1038 Inhibition of G protein-coupled receptor kinase 2 (GRK2) as a strategy to modulate leukemic cell homing and activation in CLL

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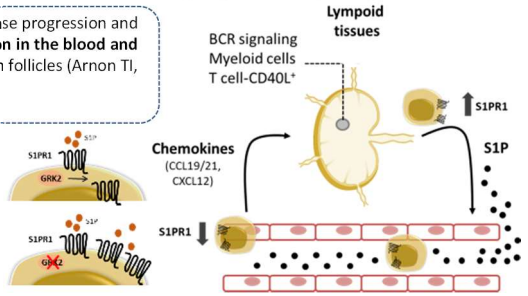
I. Background : Proliferation of leukemic cells and resistance to therapy occur within lymphoid tissues, supporting the idea that the retention of leukemic cells within this tumour microenvironment contributes to disease progression and relapse. **GRK2 plays a central role in B cell homing to lymphoid organs by inducing Sphingosine-1 phosphate receptor-1 (S1PR1) downregulation, which allows lymphocytes to overcome the S1P-mediated retention in the blood and to enter into lymphoid tissues.** In mouse models, GRK2-deletion on B cells leads to an increased response to S1P and thus to a higher presence of B cells in peripheral blood over bone marrow, lymph nodes and spleen follicles (Arnon TI, et al. 2011. Science). In addition, GRK2 has been implicated in signalling pathways related to cancer progression (Nogués L, et al. 2017. Molecular Pharmacology).
Our aim was to evaluate the role of GRK2 in leukemic cell migration, activation and survival.



II. Methods: Leukemic cells were purified by immunomagnetic cell separation from peripheral blood of untreated CLL patients. GRK2 expression was evaluated by western blot and qRT-PCR. For *in vitro* GRK2 inhibition the small molecule inhibitor, CMPD101, was used. CLL cell activation was induced by BCR stimulation with immobilized anti-IgM (25 µg/ml), then CD69 and CD86 expression was evaluated by flow cytometry (FC) after 24hs and 48hs respectively. T cell activation was induced with immobilized anti-CD3 (0.5 µg/ml). CD40L was evaluated after 24hs on T cells by FC. Chemotaxis towards S1P, CXCL12 or CXCL13 was evaluated *in vitro* using a Transwell cell migration assay. Migration index: cells that had migrated in response to the stimuli/cells that had migrated spontaneously after 3 h. GRK2-deficient murine leukemic cells were generated by using CRISPR/Cas9 with the Alt-R system (IntegratedDNA Technologies [IDT]) on the mouse cell line TCL1-355 TKO as previously described (Chakraborty S, et al. 2021. Blood). TCL1355-TKO cells were electroporated with Cas9 alone (MOCK) or with ribonucleoparticles (RNP) containing recombinant Cas9 enzyme + GRK2 guide RNA (GRK2KO). INDEL analysis of the PCR products of edited populations was performed by capillary electrophoresis and mutant allele frequency (MAF) was determinate by peak quantification. For *in vivo* migration assays, GRK2KO or MOCK (control) cells were labeled with CTV or CFSE, mixed in a ratio 1:1 and injected through the tail vein. 20h later, their localization in peripheral blood, bone marrow and spleen was evaluated by FC. Statistical analysis was performed with Prism v7 (GraphPad).

III. Results

Figure 1. CLL cells express GRK2 at levels comparable to those of normal B cells.

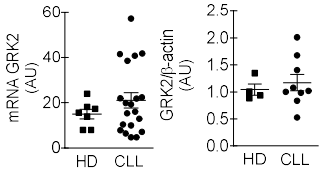


Fig 1. GRK2 mRNA and protein on B cells from healthy donors (HD) and CLL patients AU: arbitrary units.

Figure 2. *In vitro* inhibition of GRK2 reduces the up-regulation of activation markers on CLL cell stimulated through the BCR, and on co-cultures with autologous activated-T cells.

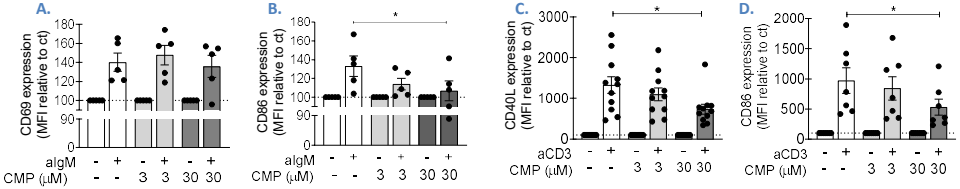


Fig 2. PBMC from CLL patients stimulated with immobilized anti-IgM (A-B) or anti-CD3 (C-D) with or without the GRK2 inhibitor, CMPD101 (CMP). CD69 and CD86) expression on CLL cells and CD40L on T cells.*p<0.05, Friedman test followed by Dunn's post-test.

Figure 3. GRK2 inhibition increases CLL cell migration to S1P, impairs S1PR1 down-regulation, and has no effect on migration to CXCL12 and CXCL13.

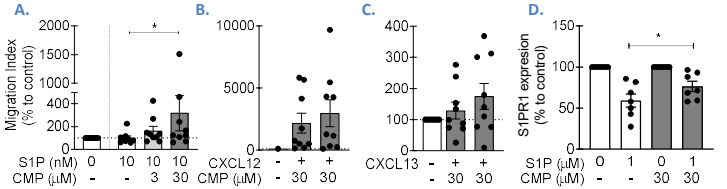


Fig 3. *In vitro* migration of CLL cells to S1P (A), CXCL12 (0.5 µg/ml) (B) and CXCL13 (1µg/ml) (C). D. Expression of S1PR1 on leukemic cells cultured with or without S1P for 24h evaluated by FC. *p<0.05, Friedman test followed by Dunn's post-test.

Figure 4. Deletion of GRK2 in the TCL1355-TKO leukemic cell line.

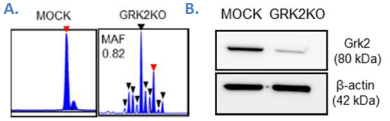


Fig. 4. Indel analysis by amplicon capillary electrophoresis of MOCK and GRK2-targeted cells. MAF=mutated alleles/total alleles. Red arrow: peak corresponding to GRK2 wt allele; black arrow: peak corresponding to GRK2 mutated allele. B. GRK2 expression by western blot.

Figure 5. GRK2 deletion increases leukemic cell migration to S1P.

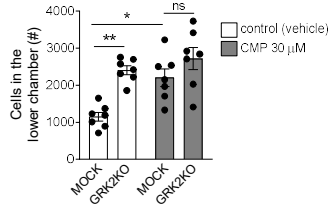


Fig 5. *In vitro* migration of GRK2KO and MOCK leukemic cells to S1P (10nM). *p<0.05, Friedman test followed by Dunn's post-test.

Figure 6. GRK2KO leukemic cells have a lower spontaneous and LPS-induced proliferation rate *in vitro*.

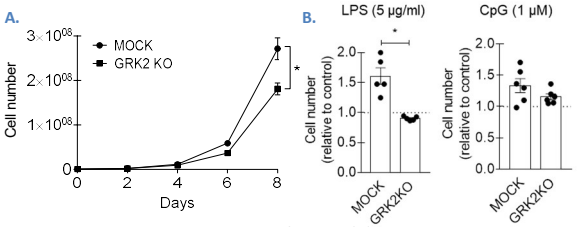


Fig. 6. *In vitro* spontaneous proliferation (A) or in response to TLR4 ligand, LPS (5 µg/ml) and TLR9 ligand, CpG (1 µM) (B). *p<0.05, Wilcoxon test

Figure 7. GRK2KO leukemic cells localization *in vivo*.

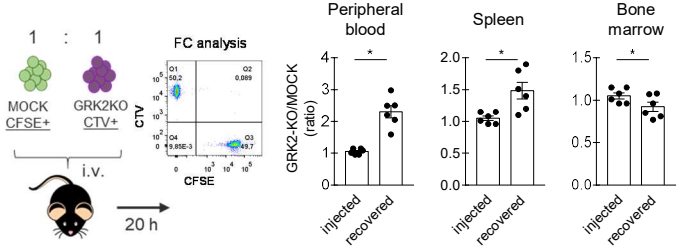


Fig. 7. The ratio of GRK2KO cells / MOCK cells in each compartment after 20hs of iv injection is shown in each compartment. *p<0.05, Wilcoxon test.

IV. Conclusion: our results suggest that GRK2 inhibition could be explored as a strategy to induce leukemic cell retention in the blood, increasing their exposure to therapeutic agents and/or to overcome resistance to treatment induced by the protective microenvironment.

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