Generation of a poor prognostic chronic lymphocytic leukemia-like disease model: PKCα subversion induces up-regulation of PKCβII expression in B lymphocytes

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Supplementary Methods

Flow Cytometric Analysis. Combinations of anti-CD23-PE (B3B4), anti-CD45-PerCP (30-F11), anti-IgM-PE-Cy7 (R6-60.2), anti-CD5-APC (53-7.3) and anti-CD19-APC-Cy7 (1D3) were used. For ZAP-70 intracellular staining, surface CD19 staining was followed by fixation with cytofix/cytoperm (BD Biosciences). The ZAP-70 antibody was either directly-conjugated with PE (1E7.2) or purified (clone 29) and detected by PE-conjugated goat anti-mouse IgG antibody. Mouse IgG2a isotype was used for control staining.

Histology and immunohistochemistry. For histological analysis, 3-μm sections were stained with haematoxylin and eosin (H&E) with a standard protocol (Glasgow Royal Infirmary, UK). For immunohistochemical staining, paraffin-embedded sections were dehydrated with xylene and ethanol then antigenic epitopes were exposed by using an EDTA solution (10 mM Tris/1 mM EDTA, pH 8.0) and microwaving. Endogenous biotin was blocked with avidin/biotin blocking kit (Vector Labs, Peterborough, UK), and sections were blocked with peroxidase blocking solution (3% H_2O_2). Thereafter, sections were blocked with 5% horse serum then stained with biotin-conjugated anti-B220 antibody (clone RA3-6B2), detected by HRP-Vectastain ABC kit (Dako, Ely, UK) and ImmPACTTM diaminobenzidine.

Determine the mutational status of IgV_H. GFP^+ splenic cells were isolated by cell sorting on a FACSAriaI (BD Biosciences), RNA was extracted using the RNAeasy kit (Qiagen, Manchester UK) and reverse transcribed with AMV (Roche Diagnostics) using oligo(dT)15 primers. cDNA was amplified with PCR primer combinations and cycles as described previously.[1] PCR products were cloned into pCRII-Blunt-TOPO (Invitrogen) and sequenced with M13 reverse/forward primers.

Western Blotting. Protein lysates containing equal protein amounts from MIEV- or PKCα-KR-HPC-co-cultures were separated by SDS-PAGE and transferred onto PVDF membrane, and blocked as described previously.[2] All antibodies were obtained from Cell Signaling Technologies (Danvers, MA) except anti-$PKC\beta_I$ (E-3) and anti-$PKC\beta_{II}$ (sc-210) antibodies, which were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were developed with Immun-Star™ Western C™ HRP chemiluminescence kit.

1. White HN. Restriction-PCR fingerprinting of the immunoglobulin VH repertoire: direct detection of an immune response and global analysis of B cell clonality. Eur J Immunol. 1998;28:3268-3279.

2. Nakagawa R, Vukovic M, Cosimo E, Michie AM. Modulation of PKC-α promotes lineage reprogramming of committed B lymphocytes. Eur J Immunol. 2012;42:1005-1015.

CLL sample ID	Treated	Sex	Stage[a]	ZAP-70 status[b]	IgV_H Mutational status[c]	Cytogenetics	PKCα expression[d] (rel to normal B)
14	Y	M	C	Pos		17p del	High
23	N	F	A		UM		High
32	N	F	B	Pos	UM	Normal	Low
34	Y	M	B	Pos	UM	11q del	NE
41	N	M	A	Neg		Normal	NE
45	Y	M	B	Pos	UM	13q del	NE
46	N	F	A	Pos	M	Normal	NE
51	N	M	A	Neg	M	Normal	Same
52	Y	F	B	Neg	M	11q del	NE
54	N	F	A	Neg	M	Normal	NE
56	N	F	A				NE
58	N	M	A				NE
60	N	F	A	Pos	M	Normal	low
62	Y	F	A		M		NE
69	Y	M	A	Pos	UM	Normal	low
70	N	F	C	Neg		Normal	Same

Table S1. Summary of the clinical parameters of the CLL samples and PKCα expression levels.

[a] CLL disease stage according to Binet staging.

[b] ZAP-70 analysis was conducted by immunohistochemistry in the regional haematology laboratory.

[c] IgV_H is considered mutated when there is a deviation of >2% from the germline sequence.

[d] PKCα expression was determined by Western blotting, and compared with expression of normal B cells isolated from PBMCs. NE – no expression detected.

Figure S1 - Nakagawa *et al.*,

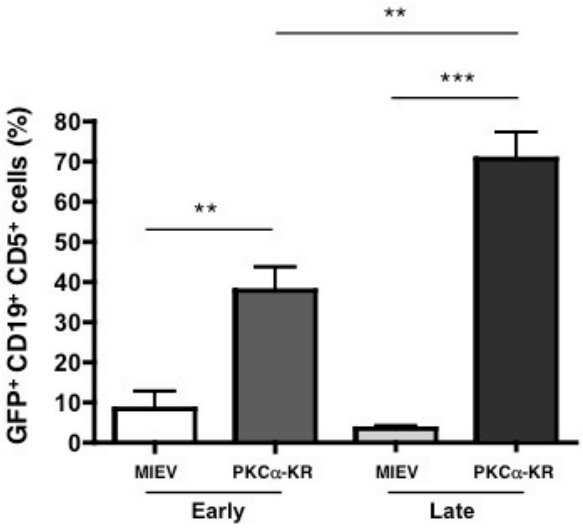

Figure S1. The percentage of the CLL-like CD19$^+$CD5$^+$ population increases over time in the PKCα-KR cocultures. HPC-OP9 co-cultures were analysed by flow cytometric analysis at early (d6 – d10) and late (d15 – d21) stages of the co-culture. The plots shown are live and size (FSC/SSC) gated and additionally gated for haemopoietic (CD45$^+$), PKCα-KR (GFP$^+$) and CLL-like (CD19$^+$CD5$^+$) cells. Student's unpaired t-test was performed; ** $p<0.005$; *** $p<0.001$. The graphs were generated from at least 5 individual experiments.

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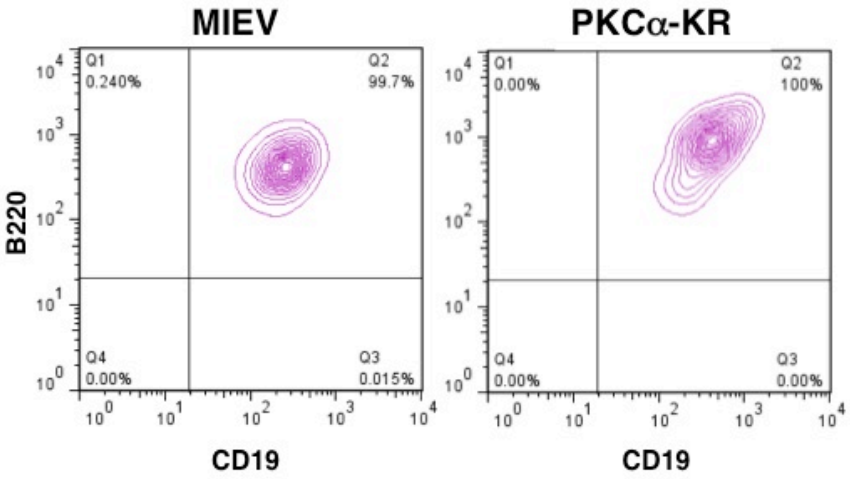

Figure S2. B lineage cells derived from MIEV- and PKCα-KR-transduced cells co-stain for CD19 and B220. HPC-OP9 co-cultures were analysed by flow cytometric analysis. The plots shown are live and size (FSC/SSC) gated and additionally gated for haemopoietic ($CD45^+$) cells.

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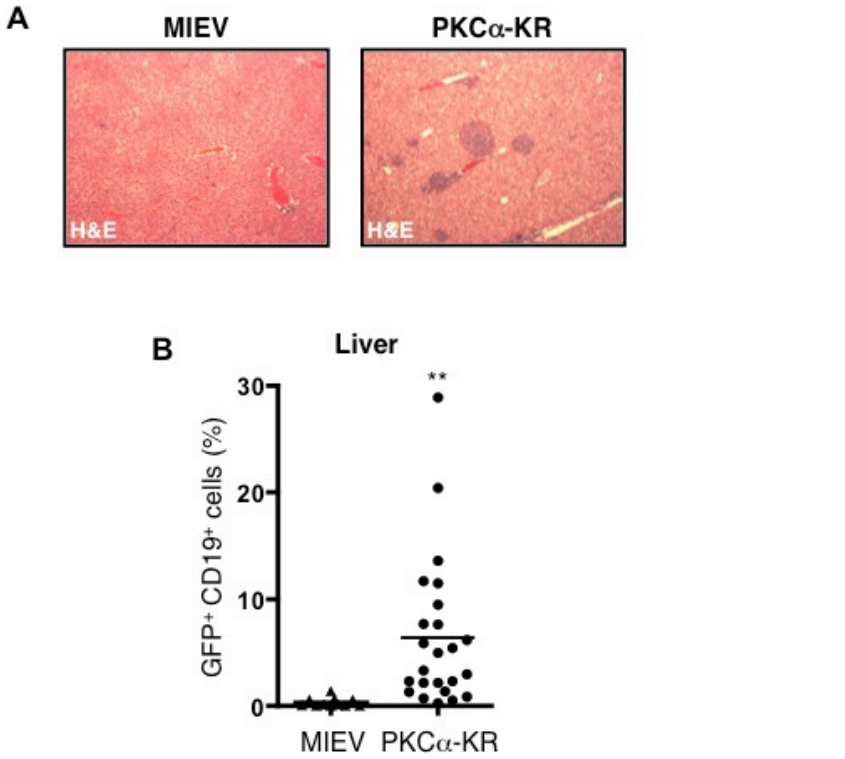

Figure S3. CLL-like cells infiltrate the liver of adoptively-transferred mice. MIEV- and PKCα-KR-transduced HPCs were i.p. injected into RAG-1$^{-/-}$ neonates. After 5 wk the reconstituted mice were sacrificed and the liver was isolated. (**A**) Paraffin-embedded spleens from MIEV (left) or PKCα-KR (right) were sectioned and stained with H&E. x 4 magnification shown. (**B**) The percentage of GFP$^+$CD19$^+$ cells within total hematopoetic CD45$^+$ cells in the liver is shown for mice reconstituted with MIEV- or PKCα-KR-HPCs. Student's unpaired t-test was performed; ** $p<0.005$. The graphs were generated from at least 10 individual mice.

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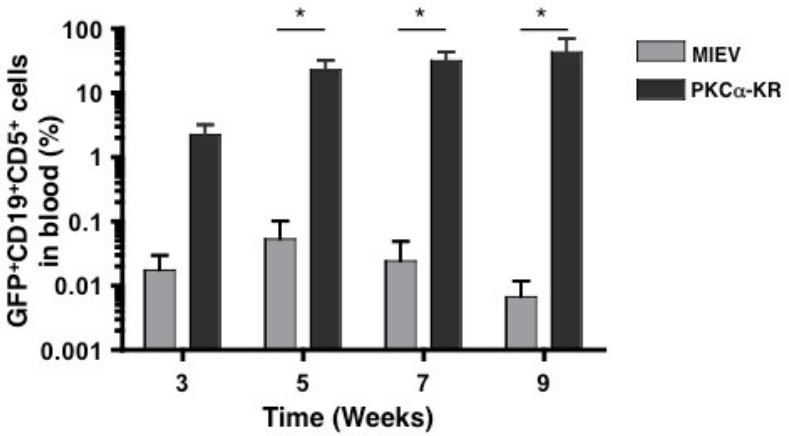

Figure S4. The percentage of the CLL-like CD19$^+$CD5$^+$ cells increases over time in the blood of PKCα-KR-HPC adoptively-transferred mice. MIEV- and PKCα-KR-transduced HPCs were i.p. injected into RAG-1$^{-/-}$ neonates. The blood was sampled 3 – 9 wk post injection and stained with CLL markers for flow cytometry. The population percentage was assessed by live and size (FSC/SSC) gating and the percentage of PKCα-KR (GFP$^+$) and CLL-like (CD19$^+$CD5$^+$) cells within the CD45$^+$ cells is shown. Student's unpaired t-test was performed; * $p<0.05$. Each data point is generated from 3 - 5 individual mice.

Figure S5 - Nakagawa *et al.*,

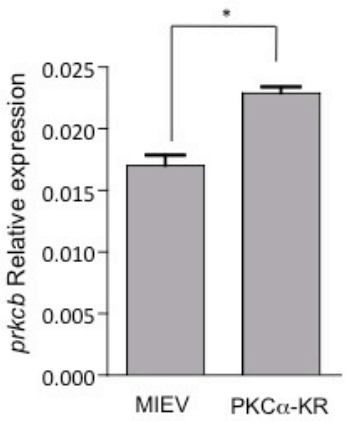

Figure S5. CLL cells isolated from the PKCα-KR mouse model exhibit altered PKCβ expression. RNA was isolated from d17 MIEV- or PKCα-KR-OP9 co-cultures and subjected to qRT-PCR to evaluate the levels of *PRKCB* expression. Results are expressed as $2^{(-\Delta CT)}$ relative to GAPDH reference gene and represent mean ± SEM (* $p<0.05$).

Figure S6 - Nakagawa *et al.*,

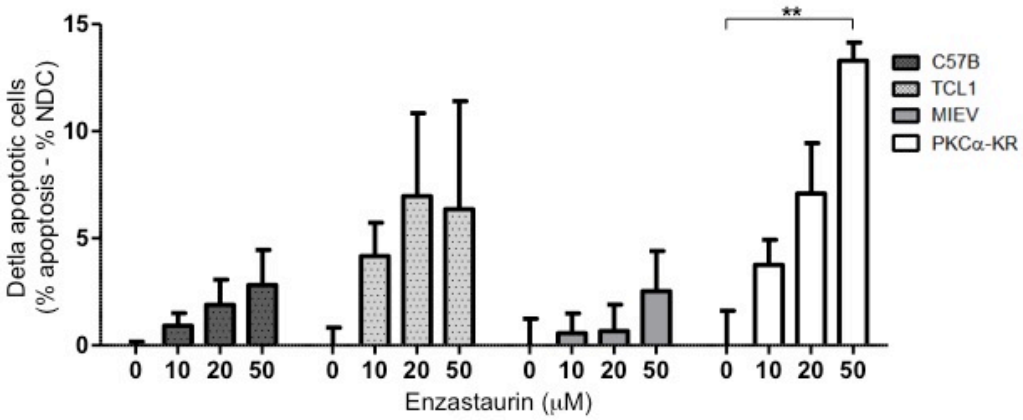

Figure S6. Enzastaurin selectively induces apoptosis in PKCα-KR-expressing CLL-like cells. Flow cytometry was used to assess the level of apoptosis induced upon enzastaurin treatment of MIEV- vs. PKCα-KR cells and splenic B lineage cells isolated from age-matched C57BL/6 vs. Eμ-TCL-1 transgenic mice. Annexin V^+DAPI^- cells represent early apoptotic cells. Data show the percentage of Annexin V^+DAPI^- cells after subtraction of background apoptosis (cells cultured in the absence of enzastaurin (0), and is referred to as the "delta apoptotic cells". Data shown are the mean (± SEM) of 3 biological replicates (** $p<0.01$).