultures and, after 24 h, cell viability was assessed by flow cytometry ($n = 8$) **(f).** Mean ± SEM is shown.

cells have a similar proliferation rate in vitro. Also, GRK2 protein expression remained suppressed in cultured GRK2-KO cells over time (Fig. 3E), suggesting that there is no selection of the wild type allele in vitro. To further validate this observation, we cultured a mix of GRK2-WT and GRK2-KO cells in a ratio 1:2 and after 16 days of culture we evaluated the mutant allele frequency (MAF) by amplicon capillary electrophoresis. As shown in Fig. 3F, the MAF did not change at the end of the culture period, confirming no selection of the wild type allele in vitro.

Although the TCL1-355 TKO cell line has the ability to spontaneously proliferate in vitro, it remains BCR dependent, as evidenced by an impairment on proliferation in the presence of BCR inhibitors[18]. Given that GRK2 has been reported to associate with proteins involved in BCR-signaling[24], we evaluated if deletion of GRK2 affected the BCR dependency of this cell line. As shown in Fig. 4A, the proliferation of both GRK2-WT and GRK2-KO cells was equally impaired in the presence of BTK inhibitors, showing that GRK2 deletion does not modify BCR-dependency of the TCL1-355 TKO cell line. We also evaluated if GRK2 deletion affected the proliferation of the leukemic cell line in response to the TLR-stimuli CpG (TLR9 ligand) and LPS (TLR4 ligand), and found that proliferation of GRK2-KO cells in response to CpG remains unaffected, while proliferation in response to LPS was lower in GRK2-KO cells than in GRK2-WT cells (Fig. 4B-C). Finally, in line with what we

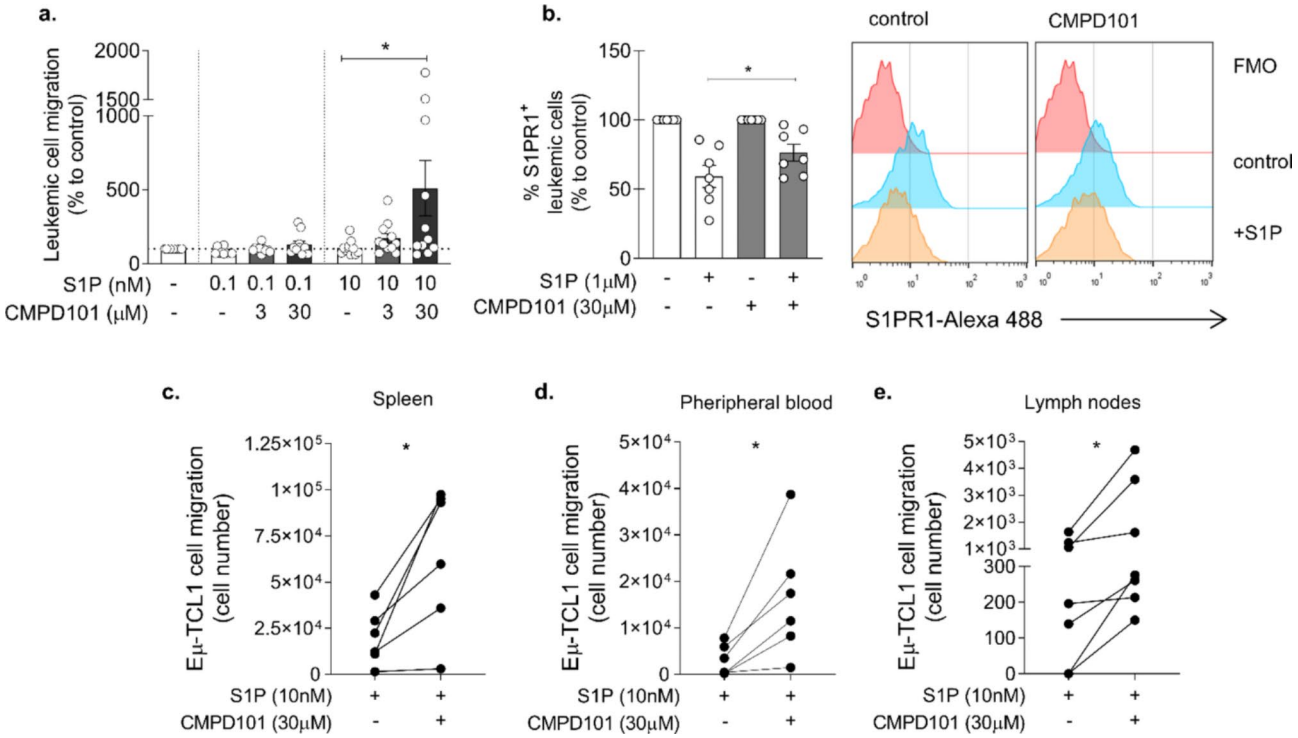


Fig. 2. GRK2 inhibition increases human and murine leukemic cell migration towards S1P in vitro. **(a)** Peripheral blood mononuclear cells (PBMC) from CLL patients were treated with CMPD101 or vehicle (DMSO), and then placed in the upper chamber of a transwell plate. The lower chamber was filled with medium alone (control) or medium with S1P. After 2 h, the number of leukemic cells that had migrated to the lower chamber was determined by flow cytometry, acquiring the samples for 60 s at a constant flow cell migration is expressed as the number of CD19 + cells that had migrated in response to S1P/ number of cells that had migrated spontaneously (without stimuli) taking spontaneous migration as 100%. ($n = 11$) The mean ± SEM is shown. *$p < 0.05$, Friedman test followed by Dunn's multiple comparisons post-test. **(b)** PBMC from CLL patients were cultured in the presence of CMPD101 or vehicle and S1P was added to cultures. After 1 h, S1PR1 expression on $CD19^+$ cells was evaluated by flow cytometry as described in methods. The mean ± SEM of the % of $CD19^+$ $S1PR1^+$ relative to control without S1P, is shown. ($n = 7$) *$p < 0.05$, Wilcoxon matched-pairs signed rank test. **(c-e)** Migration of Eμ-TCL1 cells towards S1P was evaluated as described in a. Cells from SP, PB and LN from leukemic Eμ-TCL1 mice were used ($n = 5$, 5 and 7 respectively). Graph shows the number of leukemic cells (CD19 + CD5+) that migrated in response to S1P minus leukemic cells that migrated spontaneously. *$p < 0.05$, Wilcoxon matched-pairs signed rank test.

observed with the GRK2 inhibitor in leukemic cells derived from CLL patients, GRK2 deletion in this cell line did not affect venetoclax sensitivity (Supplementary Fig. 2C).

GRK2 deletion increases leukemic cell migration to S1P

We next evaluated the migratory response of GRK2-WT and GRK2-KO cells to S1P. To this aim, we performed a transwell chemotaxis assay with a mix of GRK2-KO cells labelled with CFSE and unlabelled GRK2-WT cells in a 1:1 ratio. As shown in Fig. 4D, GRK2-KO leukemic cells had a higher migratory capacity towards S1P than GRK2-WT cells, and also a higher expression of S1PR1 (Fig. 4E). As expected, CMPD101 increased the response towards S1P of GRK2-WT but not of GRK2-KO leukemic cells (not shown). We also evaluated the migratory capacity of both cell lines towards CXCL12 and CXCL13, and consistent with what we observed with leukemic cells from CLL patients treated with CMDP101, we found that the migration towards CXCL12 and CXCL13 was similar on GRK2-WT and GRK2-KO leukemic cells (Supplementary Fig. 2D).

GRK2 deletion modifies in vivo localization of leukemic cells

To investigate the effect of GRK2 deletion on the in vivo localization of leukemic cells, we labelled GRK2-WT and GRK2-KO cells with different cell tracers and intravenously injected them into syngeneic (C57BL/6) mice in a 1:1 ratio. After 20 h we analysed their relative presence in PB, SP and BM by flow cytometry (Fig. 5A). As shown in Fig. 5B, GRK2-KO leukemic cells preferentially localized in the PB and SP, and were underrepresented in BM compared to GRK2-WT leukemic cells.

Given that the GRK2-S1P axis is also relevant for the positioning of B cells within spleen follicles[14], we next analysed the localization of the GRK2-KO and GRK2-WT cells in the spleen by fluorescence microscopy. To this aim, we injected mice as previously described and after 20 h the spleen was obtained and analysed by confocal

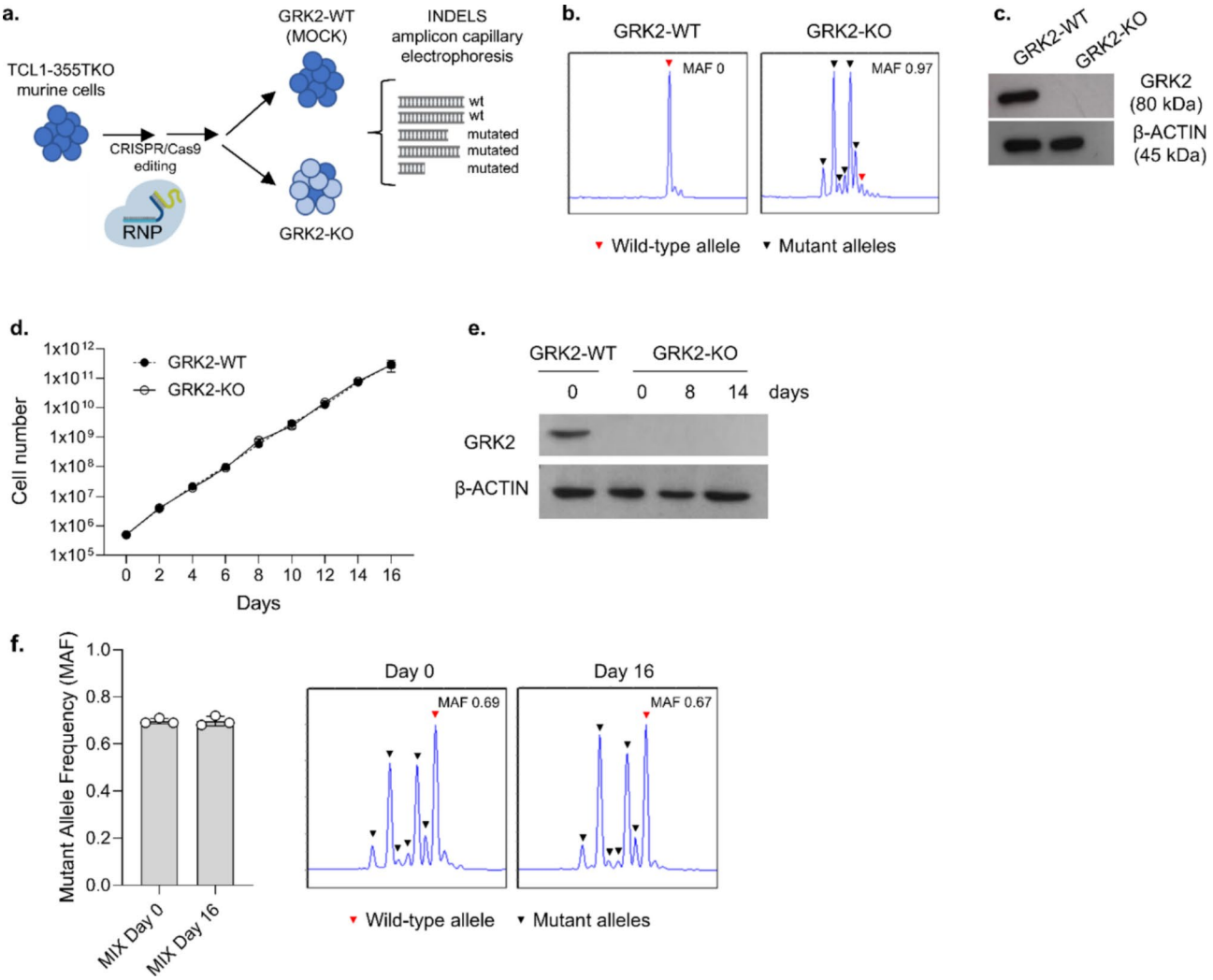


Fig. 3. GRK2 deletion on leukemic cells by CRISPR/Cas9 does not affect in vitro cell growth. **(a)** Schematic representation of the CRISPR/Cas9 procedure to target the GRK2 gene on the mouse cell line TCL1-355 TKO. GRK2-deficient murine leukemic cells were generated by using CRISPR/Cas9 with the Alt-R system (Integrated DNA Technologies [IDT]) on the TCL1-355 TKO cell line. TCL1355 TKO cells were electroporated with Cas9 alone (GRK2-WT) or with ribonucleoparticles (RNP) containing recombinant Cas9 enzyme + GRK2 guide RNA (GRK2-KO). INDEL analysis of the PCR products of KO and WT populations was performed by capillary electrophoresis. Mutant allele frequency (MAF) was determinate by peak quantification. MAF = mutated alleles/total alleles **(b)** GRK2 MAF analysis of GRK2-WT and GRK2-KO leukemic cells. Red arrow: peak corresponding to GRK2 wild type (WT) allele; black arrow: peak corresponding to GRK2 mutated allele **(c)** Protein extracts from the GRK2-KO and GRK2-WT leukemic cells were done and GRK2 and β-actin (loading control) expression was evaluated by western blot. **(d-e)** In vitro proliferation of leukemic cells was analysed by cell count and GRK2 expression was assessed at different time points by western blot. Each point represents 3 independent experiments. Images of full length blots are shown in Supplementary Figs. 3–5. **(f)** A mix of GRK2-WT and GRK2-KO leukemic cells (ratio 1:1) was cultured and number of cells and GRK2 MAF was analysed after 16 days of culture (mean ± SEM of 3 independent experiments).

microscopy. CD3 and CD169 staining was used to identify follicles, and GRK2-WT and GRK2-KO leukemic cells were counted in each region (inside follicles/white pulp and outside follicles/red pulp)[14]. As shown in Fig. 5C-D, and as previously reported for normal murine B cells[14], there was a decreased presence of GRK2-KO leukemic cells compared to GRK2-WT leukemic cells within spleen follicles.

GRK2-KO leukemic cells have a growth disadvantage in vivo

Finally, we investigated whether GRK2-WT and GRK2-KO leukemic cells were differentially selected in vivo. To this aim, we transferred by intraperitoneal injection mixtures of GRK2-WT and GRK2-KO leukemic cells into C57BL/6 mice, and after 9 to 15 days we evaluated the GRK2 MAF by amplicon capillary electrophoresis on purified B cells (> 97% of leukemic cells) from the PC, SP and PB of the transferred mice. As shown in Fig. 5E, we found that the MAF of recovered cells from PC, SP and PB was lower than the MAF of injected cells, showing an

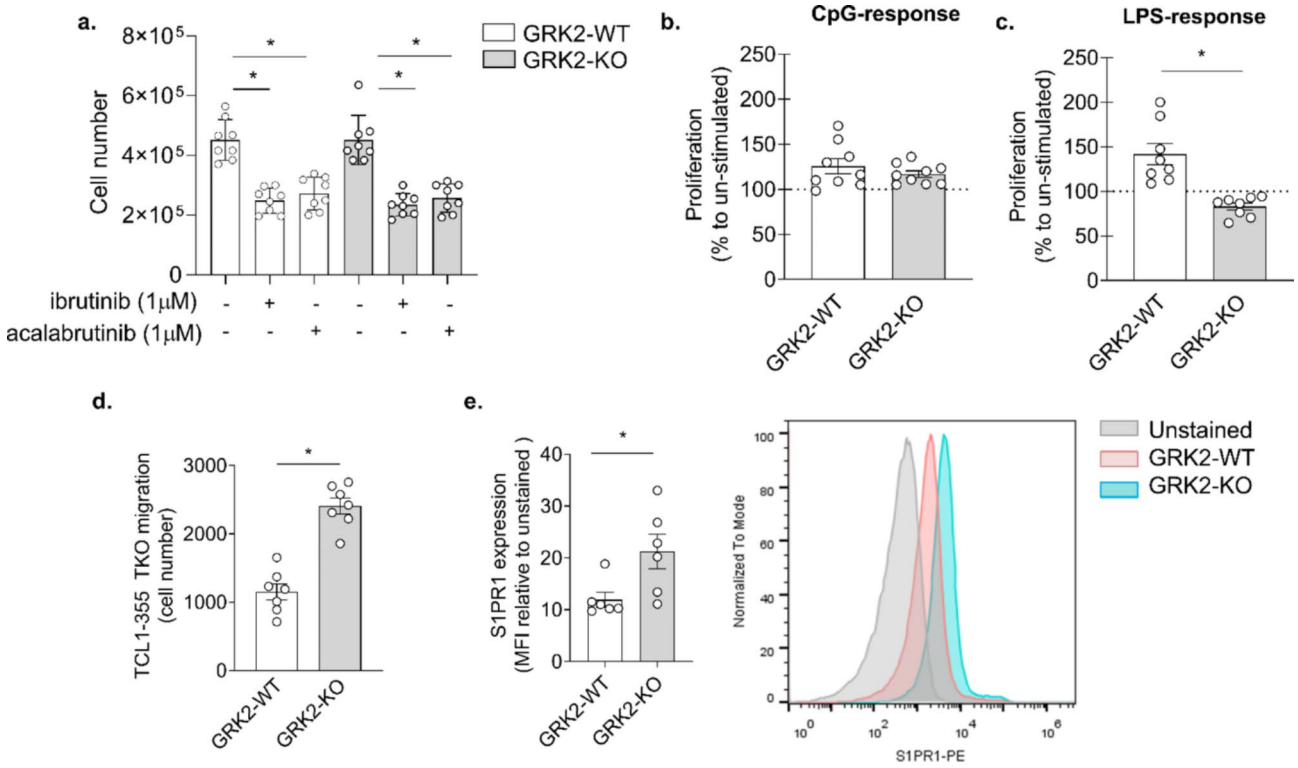


Fig. 4. GRK2 deletion has an impact on TLR4-mediated proliferation and S1P-induced migration of leukemic cells. **(a-c)** GRK2-WT and GRK-KO leukemic cell were cultured in presence of the BTK inhibitors ibrutinib (1 μM) or acalabrutinib (1 μM), or in presence of the TLR4 or TLR9-ligands LPS (5 μg/ml) or CpG (1 μM), respectively. In vitro proliferation was analysed by cell count after 24 h (mean ± SEM) ($n = 8$). *$p < 0.05$, Mann Whitney test. **(d)** GRK2-WT and GRK-KO leukemic cell migration towards S1P (10 nM) in vitro was evaluated. The graph shows the number of cells that had migrated to the lower chamber of the transwell in response to S1P minus number of cells that had migrated to the lower chamber of the transwell spontaneously (medium alone). The mean ± SEM is shown, *$p < 0.05$, Friedman test followed by Dunn's multiple comparisons post-test ($n = 7$). **(e)** S1PR1 expression was evaluated by flow cytometry in GRK2-WT and GRK2-KO leukemic cells. The mean ± SEM and a representative histogram are shown. MFI: mean fluorescence intensity. *$p < 0.05$, Mann Whitney test ($n = 6$).

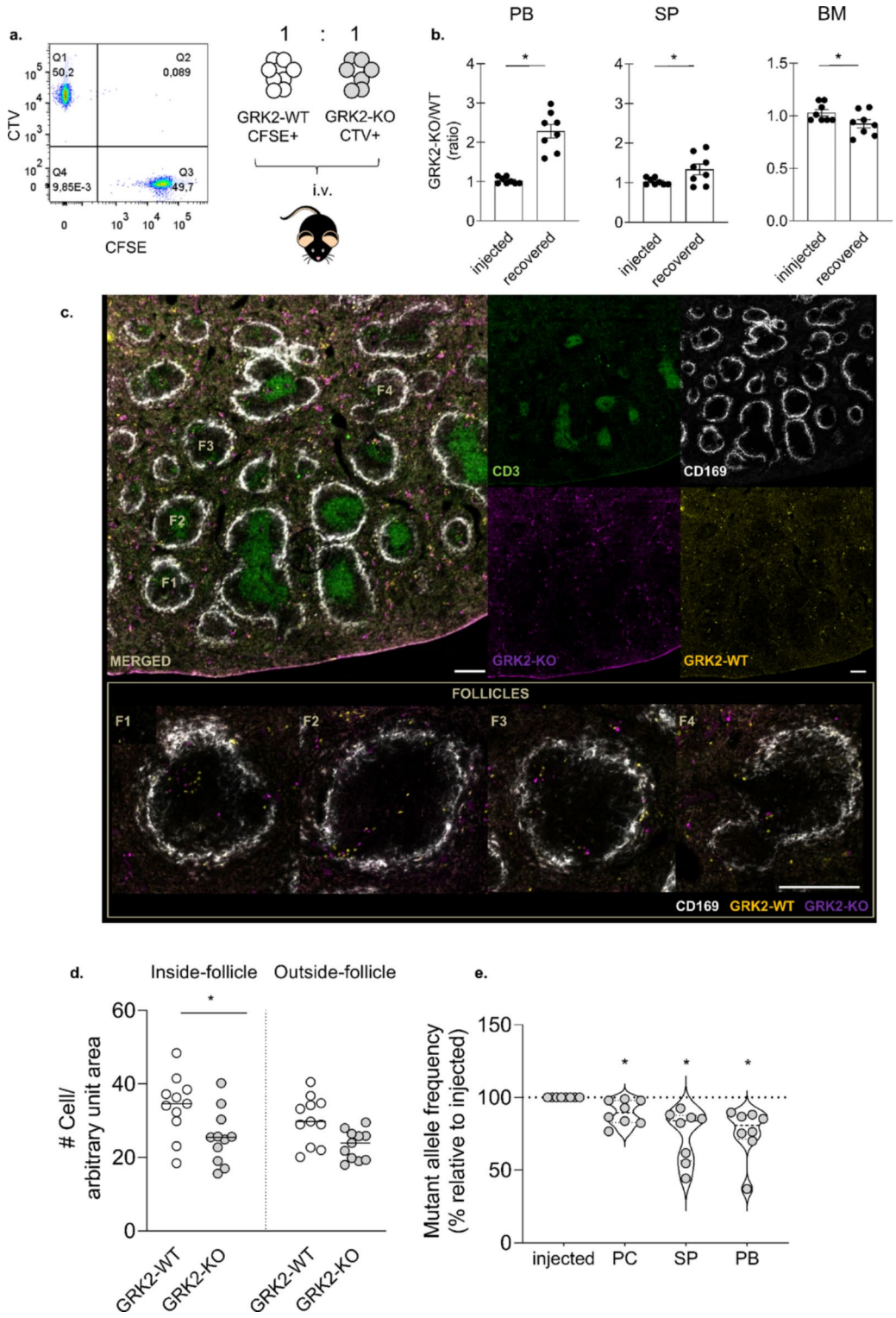
enrichment of leukemic cells with GRK2 wild type allele. Considering the lack of enrichment of leukemic cells with GRK2 wild type allele in the experiments in vitro (Fig. 3D), this result suggests that GRK2-KO cells have a selective growth disadvantage in vivo compared to GRK2-WT cells.

Discussion

The relevance of the interactions of leukemic cells with the tumour microenvironment, in both disease progression and treatment resistance, has been extensively demonstrated in CLL. In fact, the mobilization of leukemic cells from lymphoid organs to peripheral blood represents an important mechanism of action of BTK inhibitors used in the clinic and a possible therapeutic strategy itself[33,34].

In this study we describe for the first time that inhibition of GRK2 increases the response of leukemic B cells towards the main "exit signal" from lymphoid organs, S1P. We found that a GRK2 inhibitor, CMPD101, significantly increased the migration of malignant cells from CLL patients towards S1P in vitro, and that this was associated with reduced down-regulation of its receptor S1PR1. This is consistent with the previously reported role of GRK2 in regulating the internalization of S1PR1 in non-leukemic lymphocytes[12]. We also found that the effect of CMPD101 on leukemic cell migration was ligand-specific, given that it did not increase the migration of leukemic cells towards CXCL12 and CXCL13, two chemokines involved in B cell homing to lymphoid organs. Moreover, an increased migration towards S1P in the presence of CMPD101 was also observed in leukemic cells from the Eμ-TCL1 murine model of CLL. CMPD101, as well as other GRK2 inhibitors that are being evaluated in preclinical and clinical settings[35–38], have off-target kinases. In particular, at the doses used in our study, CMPD101 also targets GRK3. Nevertheless, considering that GRK2, and not GRK3, was previously reported to modulate the S1P-response in B cells[12,14,39], it is likely that the effect of CMPD101 on GRK2 is, at least in part, responsible for the increased migration towards S1P in our assays.

The latter possibility was additionally validated in experiments with a cell line derived from the murine Eμ-TCL1 model, which showed that deletion of GRK2 by CRISPR/Cas9 gene editing increases the in vitro response to S1P. Furthermore, we show that GRK2 deletion in this cell line affects the in vivo homing of the leukemic



cells, with an increased presence of GRK2-deficient leukemic cells in the peripheral blood and lower presence in the bone marrow, which is consistent with previous reports with healthy murine B cells[12,14]. Although the flow cytometry analysis also indicated a higher proportion of GRK2-KO leukemic cells compared to WT cells in the spleen, this finding was not corroborated by confocal microscopy analysis. Specifically, we observed fewer GRK2-KO cells compared to WT cells within the spleen follicles and found no significant difference in the red pulp. Thus, the higher proportion of GRK2-KO leukemic cells detected in the flow cytometry compared to the confocal microscopy analysis is likely to result from contamination with peripheral blood B cells, as previously reported by others[40]. Nevertheless, despite the discrepancy between the flow cytometry and imaging findings, the reduced presence of GRK2-KO compared to WT leukemic cells within the splenic follicles was consistent and aligned with findings reported by Huang et al. with normal B cells[14]. Considering that within follicles B cells receive signals that promote activation and survival[41], and also that proliferating CLL cells preferentially

◀**Fig. 5**. GRK2 deletion alters leukemic cells localization and leukemic cell growth in vivo. **(a-b)** GRK2-WT (CFSE+) and GRK-KO (CTV+) cells mix (1:1) were transferred intravenously into C57BL/6J mice. After 20 h, peripheral blood (PB), spleen (SP) and bone marrow (BM) were collected and the ratio of GRK2-WT/GRK2-KO cells was evaluated by flow cytometry (mean ± SEM) ($n = 8$). **(c)** Confocal image of a C57BL/6J mice's spleen transferred with GRK2-WT (CTY+) and GRK-KO (CTV+) cells (1:1), as previously described. Splenic sections were fixed and immunostained for CD3 (green) and CD169 (white). Scale bar, 200 µm. **(d)** Quantification of GRK2-WT (yellow) and GRK-KO (violet) cells inside and outside the follicle is shown. Each dot represents one spleen section where 6–10 images were quantified. The number of cells counted in each region, was then relativized to the total of area analysed (inside or outside follicles) for each image (mean ± SEM) ($n = 4$ mice). **(e)** A mix of GRK2-WT/GRK2-KO cells was injected intraperitoneally into C57BL/6J mice. After 9 to 15 days, GRK2 mutant allele frequency (MAF) was determinate by amplicon capillary electrophoresis on purified B cells (> 97% leukemic cells) from the peritoneal cavity (PC), SP, and PB of transferred mice injected. For the mix two different ratios were used (KO: WT): 95:5 ($n = 4$) and 60:40 ($n = 4$). Graph shows the MAF in each sample relative to MAF of the injected mix. $p < 0.05$, Friedman test followed by Dunn's multiple comparison test.

localize in the white pulp[4,42], it would be expected that the defective localization of leukemic cells in the white pulp will also have an impact on disease progression. Importantly, while in vitro proliferation of the TCL1-355 TKO cell line was not affected by GRK2 deletion, when injected into mice we found a growth disadvantage of GRK2-KO leukemic cells compared to GRK2-WT cells. Given the central role of signals provided by the tumour microenvironment within lymphoid tissues and the defective localization of GRK2-KO leukemic cells, it is tempting to speculate that the in vivo selection of GRK2-WT cells in the mouse is a consequence of a decreased interaction of GRK2-KO leukemic cells with the tumour microenvironment. Nevertheless, some important limitations of our study must be considered. First, while lymph nodes represent the most relevant lymphoid tissue for leukemic cell proliferation in CLL patients[2], in this study the migration of leukemic cells to the lymph nodes could not be assessed because the TCL1-derived leukaemia typically do not home to lymph nodes. Nevertheless, previous studies with normal murine B cells have already shown that GRK2 deletion affects B cell homing to lymph nodes[14], suggesting that GRK2 is likely to also regulate the lymph node trafficking of the leukemic cells in patients with CLL. Another potential limitation of this study is that in order to generate a stable leukemic cell line with GRK2 deletion for in vivo experiments, we used the TCL1-355 TKO cell line which differs from primary human and murine CLL cells by proliferating spontaneously in vitro[18]. Nevertheless, this cell line remains responsive to various microenvironmental signals[32], allowing us to evaluate how GRK2 deletion affects both migration and proliferation.

Besides its role in lymphocyte homing, GRK2 has also been reported to participate in signaling pathways that can either promote[21,31,43] or suppress[20,24,44] tumour progression through its interaction with several intracellular proteins. Moreover, an altered expression of GRK2 was reported on malignant cells from different tumours, including thyroid, breast, pancreas and ovarian cancer[20–22,24,45,46]. Here we found that leukemic cells from CLL patients express GRK2 at similar levels as B cells from healthy donors. Also, in this study we did not observe an impact of GRK2 deletion on the in vitro cell viability or on spontaneous or CpG-induced proliferation. We also described that GRK2 deletion or inhibition does not impact the in vitro sensitivity of leukemic cells to venetoclax and BTK inhibitors. This finding is particularly relevant, as GRK2 deletion in normal murine B cells has been linked to increased activation of pathways such as AKT and ERK[12], which are associated with resistance to these targeted therapies in CLL patients[47–49]. We did observe, however, that GRK2-KO leukemic cells have a lower proliferation rate in response to LPS compared to WT cells, which is in line with a previous report showing that GRK2 participates in TLR4 signaling in other cell types[50]. While an inhibitory effect on leukemic cell proliferation in response to TLR-stimulation could be desirable and TLR-inhibitors have been proposed as a therapeutic strategy[51], the relevance of TLR signaling on tumour progression in CLL has been questioned[52].

In conclusion, in this study we show that GRK2 inhibition/deletion in leukemic cells increases their response to S1P in vitro and alters their localization and growth in vivo. We propose that GRK2 inhibition could be explored as a strategy to diminish the localization of CLL cells in growth and survival niches. This would result in reduced interactions between the leukemic cells and the tumour microenvironment, responsible for disease progression and drug-resistance. Further studies using pharmacological inhibition of GRK2 in the EµTCL-1 mouse model could help to establish GRK2 as a possible new therapeutic target in CLL with the potential to increase the efficacy of current therapies.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

MB, DGE and MG designed the study and wrote the manuscript. CC, AC, VSM, MB, CM, AB, PEM performed the experiments. CC and AC generated the figures and designed experimental procedures. MV, DGE and PEM supervised certain experiments and contributed with experimental design. MB, FRB, MP and RC provided vital patient specimens. All authors analysed data and reviewed and approved the final version of the manuscript.

Declarations

Conflict of interest

MP received honoraria from: AstraZeneca, Janssen and Merck. The remaining authors declare no competing financial interests regarding this work.

Additional information

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