Stromal cell protein kinase C-β inhibition enhances chemosensitivity in B cell malignancies and overcomes drug resistance

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Overcoming drug resistance remains a key challenge to cure patients with acute and chronic B cell malignancies. Here, we describe a stromal cell–autonomous signaling pathway, which contributes to drug resistance of malignant B cells. We show that protein kinase C (PKC)-β–dependent signals from bone marrow–derived stromal cells markedly decrease the efficacy of cytotoxic therapies. Conversely, small-molecule PKC-β inhibitors antagonize prosurvival signals from stromal cells and sensitize tumor cells to targeted and nontargeted chemotherapy, resulting in enhanced cytotoxicity and prolonged survival in vivo. Mechanistically, stromal PKC-β controls the expression of adhesion and matrix proteins, required for activation of phosphoinositide 3-kinases (PI3Ks) and the extracellular signal–regulated kinase (ERK)–mediated stabilization of B cell lymphoma–extra large (BCL-XL) in tumor cells. Central to the stroma-mediated drug resistance is the PKC-β–dependent activation of transcription factor EB, regulating lysosome biogenesis and plasma membrane integrity. Stroma-directed therapies, enabled by direct inhibition of PKC-β, enhance the effectiveness of many antileukemic therapies.

INTRODUCTION

Over the past decade, next-generation sequencing technologies provided opportunities to comprehensively describe the spectrum of genomic abnormalities found in different B cell malignancies (1–4). Ultimately, this has improved our understanding of the underlying genetic mutations contributing to uncontrolled proliferation and extended cell survival while enabling the development of targeted therapies. Drug resistance remains one of the most challenging clinical problems, reflected by the invariable disease recurrence in all patients with low-grade lymphoma and chronic lymphocytic leukemia (CLL) and a substantial fraction of patients suffering from acute lymphoblastic leukemia (ALL) and high-grade lymphoma. Thus, although a majority of patients achieve disease remissions, relapses occur from cells surviving cancer therapies. Identifying and targeting cells that have acquired the ability to survive these treatments may ultimately allow full eradication of these tumor cells and achievement of cure.

The tumor microenvironment plays a pivotal role in drug resistance by providing antiprotective signals to tumor cells in protective niches (5). The bone marrow (BM) microenvironment appears to be of crucial importance for lymphoid diseases because minimal residual disease (MRD) detected in this compartment has a strong prognostic power to predict disease relapse in acute and chronic leukemias and lymphomas (6, 7). Several intermediates of the BM microenvironment to tumor cell communications have been identified, but their targeting has been proven to be difficult and to result in minor effects at best (8, 9).

Here, we describe a stromal cell–autonomous signaling pathway, dependent on the expression and activation of protein kinase C-β (PKC-β) and subsequent activation of the transcription factor EB (TFEB), both being of key importance to protect malignant B cells from cytotoxic therapies. The dependency of malignant B cells on PKC-β activity in the microenvironment can be therapeutically exploited with small-molecule inhibitors to treat patients with different B cell malignancies, including CLL, mantle cell lymphoma (MCL), and B cell ALL (B-ALL).

RESULTS

Stromal PKC-β is essential for survival, but not homing or proliferation of Eμ-TCL1-tg B cell tumors

PKC-β activity in mesenchymal stromal cells (MSCs) is essential for the engraftment of malignant B cells derived from Eμ-TCL1 transgenic (tg) mice (hereafter named TCL1-tg) (10), a model resembling human CLL (11). To further investigate the mechanisms underlying this phenotype, we set out to characterize the role of stromal PKC-β...
for tumor cell homing, proliferation, and survival. Carboxyfluorescein diacetate succinimidy l ester (CFSE)–labeled CD5⁺CD19⁺ tumor cells from diseased TCLI-tg mice were transplanted intravenously and intraperitoneally into either PKC-β knockout (KO) or wild-type (WT) recipient mice. In addition, malignant cells were transplanted into WT mice, which were pretreated for 48 hours with the PKC-β inhibitor enzastaurin [60 mg/kg, twice a day [bis in die (BID)]] or vehicle control and continued to receive treatment for 48 hours after transplantation (fig. S1A). Tumor cells homed to the spleen and BM of all recipient mice, irrespectively of PKC-β expression in the microenvironment or its pharmacological inhibition (fig. S1, B and C). A separate cohort of PKC-β KO and WT recipient mice was followed for 2 weeks, and tumor engraftment was assessed in the peripheral blood, lymphatic tissues, and the peritoneal cavity. A similar number of CFSE-labeled tumor cells were detectable in the peripheral blood of KO and WT mice on day 2. However, from day 8 onward, we observed a marked increase in tumor cells in the peripheral blood of WT, but not KO, mice (fig. S1D). Two weeks after transplantation of tumor cells, malignant B cells were virtually absent in the BM of KO mice and decreased in the spleen and peritoneal cavity of KO mice, in contrast to WT control mice where tumor cells were maintained (Fig. 1A and fig. S1E). During the first 2 weeks, disease progression was more pronounced in the BM, which showed a stronger dependency on PKC-β compared with the spleen or peritoneal cavity (Fig. 1B). The continuous decay of the CFSE label with each cell division did not differ in KO and WT recipient mice (Fig. 1, C and D), indicating that PKC-β expression in the tumor microenvironment is dispensable for tumor cell homing or proliferation but required for cell survival.

**PKC-β expression in stromal cells is essential for tumor cell engraftment and normal B1 cell development**

We then investigated whether the lack of leukemic engraftment in PKC-β KO mice was attributable to its absence in stromal cells or whether microenvironment hematopoietic cells also contributed. Germline deletion of PKC-β in mice causes immunodeficiency with a marked reduction in peritoneal B1 cells and a reduction in serum immunoglobulin M (IgM) and IgG3 (12). No differences in white blood cells, hemoglobin, or platelets were observed between WT and KO cells (fig. S2, A and B), also reflected by the presence of similar numbers of Lin− Sca1 + C-Kit + (LSK) and CD45 + EPCR + CD150 + CD48 − cells also differentiated equally well in the spleens of either WT or KO recipient animals, with no differences between CD138⁺ and CD138⁻ cells (fig. S3, C to E). PKC-β WT donor cells also differentiated equally well in the spleens of either WT or KO recipient animals, whereas PKC-β KO donor cells gave rise to fewer splenic CD45⁺CD138⁺ cells in a WT background (fig. S3, C, D, and F).

To address whether PKC-β expressed in hematopoietic cells could rescue tumor cell survival in PKC-β KO recipient animals, we injected TCLI-tg tumor cells into KO animals, previously transplanted with KO or WT CD45⁺ BM cells (Fig. 1H). All recipient chimeric mice were deficient for PKC-β in nonhematopoietic cells, but either did (WT→KO) or did not (KO→KO) contain PKC-β-expressing hematopoietic cells. Similar to our transplantation studies into nonchimeric mice, engraftment of tumor cells in the peripheral blood of WT→KO animals compared with WT control mice was significantly (P = 0.001) impaired (Fig. 1, I and J). On the other hand, WT→KO recipients contained only slightly more malignant cells than KO→KO recipients, indicating that PKC-β-mediated survival signals by hematopoietic cells play a predominant role in tumor maintenance.

**Inhibition of stromal PKC-β mitigates EMDR**

Signals derived from the microenvironment contribute not only to the development of normal hematopoietic cells but also to drug resistance of malignant cells [termed environment-mediated drug resistance (EMDR)] (5). The marked dependency of malignant B cell survival on PKC-β activity in the microenvironment raised the possibility that this interaction could be exploited therapeutically. To test this hypothesis, we cultured primary human CLL cells on BM-derived MSCs from either KO or WT mice. Human MSCs were indistinguishable from mouse MSCs with regard to PKC-β activation and survival support (10). In line with our previous observations, PKC-β deficiency in BM-derived MSCs mitigated the antiapoptotic effects of stromal cells on CLL cells (Fig. 2, A and B). To test the role of stromal PKC-β in EMDR, parallel cocultures were exposed 24 hours after CLL seeding to increasing doses of venetoclax [B cell lymphoma 2 (BCL2) inhibitor], bendamustine (alkylating agent), fludarabine (purine analog), or ibrutinib or idelalisib [inhibitors of B cell receptor (BCR)–induced kinases] before assessing the viability of CLL cells 48 hours later. Expectedly, contact with WT MSCs enhanced the resistance of CLL cells to these cytotoxic drugs when compared with suspension cells in monoculture (Fig. 2C). In particular, we observed strong protective effects of MSCs on CLL cells for venetoclax and fludarabine treatments, whereas EMDR to BCR inhibitors...
Fig. 1. Normal and malignant B1 cells require microenvironment PKC-β for survival. (A) Quantification of CFSE-labeled TCL1-tg cells as a percentage of total CD19+ cells detected in the indicated tissues. Analyses are pooled from two independent experiments using two primary tumors. ns, not significant. (B) Ratios of mean percentages CFSE+ of CD19+ cells detected in the indicated tissues. Analyses are pooled from two independent experiments using two primary tumors. ns, not significant. (C) Representative fluorescence-activated cell sorting (FACS) histograms of various tissues, 2 and 14 days after transplantation. Chimeras are labeled with genotype of donor cells in bold, and recipient genotype is also indicated. (D) Quantification of CFSE MFI in CD19+ TCL1-tg cells detected in BM, spleen, and peritoneal cavity of PKC-β WT and KO mice. Analyses are pooled from two independent experiments using two primary tumors. (E) An experimental schematic to assess the functional consequence of adoptive transfer of CD45+ selected PKC-β WT BM or KO BM into lethally irradiated (10 Gy) PKC-β WT or KO recipients. (F) Nonirradiated WT control (n = 3), WT:KO (n = 7), and four individuals of each WT:WT, WT:KO, and KO:KO were assessed for peritoneal CD19+CD5+IgM+ cells. Statistical significance was assessed using unpaired, two-tail Student t tests. (G) Concentrations of serum immunoglobulins 9 weeks after transplantation are shown, with individual means shown ±SEM. (H) Schematic of secondary transplantation of TCL1-tg cells into PKC-β chimeras. IV, intravenously. (I) Representative flow cytometry plots of CFSE detection and CD19 staining from the peripheral blood of chimeras receiving TCL1-tg transplants. Chimeras are labeled with genotype of donor cells in bold, and recipient genotype is also indicated. (J) Quantification of CFSE-labeled CD19+ TCL1-tg cells as a percentage of total CD19+ cells detected in the peripheral blood from TCL1-tg recipients. *P < 0.05, **P < 0.01, ***P < 0.001, and not significant (ns) P > 0.05.

The absence of PKC-β in MSCs completely abolished the protective effects seen with WT stromal cells under all treatments except for bendamustine, where PKC-β deficiency nevertheless also strongly decreased stromal protection. PKC-β expression in monocytes, which are derived from the hematopoietic system and also support leukemogenesis (15), was dispensable for...
Fig. 2. Stromal PKC-β confers drug resistance to malignant B cells via BCL-XL expression.
(A) Representative annexin V and 4′,6-diamidino-2-phenylindole (DAPI) stains of monocultured or cocultured CLL cells 72 hours after seeding of CLL. FITC, fluorescein isothiocyanate. (B) Percentage of live (annexin V−, DAPI−), apoptotic (annexin V+, DAPI−), and dead (DAPI+, annexin V+−) stained cells are shown for individually cultured primary CLL (n = 7) for the three culture conditions. Statistical significance for differences in live cells between conditions was assessed using paired two-tailed Student's t tests. (C) IC50 (median inhibitory concentration) graphs of human CLL cells after 72 hours of monoculture or coculture with either PKC-β WT stroma or PKC-β KO stroma in the presence of venetoclax, bendamustine, fludarabine, ibrutinib, or idelalisib treatment administered 24 hours after seeding of CLL (n = 5 patients per culture condition). CLL viabilities were normalized to dimethyl sulfoxide (DMSO) controls. Statistical significance of the differences between PKC-β WT and PKC-β KO is shown. (D) Annexin V− measured viability of CLL cells (n = 6) normalized to DMSO control after 72 hours of coculture with PKC-β WT stroma in the presence of increasing concentrations of enzastaurin, sotraductin, or midostaurin administered 24 hours after seeding of CLL. Individual values from (B) are plotted for reference. (E) Viability of CLL cells normalized to DMSO controls after 72 hours of PKC-β WT coculture with 48 hours of exposure to increasing doses of venetoclax (n = 9; n = 21 at 5 nM) or fludarabine (n = 9; n = 8 at 100 μM) ± enzastaurin treatment. (F) Linked graphs of patient-derived CLL cell viability (n = 7) in the presence of labeled treatments cocultured on PKC-β KO stroma. Statistical significance was assessed using ratio-paired, two-tailed Student's t tests. (G) Synergism was calculated using Combenefit software, within the Bliss model, for venetoclax combined with enzastaurin, sotraductin, or midostaurin (n = 6). Heat maps reflect values for the respective compound combinations, with error (±SD) indicated below. A scale of 50 to −50 represents maximal synergism and −50 being maximal antagonism (for this panel, *P < 5 × 10−2; **P < 10−3; ***P < 10−6). (H) In vitro experiment with mono- or cocultured primary CLls (n = 10) on WT splenic FRC stroma (gp38+CD31+) treated with vehicle control, enzastaurin, or venetoclax ± enzastaurin. Statistical significance was determined using a paired two-tailed Student's t test. (I) BCL2 family proteins and β-actin immunoblots of primary CLL at the indicated time points of venetoclax treatment, cocultured with either PKC-β WT stroma or PKC-β KO stroma. (J) BCL2 family proteins and β-actin immunoblots of primary CLL at the indicated time points of venetoclax, fludarabine, or bendamustine treatment, cocultured with PKC-β WT stroma ± enzastaurin. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and not significant (ns) P > 0.05.
PKC-β expression in MSCs is crucial for EMDR of primary CLL cells. We previously demonstrated that PKC-β kinase activity in MSCs is essential for microenvironment-mediated survival support of leukemia cells (10). Therefore, we tested whether ablation of EMDR could be achieved with small-molecule PKC inhibitors. Enzastaurin and sotrastaurin are orally bioavailable, reversible adenosine triphosphate (ATP)–competitive inhibitors of PKCs, with enzastaurin being more specific for the β isoform (16, 17). Midostaurin is a multi kinase inhibitor with activity against PKC isoforms and was recently approved for the treatment of FLT3-ITD + acute myeloid leukemia (AML) (18). Cocultures of WT MSCs and primary CLL cells were exposed to increasing low doses of these inhibitors for 48 hours. Similar to coculturing CLL cells on KO MSCs, each PKC inhibitor reduced the viability of CLL cells (Fig. 2D). The cytotoxic effects of enzastaurin and sotrastaurin did not exceed the survival disadvantage of KO MSCs and were dose dependent, whereas midostaurin induced CLL cell death beyond this effect similarly across all doses. This suggests that midostaurin also inhibits other kinases in MSCs and/or has direct cytotoxic effects on primary CLL cells. To test whether PKC-β inhibitors can sensitize malignant B cells to cytotoxic agents, we treated MSC/CCL cocultures for 48 hours with low-dose (5 μM) enzastaurin and increasing doses of different cytotoxic agents. Assessment of apoptotic CLL cells after 48 hours in coculture indicated that enzastaurin sensitized CLL cells to venetoclax, benzamustine, and fludarabine, phenocopying the experiments with KO stromal cells (Fig. 2E and fig. S4, E and F; patient characteristics are provided in table S1), but not consistently to ibritinib andidelalisib (fig. S4F). PKC-β expression is not only restricted to MSCs but also found at high amounts in malignant B cells (19). To prove that the synergistic effects are mediated by the inhibition of stromal PKC-β and not attributed to off-target effects or inhibition of PKC-β expressed in tumor cells, we cultured primary CLL cells on PKC-β KO stromal cells and then exposed cocultures to enzastaurin in the absence or presence of venetoclax. Analyses of apoptotic B cells after 48 hours demonstrated that enzastaurin did not affect the survival of CLL cells cultured on PKC-β KO stromal cells. Furthermore, under these conditions, enzastaurin also did not enhance the cytotoxic effects of venetoclax (Fig. 2F). Therefore, by genetically removing the target protein for the kinase inhibitor, these data demonstrate that the PKC-β inhibitor sensitizes malignant B cells to cytotoxic drugs by ablating microenvironment-mediated, PKC-β–dependent survival signals and drug resistance.

Using the Combenefit platform (20) for the assessment of drug synergy, the Bliss independence and the Loewe additivity models demonstrated that the cytotoxic effects of combinational treatments with venetoclax and PKC-β inhibitors were synergistic (Fig. 2G and fig. S4G). Similar to enzastaurin, the PKC inhibitors sotrastaurin and midostaurin also chemosensitized malignant B cells to cytotoxic agents (Fig. 2G), indicating a class-specific, rather than a drug-specific, effect. To assess whether PKC-β dependency extends to stromal cells other than MSCs, we isolated fibroblastic reticular cells (FRCs) from spleens of WT mice. Similar to MSCs, FRCs provide constitutive survival signals to primary CLL cells dependent on PKC-β activity. Enzastaurin enhanced the cytotoxic effects of venetoclax in FRC cocultures (Fig. 2H), indicating that PKC-β is important for EMDR provided by different stromal cell types. The chemosensitizing effects of enzastaurin were most pronounced in combination with venetoclax. The efficacy of this BCL2 inhibitor is largely dependent on the relative expression of other antiapoptotic proteins. To understand how stromal PKC-β inhibits the cytotoxicity of venetoclax, we analyzed the expression of antiapoptotic proteins in CLL cells cultured on either WT or KO stromal cells. In CLL cells cultured on WT stromal cells and treated with a low dose of venetoclax, BCL–extra large (BCL-XL) was significantly (P = 0.009 at 48 hours after treatment) up-regulated, whereas expression of BCL2, BCL2A1, and MCL-1 decreased after 48 hours. In contrast to WT stromal cells, the enhanced expression of BCL-XL was markedly mitigated in CLL cells cultured on KO stromal cells (Fig. 2I; for protein quantification, see fig. S5A). Similar to coculture on KO stromal cells, treatment of WT stromal CLL cocultures with venetoclax or other chemotherapies in combination with enzastaurin also blocked the up-regulation of BCL-XL in CLL cells (Fig. 2J). The stroma-dependent stabilization of BCL-XL was not regulated by enhanced transcription, because BCL-XL transcripts were readily detectable in cocultured CLL cells and remained stable after venetoclax treatment (fig. S5, B and C). In conclusion, the activation of PKC-β in the microenvironment contributes to drug resistance by controlling the posttranscriptional regulation of BCL-XL in malignant B cells.

**Stromal PKC-β is essential for ERK activation and BCL-XL stabilization in CLL cells**

To further understand the role of stromal PKC-β in EMDR, RNA sequencing (RNA-seq) expression profiling was performed on primary CLL cells cultured on either WT or KO stroma in the presence of venetoclax (used at a minimal concentration of 1.25 nM to avoid substantial cell death; see Fig. 2C). Clustering of the global expression changes between conditions demonstrates a considerable difference in the CLL response to venetoclax when cocultured with WT versus KO stroma (Fig. 3A). Pairwise comparison of gene expression in CLL cells cultured on either WT or KO stroma in the presence of venetoclax identified 810 differentially expressed genes; of these, 755 were significantly up-regulated in CLL cells cultured on WT stroma compared with cells cocultured on KO stroma, whereas 55 genes were down-regulated (adjusted P value, <0.01; log2 fold change, >1). Gene set enrichment analysis (GSEA) indicates that coculture on WT stromal cells enhanced the expression of genes required for extracellular matrix (ECM) remodeling and cell-cell interactions (Fig. 3B and data file S1). We then used Ingenuity Pathway Analysis to identify canonical pathways and regulators operating upstream of these transcriptional changes. These analyses indicated that phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling was inhibited in CLL cells cultured on KO stroma (Fig. 3C), which was associated with effects on integrin and transforming growth factor–β (TGF-β) pathways (fig. S6A). Immunoblot analysis showed extracellular signal–regulated kinase (ERK), but not p38 or JNK (c-Jun N-terminal kinase), pathway activation in CLL cells cultured on WT stroma under venetoclax exposure. This activation was severely suppressed in venetoclax-treated tumor cells cultured on WT stroma (Fig. 3D and fig. S6B) or cultured on WT stroma in the presence of enzastaurin (fig. S6C). ERK activation was associated with enhanced BCL-XL expression, suggesting a functional link between the two. The MEK1/2 (MAPK kinase 1/2) inhibitor trametinib antagonized venetoclax-induced ERK activation and was associated with decreased BCL-XL protein expression (Fig. 3E) and enhanced cytotoxicity (Fig. 3F).
KO Co-PKC-ns phosphorylated p38 (Thr180/ Tyr182), and with either PKC-βCLL at indicated time points of PKC-βCLL (right half of the map). Red and blue indicate relative high and low expression, respectively. Each condition analyzed depicts three distinct primary CLL samples. (B) GSEA evaluating canonical pathways enriched in CLL cells cocultured with PKC-β WT stroma or PKC-β KO stroma. (C) Ingenuity Pathway Analysis (IPA) (Qiagen NV) of predicted upstream regulators of observed differential CLL expression based on computed activation Z score. (D) BCL-XL, phosphorylated ERK (Thr202/Thr204), total ERK, phosphorylated JNK (Thr183/Thr185), phosphorylated p38 (Thr180/Tyr180), and β-actin immunoblots of primary CLL at indicated time points of venetoclax treatment cocultured with either PKC-β WT stroma or PKC-β KO stroma. (E) BCL-XL, phosphorylated ERK (Thr202/Thr204), total ERK, and β-actin immunoblots of primary CLL under venetoclax treatment cocultured with PKC-β WT stroma ± trametinib (1 μM). (F) Viability of CLL cells (n = 9) after 72 hours of PKC-β WT stromal coculture with 48 hours of exposure to venetoclax ± trametinib treatment. Annexin V measured viability of CLL cells normalized to DMSO controls. Statistical significance was assessed using a paired two-tailed Student’s t test. (G) Annexin V measured CLL cell viability (n = 5) after 72 hours of PKC-β WT stromal coculture with 48 hours of exposure to venetoclax ± idelalisib treatment ± enzastaurin treatment. (H) Total AKT, phosphorylated AKT (T308), phosphorylated AKT (S473), and β-actin immunoblots of primary CLL under venetoclax treatment cocultured with PKC-β WT stroma ± idelalisib (7.5 μM). (I) Total AKT, phosphorylated AKT (T308), phosphorylated AKT (S473), BCL-XL, and β-actin immunoblots of primary CLL cocultured with either PKC-β WT stroma or PKC-β KO stroma under venetoclax treatment. (J) Heat map displaying mRNA expression profiles of patient samples (n = 51) after clustering of six CLL genes regulated by microenvironment PKC-β. Two major clusters, classified as gene signature “high coexpression” and “low coexpression,” segregate the cohort. (K) Kaplan-Meier curves (x axis shows time since starting treatment) according to the clusters high coexpression versus low coexpression, showing significant differences (pairwise log-rank tests) for median overall survival (high coexpression: 413 days versus low coexpression: 1319 days; P < 0.004). MST, median survival time. *P < 0.05, **P < 0.01, ***P < 0.001, and not significant (ns) P > 0.05.
Because activation of PI3K limits the efficacy of venetoclax (21, 22), we investigated whether the PI3K inhibitor idelalisib combined with venetoclax induced greater apoptosis in CLL cells than venetoclax alone. In combination with the PI3K inhibitor, venetoclax-induced apoptosis was enhanced (Fig. 3G), and AKT phosphorylation, used as a surrogate to assess PI3K activity in malignant B cells, was abolished (Fig. 3H). To distinguish between the effects of idelalisib in tumor or stromal cells, we performed immunoblotting on CLL cells cultured in the presence of venetoclax on either WT or KO stromal cells. Results from this experiment indicated that PI3K is activated in venetoclax-treated tumor cells and suggested that stromal PKC-β activity partly contributes to this activation, although this dependency was far less pronounced than the effects observed on MAPK signaling (Fig. 3I and fig. S6D).

To provide further evidence that the up-regulation of PKC-β–mediated and EMDR-associated genes has clinical relevance, we retrospectively assessed the impact of stroma-mediated, deregulated pathways on progression-free and overall survival in a cohort of patients with fludarabine-refractory CLL (23). Coexpression of genes regulated by stroma PKC-β was found as a signature in a majority of the drug-resistant cohort (Fig. 3J). Moreover, patients with high coexpression signatures demonstrated a worse prognosis to salvage treatment compared with patients with low coexpression signature (Fig. 3K and fig. S6E), emphasizing the relevance of these genes, identified in coculture, to clinical observations. In conclusion, our in vitro data show that stroma-mediated drug resistance to venetoclax is mediated by ERK and PI3K signaling, dependent on PKC-β activity in stromal cells.

PKC-β–dependent lysosome biogenesis is required for EMDR of B cells

These results prompted us to also investigate the molecular events orchestrated by PKC-β in stromal cells. RNA-seq analysis was performed using PKC-β WT and KO stromal cells cocultured with primary CLL cells for 48 hours. A total of 3352 genes with significantly changed expression (fold change, >2; adjusted P value, <0.05) were identified in PKC-β KO cells compared with WT cells (Fig. 4A). This demonstrates a broad impact of PKC-β on stromal cell characteristics, including stark differences in the expression of genes encoding plasma membrane and intracellular vesicular proteins. Nearly half of all annotated plasma membrane and intracellular vesicular protein genes were differentially regulated between PKC-β WT and KO stroma, with 438 and 287 genes exhibiting greater than a twofold change in the two gene sets, respectively (adjusted P value, <0.05). GSEA revealed PKC-β WT stromal enrichment of genes involved in ECM interactions and lysosome vesicle biogenesis (Fig. 4, B and C). Analysis of lysosome biogenesis by lysosome staining showed a substantially reduced lysosomal content in PKC-β KO cells compared with WT stroma (fig. S7A). Moreover, PKC-β KO stroma was also resistant to chloroquine-induced lysosomal accumulation compared with WT stroma. In line with our observations, lysosome biogenesis in osteoclasts is controlled by PKC-β–mediated serine phosphorylation of the TFEB C-terminal motif (24). Phosphorylation of TFEB increased its nuclear abundance and enhanced transcriptional activity (25).

TFEB is a member of the microphthalmia–transcription factor E (MiT-TFE) family of transcription factors, which are major factors regulating lysosome biogenesis and autophagy (26). To ascertain whether lysosome deregulation extended to stroma in coculture with CLL under stress conditions, we conducted immunoblots for lysosome component protein, lysosomal-associated membrane protein 1 (LAMP1), and lysosome biogenesis transcription factor TFEB. Immunoblots showed markedly increased LAMP1 and TFEB protein expression in WT stroma at 24 and 48 hours, as compared with KO stroma in similarly treated CLL cocultures (Fig. 4D). Further analysis found increased nuclear expression of TFEB in cocultured PKC-β WT compared with KO stroma (Fig. 4E and fig. S7B). To assess the importance of TFEB for stroma-mediated drug resistance, we generated TFEB-deficient cells using CRISPR-Cas9 deletion (Fig. 4F). Although TFEB-deficient stromal cells and control counterparts provided equal survival support for primary CLL cells in the absence of drugs, exposure of cocultures to venetoclax demonstrated that TFEB is an essential factor for EMDR (Fig. 4G). We used chloroquine and bafilomycin to interrogate whether the inhibition of lysosome function also inhibits EMDR. Chloroquine-treated cocultures did not show impaired survival of malignant B cells in contrast to bafilomycin-treated ones, which showed reduced antiapoptotic effects from stroma on CLL cells. However, both compounds markedly enhanced the cytotoxic effects of venetoclax (Fig. 4H). To disentangle direct inhibitory effects of chloroquine and bafilomycin on CLL and stromal cells, we pretreated stromal cells with both compounds before their washout, preceding primary CLL coculture and subsequent venetoclax exposure. Pretreatment of stromal cells similarly mitigated EMDR, indicating that the observed effects were attributed to the inhibition of stroma and not CLL lysosomes (fig. S7C). Last, complementary to the deletion of TFEB from stromal cells, parental KO stromal cells were transduced with a constitutively active TFEB (caTFEB) variant (27) or vector control. CLL cocultured with PKC-β KO stroma expressing caTFEB demonstrated a significantly (P = 3.71 × 10⁻⁷) higher resistance to venetoclax compared with cells cultured on control stroma (Fig. 4I and fig. S7D). In addition, venetoclax-exposed CLL cells, cocultured on PKC-β KO stroma expressing caTFEB, demonstrated increased expression of BCL-XL and increased ERK phosphorylation compared with CLL cells cocultured on control KO stroma (Fig. 4J). To ascertain whether PKC-β inhibition impairs in vivo lysosomal biogenesis, TCL1 tumor–transplanted recipient mice were treated with enzastaurin. Stroma–restricted down-regulation of both LAMP1 and LAMP2 was observed in the enzastaurin cotreated cohort, in comparison to venetoclax-treated and vehicle control cohorts (Fig. 4K). In contrast, these lysosome-associated proteins were unchanged across all cohorts in the nonstromal compartment, signifying a stroma-specific response to PKC-β inhibition in vivo. These data demonstrate that PKC-β–mediated activation of TFEB and lysosome biogenesis in stromal cells are central for EMDR and the reciprocal stabilization of BCL-XL in tumor cells.

Plasma membrane protein composition of stromal cells is regulated by PKC-β

Our gene expression profiling of stroma cells indicated a disparate cell surface phenotype between PKC-β WT and KO cells. To define the contribution of cell-cell interactions and response to venetoclax, we separated CLL cells from stromal cells using Transwells.Disruption of cell–cell contact increased spontaneous apoptosis and venetoclax-induced cell death, indicating that cell-cell contact is of predominant importance for EMDR (Fig. 5A). To identify proteins on the surface of stromal cells relevant for contact-dependent survival of CLL cells, we performed plasma membrane profiling (PMP) of MSCs, cultured...
Fig. 4. PKC-β-dependent lysosome biogenesis is central for stroma-mediated drug resistance. (A) Differential gene expression between PKC-β WT and KO stroma cocultured with patient CLL cells (n = 3) for 48 hours. The differentially expressed genes encoding plasma membrane and intracellular vesicle proteins are highlighted in blue and red, respectively. Significantly deregulated genes (adjusted P value, <0.05; fold change, >2; 3352 genes) were compared to the lists of annotated gene sets for plasma membrane proteins (Protein Atlas; 1734 genes) or intracellular vesicle genes (Gene Ontology Consortium; 1259 genes). (B) GSEA enrichment of canonical pathways associated with PKC-β WT stromal coculture with patient CLL cells (n = 3). KEGG, Kyoto Encyclopedia of Genes and Genomes. (C) Heat map depicting differentially expressed genes between PKC-β WT and KO from a Lysosomal Biogenesis gene set (Reactome). (D) LAMP1, TFE3, and β-actin immunoblots of PKC-β WT stroma and PKC-β KO stroma, cocultured with primary CLL under venetoclax treatment. (E) Immunoblots (IB) of TFE3 and TATA-binding protein (TBP) from nuclear lysates of PKC-β WT stroma and PKC-β KO stroma ± primary CLL coculture. (F) Immunoblot of TFE3 for Tfeb-deleted stroma (sgRNA_3) and sgRNA control stroma and β-actin under CLR coculture conditions. (G) Linked viability plots of patient CLL cells (n = 9) treated with vehicle or venetoclax under coculture with PKC-β WT control or Tfeb-deleted stroma. (H) CLL viability assay (n = 5) after 72 hours of PKC-β WT coculture with 48 hours of exposure to enzastaurin, chloroquine, or bafilomycin ± venetoclax treatment. (I) Linked viability plots of patient CLL cells (n = 10) treated with vehicle or venetoclax under coculture with PKC-β WT control or Tfeb-deleted stroma ± venetoclax treatment. (J) Immunoblots of BCL-XL, p-ERK, and β-actin of CLL cocultured on either PKC-β KO control or caTFEB-transduced stroma ± venetoclax treatment. (K) TCL1 tg-transplanted PKC-β WT recipients were treated with the indicated treatments 3 days after transplantation for the duration of 3 days. BM was subsequently analyzed for LAMP1 and LAMP2 expression in viable MSCs (CD45−Ter119−; left graph) and non-MSCs (CD45−Ter119+; right graph), with representative flow cytometry gating shown. MFIs of LAMP1 and LAMP2 are depicted for individual mice in both graphs, with statistical significance determined using an unpaired two-tailed Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and not significant (ns) P > 0.05.
Fig. 5. Stroma PKC-β regulates plasma membrane integrity and VCAM1 expression. (A) Linked viability plots of CLL patient samples (n = 11) ± Transwell sequestration in the presence of PKC-β WT stroma under vehicle or venetoclax treatment. CLL cell viabilities are normalized to DMSO controls. (B) Global heat map of plasma membrane proteins from PKC-β WT stroma ± primary CLL coculture treated with either vehicle or enzastaurin (5 μM) analyzed by mass spectrometry. The heat map presents clustering of log2-transformed plasma membrane protein abundance for each of three replicates (k-means clustering; k = 5), whereas the side plot depicts the mean protein abundance value for each treatment condition (maximum value of 225). (C) Heat map of significantly altered plasma membrane proteins on the surface of PKC-β WT stroma cocultured with CLL and treated with either vehicle or enzastaurin (5 μM) (q value, <0.05). (D) FACS histograms of VCAM1 (CD106) expression on PKC-β WT and PKC-β KO stromal monocytes. (E) Linked viability plots of CLL patient samples (n = 10) normalized to DMSO controls, in the presence of PKC-β WT stroma ± genetic deletion of Vcam1, under vehicle or venetoclax treatment. (F) Linked viability plots of CLL patient samples (n = 9) normalized to DMSO controls, in the presence of PKC-β WT stroma ± Vcam1 neutralizing or control antibody, under vehicle or venetoclax treatment. (G) FACS analysis of VCAM1 expression in PKC-β WT and PKC-β KO stroma, transduced with either caTFEB or control vector. (H) FACS analysis of VCAM1 expression of cocultured PKC-β WT stroma with CRISPR-modified cells transduced with either Tfeb sgRNA or sgRNA control, APC, allophycocyanin. (I) Percentage plots of adhesion (blue) and mobilization (red) of CLL cells under the various indicated coculture and treatment conditions.

(J) Experimental schematic to assess whether enzastaurin mobilizes CLL from leukemic TCL1-tg mice. (K) CD19 + CD5+ cells in the peripheral blood of vehicle-treated TCL1-tg mice (n = 4), enzastaurin-treated mice (n = 5), and nonleukemic controls (n = 4). (L) CD19 + CD5+ cells in the indicated compartments of vehicle-treated TCL1-tg mice (n = 4), enzastaurin-treated mice (n = 5), and nonleukemic controls (n = 4). (M) Representative flow cytometry gating depicting BM stromal cells (CD45+ Ter119+) assessed for VCAM1 expression in vivo. PE, phycoerythrin. (N) Representative FACS histograms of VCAM1 MFI from DAPI− CD106− cells of BM of TCL1-tg and PKC-β KO mice. (O) Graph of DAPI− CD45+ Ter119− CD106+ MFI cells from BM of indicated TCL1-tg mice treated with vehicle (n = 4), enzastaurin (n = 5), and PKC-β KO mice (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and not significant (ns) P > 0.05.

In the absence or presence of CLL cells ± enzastaurin. Quantitative proteomic analysis identified changes in the composition of cell surface proteins of MSCs induced by contact with CLL cells (Fig. 5B and data file S2). Inhibition of PKC-β with enzastaurin altered the expression of cell surface proteins of CLL-activated MSCs, whereby the expression of 79 and 123 proteins was up- and down-regulated.
by enzastaurin (q value, <0.05), respectively. A cluster of proteins with increased CLL-dependent cell surface expression was appreciably reduced by enzastaurin (cluster 2), whereas the surface expression of other proteins was increased after treatment with the PKC-β inhibitor (clusters 1 and 3). Enzastaurin altered the expression of proteins that have potential roles in adhesion, including collagen1A2 and several matrix glycoproteins (Fig. 5C). In addition, surface proteins with a potential role for activating MSCs themselves were detected. The PKC-β-dependent expression of IL1R1, ADAM17, and IL6ST may contribute to the observed inflammatory gene signature of tumor cell–activated MSCs (10). PMP of MSCs indicated that vascular cell adhesion molecule 1 (VCAM1) was also down-regulated by enzastaurin. Flow cytometric analysis on primary MSCs from WT and KO mice demonstrated a much higher expression of VCAM1 on WT cells (Fig. 5D), which was down-regulated on CLL-activated stromal cells upon PKC-β kinase inhibition (fig. S7E). VCAM1 can bind to integrins expressed on a variety of leukocytes, contributing to cell adhesion and survival (28). We therefore hypothesized that diminished EMDR observed in KO stromal cells was partly attributed to a reduced expression of VCAM1. To test this, we generated VCAM1-deficient BM MSCs using CRISPR-Cas9. Assessment of viability of CLL cells, cocultured on VCAM1-proficient or VCAM1-deficient stromal cells, indicated that VCAM1 contributes to the antiapoptotic effects on malignant B cells. This dependency was further augmented by treatment with venetoclax (Fig. 5E). Similarly, a blocking antibody against VCAM1 enhanced spontaneous and venetoclax-induced apoptosis of CLL cells, indicating that VCAM1 is important for EMDR (Fig. 5F). Overexpression of caTfEB not only rescued EMDR (Fig. 4) but also partially restored VCAM1 expression on KO cells (Fig. 5G). In support of this finding, deletion of Tfeb caused a marked down-regulation of VCAM1 on stromal cells (Fig. 5H and fig. S7F), indicating that PKC-β-dependent activation of TfEB and lysosome biogenesis is important for plasma membrane integrity.

Because a number of adhesion molecules were affected by enzastaurin, we investigated whether blocking PKC-β resulted in a diminished capacity for adhesion of CLL cells in vitro. Patient-derived CLL cells were flowed over PKC-β WT and KO stroma in defined channel slides (fig. S8A). Results indicated no difference between the adhesive capacity of both stromal genotypes in this assay (Fig. 5I, blue), with both types of stroma able to retain flowing CLL cells and concordant with the in vivo homing data previously reported (fig. S8B). In stark contrast, combination treatment reduced tumor burden and terminal spleen weights while prolonging the life expectancy of mice (Fig. 6, A and B, and fig. S9D). A separate assessment of BCL-XI in vivo in tumor cells of diseased animals showed that venetoclax induced its expression, as anticipated from in vitro experiments (Fig. 6C).

As with venetoclax, mice were also treated with fludarabine in combination with enzastaurin (fig. S9E). Disease progression, monitored in the peripheral blood 25 to 28 days after initial treatment, showed tumor cells in both cohorts, with a difference between treatment groups (32.4 ± 3.4% of circulating tumor cells in the fludarabine group versus 9.6 ± 6.7% in the combination treatment group; fig. S9F). Mice were then treated with a second cycle of therapy 54 days after transplantation. Concurrent treatment with fludarabine and enzastaurin markedly prolonged survival of mice compared with fludarabine monotherapy (Fig. 6D). No increased hematotoxicity was observed in mice concurrently treated with enzastaurin (fig. S9, G and H).

Our in vitro data demonstrated that the chemosensitizing effects of enzastaurin were ablated in cocultures of tumor and PKC-β KO stromal cells, indicating that no off-target effects contributed to the enhanced cytotoxicity of venetoclax (Fig. 2F). To prove that no off-target effects of coadministered enzastaurin contribute to the survival benefits observed in vivo, we generated TCLI-driven B cell lymphoma, which could overcome the strong dependency on microenvironment PKC-β (Fig. 1, A to D). TCLI-tg mice were crossed onto transposon- (GrOn)–harboring mice also carrying a hyperactive PiggyBac transposase (further referred to as Hyper-GrOn/ (HGI)) (30, 31). Transplanted TCLI-HG tumors overcame loss of microenvironment PKC-β, although leukemia progression was delayed compared with PKC-β WT recipients receiving the same TCLI-HG cells (fig. S9I). After engraftment of B cell tumors from this mouse model in PKC-β KO animals, mice were subsequently treated with

**Enzastaurin enhances chemosensitivity and prolongs survival in vivo**

Encouraged by these results, we tested whether the chemosensitizing effects of PKC-β inhibitors observed in vitro could be translated into the treatment of murine B cell malignancies. To this end, we transplanted 3.5 × 10⁶ to 5 × 10⁶ splenocytes from several individual diseased TCLI-tg mice intraperitoneally into four cohorts of syngeneic C57B/6 mice. Three days after transplantation, mice were orally treated for 16 consecutive days with enzastaurin, venetoclax, or a combination of both (treatment scheme is shown in fig. S9A). Treatment was well tolerated without hematotoxicity (fig. S9B) or weight loss. However, venetoclax-treated mice developed a loss of hair pigmentation, which was markedly enhanced with enzastaurin coadministration (fig. S9C). This is a known on-target side effect of venetoclax, explained by the exquisite dependency of melanoblasts on BCL2 (29). Monitoring of leukemogenesis in the peripheral blood revealed that single-agent treatment with enzastaurin or venetoclax, given over the course of 16 days, had no effect on the number of tumor cells in the peripheral blood or on disease progression. In stark contrast, combination treatment reduced tumor burden and terminal spleen weights while prolonging the life expectancy of mice (Fig. 6, A and B, and fig. S9D). A separate assessment of BCL-XI in vivo in tumor cells of diseased animals showed that venetoclax induced its expression, as anticipated from in vitro experiments (Fig. 6C).
Fig. 6. Enzastaurin enhances the therapeutic effects of chemotherapy in vivo. (A) Percentages of TCL1-tg CLL cells (CD5+ B220+) in peripheral blood from treatment cohorts at the indicated post-transplantation time points. PB, peripheral blood. (B) Kaplan-Meier survival curves of leukemic mice on vehicle therapy, single-agent therapies, or combined therapy with enzastaurin (60 mg/kg) and venetoclax (100 mg/kg) after intraperitoneal injection of TCL1-tg tumors. Two individual primary tumors were used, transplanted into 19 or 20 mice. Statistical significance was determined using log-rank (Mantel-Cox) analysis. Dosing scheme is shown in fig. S9A. (C) BCL-XL expression measured by flow cytometry as MFI of CD19+ splenocytes isolated from TCL1-engrafted WT mice treated for 72 hours with either vehicle (n = 6) or venetoclax (n = 7). (D) Kaplan-Meier survival curves of leukemic mice on single-agent fludarabine therapy or combined therapy with enzastaurin (60 mg/kg) and fludarabine (34 mg/kg) after intraperitoneal injection of TCL1-tg tumors. Two individual primary tumors were used, transplanted in seven mice each. Schematic of dosing is shown in fig. S9E. (E) PKC-β-deficient (KO) mice received transplants of TCL1-HG splenocytes. Respective treatments began 72 hours after transplantation. PB, peripheral blood from treatment groups. (F) Linked viability graphs of primary MCL cells, normalized to DMSO controls, after coculture on WT stroma with 48 hours of exposure to indicated doses of venetoclax (n = 9) ± enzastaurin treatment. Statistical significance was assessed using a paired two-tailed Student’s t test. (G) Linked viability graphs of primary ALL cells after 72 hours of PKC-β WT stromal coculture with 48 hours of exposure to indicated doses of dexamethasone or vincristine (n = 9) ± enzastaurin treatment. ALL viabilities were normalized to DMSO controls. (H) Linked viability graphs of ALL cells, normalized to DMSO controls, after 72 hours of PKC-β KO stromal coculture with 48 hours of exposure to indicated doses of dexamethasone or vincristine (n = 5) ± enzastaurin treatment. Representative bioluminescent imaging of ALL PDX-engrafted NSG mouse treatment cohorts from three independent experiments with two individual patient-derived xenografts (vehicle control, MST = 69 days; enzastaurin treated, MST = 68 days; vincristine treated, MST = 88 days; vincristine + enzastaurin treated, MST = 101 days). *P < 0.05, **P < 0.01, ***P < 0.001, and not significant (ns) P > 0.05.
vincristine treatment or a combination of enzastaurin and venetoclax. Venetoclax treatment prolonged life expectancy of diseased KO recipient mice. The addition of enzastaurin did not result in extended survival (Fig. 6E), indicating that the chemosensitizing effects of the PKC-β inhibitor were not attributed to off-target effects or on-target inhibition of tumor PKC-β.

On the basis of our previous observation that PKC-β was also activated in stromal cells after contact with tumor cells from patients with ALL and MCL (10), we investigated whether PKC-β inhibition in WT stromal cells mitigated EMDR of cocultured primary B cells from patients with ALL and MCL. Analogous to CLL, enzastaurin enhanced the therapeutic effect of venetoclax on MCL cells (Fig. 6F and table S1). To demonstrate a chemosensitizing effect of PKC-β inhibition on primary cells from patients with ALL, enzastaurin was used in combination with dexamethasone or vincristine, both established chemotherapies in the treatment of patients with ALL. Inhibition of stromal PKC-β with enzastaurin increased the cytotoxicity of both drugs (Fig. 6G and table S1). This effect was completely abolished in cocultures with KO stromal cells (Fig. 6H), confirming that the PKC-β inhibitor sensitized B-ALL cells by targeting stromal PKC-β and excluding the contribution of off-target effects or inhibitory effects on PKC-β expressed in ALL cells. To assess whether PKC-β inhibition also sensitizes primary ALL cells to chemotherapy in vivo, NOD.Cg-Prkd–/–H2rg–/–B6.SJL/J (NSG) mice xenografted with luciferase-labeled ALL-PDX cells were treated for 3 days with vincristine ± enzastaurin. Bioluminescence imaging 3 to 6 weeks after transplantation revealed comparable leukemic burdens between vehicle- and enzastaurin-treated mice (Fig. 6I and fig. S9J). As expected from our in vitro data, enzastaurin monotherapy did not extend survival of ALL-xenografted mice compared with vehicle controls, whereas the concomitant administration of enzastaurin and vincristine increased life expectancy compared with single-agent vincristine treatment (Fig. 6J).

**DISCUSSION**

A growing amount of research has identified tumor-host interactions as essential processes regulating tumor cell immune evasion, metabolic adaptations, survival, and proliferation (32). Here, we describe a crucial dependency for survival of normal B1 and malignant B cells on PKC-β expressed in stromal cells. Under conditions of cytotoxic stress, PKC-β regulates lysosome biogenesis by stabilizing TFEB, which is essential for plasma membrane composition and EMDR, effectuated by BCL-XL expression in tumor cells. Genetic and pharmacological interference with this stress response blunts this adaptation and increases the efficacy of chemotherapies (fig. S10).

The temporal and spatial requirements for EMDR remain to be defined. It appears that the BM is of particular importance for malignant B cells because the absence of PKC-β results in a rapid loss of adoptively transferred cells in this compartment. This is in consonance with the clinical importance of this compartment for many B cell diseases, as reflected by the negative impact of BM MRD positivity on progression-free survival (7, 33). However, this conclusion is partly based on a lack of data, because MRD assessment in patients is restricted to easily accessible locations. Therefore, it remains unknown whether chemoprotective niches exist in other organs. Although we have observed the strongest dependency on PKC-β in the BM compartment during early disease stages, it is reasonable to assume that the spatial requirements and cellular composition of drug-protective niches are dynamic and change over time. In support of this hypothesis, in the TCF1 model, the spleen appears to be the predominant site of disease at a terminal stage. The marked reduction in the spleen size in enzastaurin-cotreated mice suggests that drug-protective niches also exist in this organ, supported by our finding of prosurvival, PKC-β–dependent signals derived from FRCs. Therefore, cell types other than BM MSCs, present in different organs, are likely to also contribute to the observed effects in vivo. Their identification and molecular characterization are important and can be achieved through the use of reporter mice.

In addition to tumor cells, we have observed that the physiological development of B1 cells also depends on PKC-β activity in the microenvironment. Although predominantly derived from fetal liver progenitors, B1 cells can also be derived from BM HSCs (34, 35). Results from our BM chimeras were unexpected and, in light of previously published data (36), indicate that PKC-β has a dual function in B cell development, maintenance, and differentiation. On the basis of the observations made in germline deleted PKC-β KO mice (12), several groups have unambiguously demonstrated that intrinsically expressed PKC-β is essential for BCR signaling through the activation of IKKα/nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) (37, 38). More recently, it was demonstrated that PKC-β–deficient B cells fail to present antigens and to differentiate into plasma cells upon immunization (36). However, the capacity to form germinal centers ex vivo was not ablated per se in PKC-β KO B cells, indicating that the essential role of PKC-β for B cell differentiation is dependent on cell-cell interactions. A dual function of PKC-β in B cell physiology is also supported by our observation that WT BM cells outperformed PKC-β KO cells in KO recipient mice with respect to the development of peritoneal B1 cells. In contrast, the capacity of KO cells to produce B1 cells was indistinguishable from WT cells in WT recipient animals. In line with this observation, we found similar titers of the natural antibodies IgM and IgG3 in WT recipient animals reconstituted with KO CD45+ BM cells. Although we cannot fully exclude the possibility that plasma cells survived lethal irradiation of recipient mice (39) and contributed to these antibody concentrations, reduced IgM and IgG3 titers in KO recipient animals reconstituted with WT CD45+ BM cells strongly suggest that extrinsically expressed PKC-β is crucial for the production of natural antibodies. These findings reveal that the exquisite dependence of malignant B cells, especially under therapy, on PKC-β–dependent functions in their environment is also an integral part of normal B cell physiology.

Central to the EMDR observed in our studies was a PKC-β–mediated increase in BCL-XL expression, in response to various chemotherapeutics. It was previously recognized that BCL-XL can confer a multidrug-resistant phenotype in hematopoietic (40) and nonhematopoietic (41) cells, making it a key target for therapy. CLL cells respond to the cellular stress of BCL2 antagonism by a microenvironment-mediated posttranscriptional increase in BCL-XL expression. However, our data show that the increased BCL-XL protein expression in CLL cocultured with PKC-β WT stroma was not attributed to enhanced transcription. Different posttranslational modifications of BCL-XL, such as ubiquitination (42) and deamidation (43), must therefore be considered as possible contributing factors.

Our experiments demonstrate that VCAM1 expression on stromal cells is exquisitely dependent on PKC-β–mediated activation of TFEB. Previous studies have reported that the acute deletion of...
Vcam1 in adult mice resulted in a reduction in immature and mature B cells in the BM (44, 45). In addition, homing of adoptively transferred mature B cells to the BM was impaired in these mice (44). In contrast, germline deletion of Vcam1, which is often embryonically lethal, gave rise to a hypomorphic mouse model with no defects in B cell maturation (46), suggesting that low expression of Vcam1 is sufficient for B cell development or that compensatory mechanisms exist. It is reasonable to assume that the reduced expression of Vcam1 on BM MSCs of PKC-β-deficient mice contributes to the PKC-β-deficient phenotypes. The low expression of constitutive Vcam1 on mesenchymal cells of PKC-β KO mice may be related to reduced activity of NF-κB, because Vcam1 is a direct transcriptional target (47). We have previously demonstrated that the PKC-β-dependent activation of NF-κB in stromal cells, essential for tumor cell survival, is dependent on essential modulator (NEMO) (10). Therefore, it is reasonable to speculate that the NF-κB deficiency observed in PKC-β KO stromal cells is, at least partially, attributed to a lack of lysosome-mediated degradation of IkB (inhibitor of nuclear factor κB), as previously described (48, 49). However, lysosomes are multifunctional organelles contributing to many cellular processes, including autophagy, exosome release, plasma membrane repair, and adhesion. Therefore, other mechanisms may operate in parallel, including ECM remodeling and enhanced integrin signaling (50, 51), which could both further contribute to EMDR.

We believe that we have generated sufficient data to justify investigating the chemosensitizing properties of PKC-β inhibitors in a clinical setting. We have observed substantial survival benefits by coadministered enzastaurin together with short courses of chemotherapy. Enhanced effects of PKC-β inhibitors are therefore likely to be observed in clinical practice with repeated treatment cycles or continuous treatments.

Conceptually similar to our proposed drug combinations, the concomitant treatment with BCL2 and BTK inhibitors is currently being investigated in patients with lymphoma (52), aiming at blocking microenvironment interactions to enhance the cytotoxicity of venetoclax. Contrary to this concept, our data indicate that PKC-β inhibitors cause an in situ chemosensitization of B cells because we have not observed an adherence deficiency or a redistribution of cells into the peripheral blood upon kinase inhibition. The reduced expression of adhesion molecules on PKC-β-deficient stromal cells likely reduces survival signals to leukemic cells, but without affecting niche residency. The dependency of different types of B cells, including normal B1 cells and immature malignant B cells, on PKC-β functions in the microenvironment further indicates that PKC-β inhibitors may be clinically used beyond the spectrum of BCR inhibitor–sensitive disease entities. The treatment of autoimmune diseases with a pathogenic contribution from B cells, such as systemic lupus erythematosus, may also benefit from the chemosensitizing effects of PKC-β inhibitors.

At present, the PKC-β inhibitor enzastaurin, an oral ATP-competitive small-molecule inhibitor with a relative specificity for PKC-β (16), is in clinical development at the stage of phase 3 trials (for example, NCT03263026). In addition, midostaurin, although less specific for the β isoform, has recently been approved for the treatment of patients with AML (18). On the basis of the idea of targeting intrinsic expression of PKC-β in malignant B cells, the PRELUDE phase 3 trial was designed to test the efficacy of enzastaurin as a maintenance monotherapy for patients with diffuse large B cell lymphoma (DLBCL) after treatment with R-CHOP. The trial failed its primary end point (disease-free survival) (53), which resulted in a temporary halt in the clinical development of the drug. Unlike in low-grade lymphomas or ALL, MRD is clinically not a problem for the vast majority of patients with DLBCL, suggesting that EMDR may not play a substantial role for the majority of patients with high-grade non-Hodgkin’s lymphoma. This is reflected by the observation that 70% of patients in the PRELUDE trial were already cured at the end of immunochemotherapy before commencing maintenance therapy.

The incorporation of PKC-β inhibitors into treatment regimens used for various B cell malignancies may have profound clinical and socioeconomic implications: Improved clinical responses may ultimately allow for the reduction in the number of treatment cycles, lowering cumulative drug doses and minimizing side effects, costs, and need for salvage therapies. Our data demonstrate that the application of PKC-β inhibitors can be limited to treatment days, minimizing their compound-specific side effects and costs. Clinical trials are now needed to address whether our data can be translated into improved patient care.

**MATERIALS AND METHODS**

**Study design**

Our primary objective was to test whether the dependency of malignant B cells from patients with CLL, MCL, and ALL on PKC-β expressed and activated in the tumor microenvironment could be harnessed therapeutically with kinase inhibitors. This dependency was investigated through ex vivo coculture experiments of tumor and stromal cells, either proficient or deficient for PKC-β (representative gating depicted in fig. S11A). Activated signaling pathways in either cell compartment were biochemically analyzed in combination with RNA-seq and mass spectrometry. Our data were validated through in vivo experiments, in which diseased C57B/6 or NSG mice were treated with cytotoxic agents in combination with PKC-β inhibitors. Furthermore, to test whether dependency on stromal PKC-β extends to normal B1 cells, we generated BM chimeric mice differing in the expression of PKC-β.

We did not use statistics to predetermine sample sizes. Sample sizes in the mouse experiments were based on our experience and published data (10, 54). Animals were randomly assigned to one of the following treatment cohorts (vehicle control, venetoclax, fludarabine, vincristine, enzastaurin, enzastaurin + venetoclax, enzastaurin + fludarabine, and enzastaurin + vincristine). Investigators were not blinded to treatment allocations, but animal technicians who delivered care and decided independently upon which animals needed to be euthanized (applying strict criteria for end points) were blinded. In vitro studies were conducted with multiple technical and biological replicates, indicated in the figure legends, to ensure reproducibility of data.

**Primary cells**

After patients’ informed consent and in accordance with the Helsinki Declaration, peripheral blood was obtained from patients with a diagnosis of CLL, ALL, or MCL. Studies were approved by the Cambridgeshire Research Ethics Committee (07/MRE05/44), the Technical University of Munich (project number 1894/07), and the Institutional Review Board of the Erasmus Medical Center.

**Extraction of murine BM stromal cells**

All in vivo experiments were conducted in accordance with the U.K. Home Office regulations (license P846C00DB). Murine BM MSCs
were established from femora and tibiae of 4- to 8-week-old mice. BM stromal cells were harvested from young female age-matched Prkcb+/+ and Prkcb−/− mice by flushing the cavities of femora and tibiae with phosphate-buffered saline (PBS). After filtration through a 70-μm filter and depletion of erythrocytes using a lysis buffer (BD Pharm Lyse; BD Biosciences), washed cells were either used further for experiments or cryopreserved.

**Generation of chimeric mice**

BM s from CD45.2 Prkcb+/+, Prkcb−/−, and CD45.1+ B6.SJL- Ptprca Pepe+/Boy (the Jackson laboratory) age-matched mice were isolated and depleted of CD45− cells with purity of >95% confirmed by flow cytometry (muCD45 microbeads; Miltenyi Biotec). CD45.2 Prkcb−/− cells (3 × 10^6) or CD45.1+ B6.SJL- Ptprca Pepe+/Boy cells purified from the BM were injected intravenously into recipients with different CD45 isotype after irradiation (10 Gy) (CD45.1+ BM into CD45.2 recipient and CD45.2+ BM into CD45.1 recipient). CD45.2 Prkcb−/− BM was also injected into irradiated CD45.2 Prkcb−/− recipients as a control. Chimerism was assessed by flow cytometry of CD45.1 and CD45.2 staining of peripheral blood withdrawn by tail vein bleeding (representative gating depicted in fig. S11B). Serum immunoglobulin isotype concentrations were assessed from peripheral blood using a mouse-specific immunoglobulin isotype panel and subsequently analyzed and quantified using commercial software (LegendPlex Mouse Ig Panel, LegendPlex Analysis Software; BioLegend). Peritoneal fluid, peripheral blood, BM, and spleen were harvested from animals and analyzed by flow cytometry. Upon confirmation of chimerism, chimeric mice of appropriate genotype were injected with 4 × 10^7 CFSE-labeled TCL1 tumor cells intravenously and 1 × 10^6 cells intraperitoneally. Assessment of labeled CLL cells in peripheral blood was performed by flow cytometry.

**In vivo models for CLL homing and engraftment**

For CLL homing experiments, primary TCL1-tg tumor cells were labeled with 5 μM CFSE (Life Technologies) per each manufacturer’s protocol. After confirmation of labeling by flow cytometry, 4 × 10^6 cells were injected intravenously and 1 × 10^6 cells were injected intraperitoneally into age-matched mice of two genotypes. Peripheral blood was drawn by tail vein bleeding and analyzed by flow cytometry after erythrocyte depletion. Animal well-being was monitored daily, and all experiments were conducted under the U.K. Home Office regulations.

**In vivo model for VCAM1 biomarker ± enzastaurin**

Leukemic TCL1-tg mice were analyzed for leukemic burden by CD5+C19 staining of peripheral blood. Cohorts were assembled to match overall leukemic burden across vehicle control and enzastaurin treatment cohorts. Enzastaurin or vehicle (5% dextrose + 10% ethanol in water) was administered at a dose of 60 mg/kg BID per os (PO) for a total of 96 hours. Analysis of BM VCAM1 expression was conducted 3 hours after the final treatment by flow cytometry. In brief, stromal cell populations were isolated from femur and tibia, crushed with mortar and pestle and digested with 2 ml of collagenase I (STEMCELL Technologies) and collagenase IV (1 mg/ml; Sigma-Aldrich) at 37°C with strong agitation for 30 min. Cells were washed with PBS + 2% fetal bovine serum and filtered through a 40-μm mesh filter. Red blood cells were lyzed with PharmaLyse (BD Biosciences) for 10 min on ice. Samples from individual mice were analyzed by flow cytometry (representative gating depicted in fig. S11C).

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**In vivo model for LAMP1 and LAMP2 biomarkers ± enzastaurin**

Seventy-two hours after transplantation, PKC-β WT recipients of TCL1-tg transplants were treated with vehicle, venetoclax (100 mg/kg per day PO), or pretreated with enzastaurin (60 mg/kg BID PO) and then treated with the combination of enzastaurin + venetoclax for 3 days. BM was harvested 3 hours after the final dose and analyzed for intracellular LAMP1 and LAMP2 expression after surface staining and subsequent fixation and permeabilization (BioLegend). Mean fluorescence intensities (MFIs) of LAMP1 and LAMP2 were assessed in viable MSCs (CD45− Ter119−) and non-MSCs (CD45− Ter119+). Statistical significance of intracellular staining was determined using an unpaired two-tailed Student t test.

**In vivo CLL study of venetoclax ± enzastaurin**

C57B/6 mice (the Jackson laboratory) were injected intraperitoneally with either 3.5 × 10^6 or 5 × 10^6 cells of two primary TCL1-tg tumors. Seventy-two hours after transplantation, the treatments began by oral gavage for 16 consecutive days. Venetoclax was solubilized as previously reported (55). In brief, venetoclax was formulated in phosal 50 propylene glycol (60%), polyethylene glycol 400 (30%), and ethanol (10%). Leukemic burden was assessed by flow cytometric analysis of erythrocyte-depleted peripheral blood. Mice in these and subsequent treatment studies were euthanized upon signs of ill health, such as piloerection, hunched posture, inactivity, or inappetence for a period of 48 hours principally by animal husbandry technicians who were blinded to allocation to treatment groups. In addition, any animal losing 15% of its body weight when compared with age-matched controls was culled.

**In vivo CLL study of fludarabine ± enzastaurin**

C57B/6 mice were injected intraperitoneally with 5 × 10^6 cells of two primary TCL1-tg tumors. Seventy-two hours after transplantation, the treatments began by oral gavage and intraperitoneal injection. Fludarabine phosphate (Sigma-Aldrich) was solubilized in sterile PBS. Mice received fludarabine treatment [34 mg/kg per day intraperitoneally (IP)] for five consecutive days for two cycles. Enzastaurin (60 mg/kg BID PO) or vehicle was administered twice daily during the same periods. Leukemic burden was assessed by flow cytometric analysis of peripheral blood.

**In vivo ALL-PDX study of vincristine ± enzastaurin**

NOD.Cg-PkdcsclIl2rgtm1Wjl/SzJ (NSG) mice were injected intravenously with 2 × 10^5 to 3 × 10^5 cells of luciferase-transduced ALL cells derived from two distinct patient samples. In three independent experiments, cohorts were treated 72 hours after transplantation. Enzastaurin (60 mg/kg BID PO) or vehicle control was administered orally 12 hours before intraperitoneal dosing of vincristine or vehicle and continued for 72 hours thereafter. Vincristine (Sigma-Aldrich) was solubilized in sterile PBS and administered once per treatment cycle at a weight-adjusted dose between 0.5 and 0.6 mg/kg for one or two cycles (54). For the combination-treated cohorts, 14 mice received one cycle of treatment (n = 7, for both 0.5 mg/kg and 0.6 mg/kg of vincristine), whereas 5 mice received a second treatment cycle (0.5 mg/kg of vincristine) 4 weeks after the initial treatment, with single-agent cohorts also receiving a second treatment cycle. Anesthesia-related morbidities on day 73 after transplantation in one independent experiment resulted in the censoring of three (vincristine) and four (combination treatment) animals. Leukemic burden...
was assessed by flow cytometry and bioluminescence imaging (Xenogen IVIS) of peripheral blood. Luciferin was administered intraperitoneally to anesthetized animals before live imaging.

Statistical analysis

All in vitro experiments were repeated at least three times, and the means ± SEM were calculated. The sample sizes for each experiment are provided in the figure legends. Statistical analyses of results were performed using one-way analysis of variance (ANOVA) followed by two-tailed Student t tests, with unpaired or paired analyses as appropriate. Statistical annotations were denoted with asterisks as follows: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, and not significant (ns) P > 0.05. In vivo studies were carried out using multiple animals (3 to 15 per group, specified in the figure legends), and Kaplan-Meier curves were generated from survival data. Original data are provided in data file S3.

SUPPLEMENTARY MATERIALS

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133 Rawlings, A. M., H. Chen, K. K. Wu, Cytokine-induced autophagy promotes long-term VCAM-1 but not ICAM-1 expression by degrading late-phase I deamidation is a


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Stromal cell protein kinase C-β inhibition enhances chemosensitivity in B cell malignancies and overcomes drug resistance

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No more help for the cancer

Just as the tumor microenvironment influences the behavior of solid tumors, hematological malignancies are affected by the microenvironment in the bone marrow stroma. Park et al. identified a pathway dependent on protein kinase C-β from the stromal cells, which promotes survival and drug resistance in B cell malignancies. The authors examined the mechanism for the observed effects and then identified small-molecule compounds for potential intervention. Inhibiting the protein kinase C-β signaling pathway in stromal cells sensitized these cancers to a variety of drugs and extended survival in both genetic and patient-derived mouse models of cancer.