Sphingosine kinase 1 participates in the activation, proliferation and survival of chronic lymphocytic leukemia cells

Sphingosine kinases (SKs) have received the most attention as important enzymes in cancer biology. They participate in the regulation of bioactive sphingolipid metabolism by producing sphingosine-1 phosphate (S1P) which mediates several biological functions, including cell growth, differentiation, cell survival, migration, and angiogenesis among other tasks.[1] S1P generation depends on the conversion of sphingosine to S1P, in a reaction catalyzed by two isoforms of SKs, SK1 and SK2, and its levels are tightly controlled *via* a rapid degradation by intracellular S1P lyases (S1PL) or dephosphorylated by S1P phosphatases.[1] Once produced, S1P may function as an intracellular second messenger and/or can be exported outside the cells, where it binds to specific S1P receptors (S1PRs) and initiates downstream signaling pathways, in a paracrine or autocrine manner, in a process known as "inside-out" signaling.[1]

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder characterized by the accumulation of clonal B lymphocytes in peripheral blood and lymphoid tissues. Given that several studies have implicated the SKs/S1P/S1PL pathway as an essential regulator of cell proliferation and survival in cancer cells,[2-4] we evaluated the role of SKs and S1PL in leukemic cells from CLL patients. To this aim we first measured the basal expression of *SKs* messenger ribonucleic acids (mRNAs) and *S1PL* mRNA by quantitative real-time polymerase chain reaction (qRT-PCR) on purified B cells from CLL patients and age-matched healthy donors. We found that CLL cells expressed heterogeneous levels of *SK1, SK2* and *S1PL* mRNA, while only *SK1* mRNA was statistically significantly higher compared to healthy donors (Figure 1A). As we observed at mRNA level, when SK1 was evaluated by western blot we found a higher expression of the protein in B cells from CLL samples compared to healthy donors (Figure 1B and *Online Supplementary Figure S1A*). In addition, within CLL patients, there was a positive correlation between *SK1* mRNA and protein expression (*Online Supplementary Figure S1B*). We also observed that the ratio between *SK1* and *S1PL* was increased for B cells from CLL patients compared to B cells from healthy donors (Figure 1C), while no statistically significant differences were found for *SK2/S1PL* ratios (*Online Supplementary Figure S1C*). Remarkably, CLL patients

Table 1. Association between *SK1/S1PL* ratios and different clinical and biological features of CLL patients.

variables		*SK1/S1PL* ratios		
		Low	High	
	n	n	n	*P**
Sex	30	16	14	ns
female		4	3	
male		12	11	
Binet	30	16	14	0.0092
A		10	2	
BC		6	12	
CD38 expression	30	16	14	0.0121
$CD38^-$		11	3	
$CD38^+$		5	11	
CD49d expression	26	14	12	0.0074
$CD49d^-$		13	5	
$CD49d^+$		1	7	
β2-microglobulin	25	14	11	ns
< 3.5 g/ml		6	5	
≥ 3.5 g/ml		8	6	
LDH	29	15	14	ns
< 350 U/l		7	6	
≥ 350 U/l		8	8	
HGB	30	16	14	ns
≤ 10 g/dl		2	8	
> 10 g/dl		14	8	
Platelets	30	16	14	ns
≤ 100 x 10^3/ul		2	3	
> 100 x 10^3/ul		14	11	
Disease progression	30	16	14	0.0355
Indolent		13	6	
Progressor		3	8	
IGVH mutational status	27	14	13	0.0412
Unmutated		3	8	
Mutated		11	5	

*Statistical significance was determined by using Fisher´s test. LDH: lactate dehydrogenase; HGB: hemoglobin; n: number of patients in each group; NS: not significant; IGVH: immunoglobulin variable region heavy chain.

with higher *SK1/S1PL* ratios preferentially belonged to Binet B or C clinical stages, were unmutated, CD38⁺, CD49d⁺ and showed a progressive disease (Table 1 and *Online Supplementary Table S1*), suggesting that SK1/S1PL molecules might participate in the clinical outcome of the patients, favoring the progression of the disease.

Malignant B cells from CLL patients mainly receive advantageous signals in lymphoid tissues, where the supportive microenvironment and B cell receptor (BCR) signaling promote their activation and proliferation, modulate cell adhesion and migration and protect CLL cells from spontaneous and drug-induced apoptosis.[5] We have previously reported that CLL cells activated by signals from the supportive microenvironment transiently impair the expression of S1PR1,[6] in a process that may extend the residency of the leukemic clone within the survival niches of lymphoid tissues. In the study herein we wanted to determine whether the activation of CLL cells has any influence on *SK1/S1PL* ratios. To this aim, purified B cells from CLL patients were cultured in the presence of immobilized Fab´2 anti-human immunoglobulin M (anti-IgM) and CD40L, CpG or HS5 cell line as a feeder layer and, as expected, after 24 hours of culture, the stimuli induced the upregulation of the activation marker CD69 on CLL cells (*data not shown*). Interestingly, we found that CLL activation enhanced *SK1/S1PL* ratios in all of the patients evaluated (Figure 1D), independently of their clinical stage or the prognosis group (*data not shown*). Ibrutinib, by impairing leukemic cell activation (*data not shown*) was able to strongly reduce the upregulation of *SK1/S1PL* ratios induced by the stimulus (*Online Supplementary Figure S2*). Moreover, in order to evaluate the expression of *SK1/S1PL* ratios within *in vivo* activated CLL cell subpopulations, we took advantage of the fact that we had previously obtained mRNA samples of *in vivo* activated CLL cells subpopulations and non-activated counterparts of the same patient.[6] When *SK1, SK2* and *S1PL* mRNA levels were evaluated in the proliferative fraction of circulating CLL cells (PF, IgG⁺ cells), described by Palacios *et al.*,[7] and the quiescent fraction from the same patient (IgM⁺IgG⁻ cells, QF), we found higher *SK1/S1PL* ratios in the PF of the three CLL patients evaluated (Figure 1E). Additionally, bone marrow leukemic cells expressing high levels of CD38, which defines a subpopulation of activated lymphocytes, showed higher *SK1/S1PL* ratios compared to their CD38 low or negative counterparts (n=3) (Figure 1F), indicating that *in vivo* activated CLL cells expressed higher ratios of *SK1/S1PL* compared with the rest of the leukemic clone.

Subsequently, to test whether the inhibition of SKs could modify the survival of CLL cells, we employed SKI-II, which is an orally bioavailable inhibitor for SK1 and SK2 that blocks S1P production and cell proliferation.[8] When peripheral blood mononuclear cells

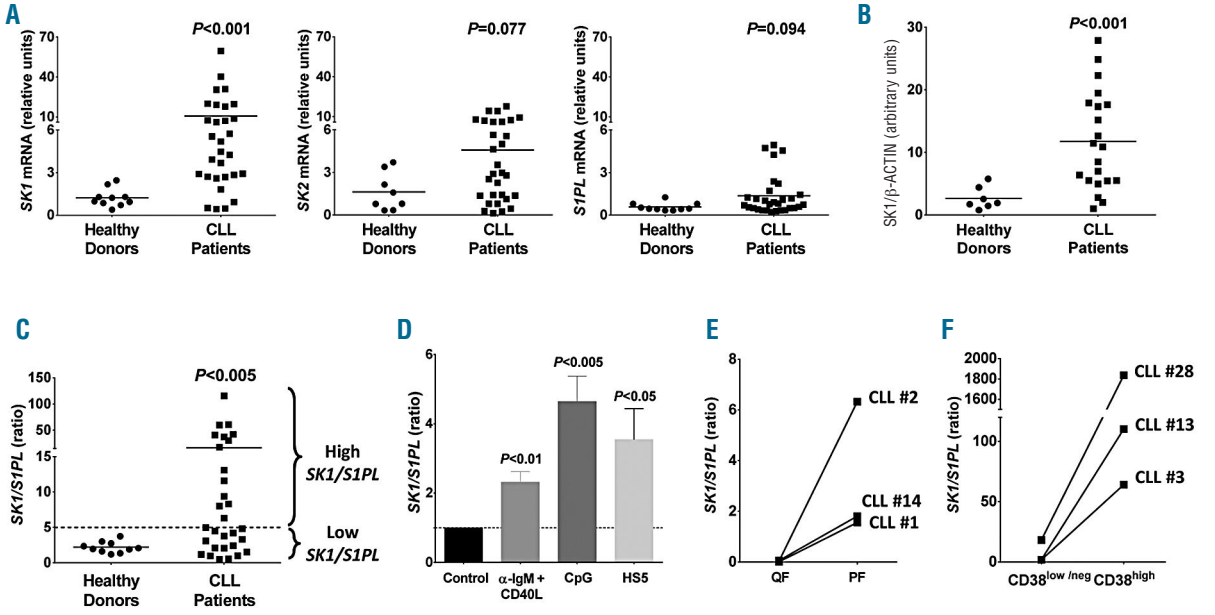

Figure 1. *SK1/S1PL* ratios in resting and activated CLL cells. (A) *SK1, SK2* and *S1PL* mRNA levels were evaluated by qRT-PCR in purified B cells from 30 CLL patients and 10 age-matched healthy donors. Results were normalized to *GAPDH* human gene and were represented as relative units ($2^{-\Delta Ct} \times 10^3$). Statistical analysis was performed using the Mann–Whitney test. (B) The graph shows the expression of SK1 relative to β-ACTIN in purified B cells from 7 age-matched healthy donors and 21 CLL patients evaluated by western blot. Statistical analysis was performed using the Mann–Whitney test. (C) The figure shows the *SK1/S1PL* ratios evaluated by qRT-PCR as described above. The dashed line segregates CLL patients with high or low *SK1/S1PL* ratios, a cutoff of 5 was established between patients with less or more than -3 standard deviation (SD) with respect to the healthy control group. Statistical analysis was performed using the Mann–Whitney test. (D) Purified CLL cells were cultured for 24 hours without (control) or with anti-IgM (α-IgM) plus CD40L (n=12), CpG (n=9) or HS5 cell line (n=4) and the expression of *SK1* and *S1PL* mRNA were evaluated as described above. Bars represent the mean ± SEM of the *SK1/S1PL* ratio relative to control cultures. Statistical analysis was performed using Wilcoxon's signed-rank test. (E) Leukemic cells from peripheral blood mononuclear cells (PBMCs) of CLL patients were segregated by IgG or IgM expression.[7] Total RNA was purified from the IgG⁺ cells (PF) and IgM⁺IgG⁻ cells (QF), and the expression of *SK1* and *S1PL* mRNA was evaluated as described above. The figure shows the *SK1/S1PL* ratio of each patient. Patients number 1, 2 and 14 in the *Online Supplementary Table S1* correspond to Patients number 39, 40 and 38, respectively, from our previous paper.[6] (F) Bone marrow mononuclear cells from 3 CLL patients were segregated by CD38 expression as previously detailed.[6] Total RNA was purified from the $CD19^+CD38^{high}$ fraction and from the $CD19^+CD38^{low/negative}$ fraction. *SK1* and *S1PL* mRNA were evaluated as described above. The figure shows the *SK1/S1PL* ratio of each patient. Patients number 3, 13 and 28 in the *Online Supplementary Table S1* correspond to Patients number 35, 36 and 34, respectively, from our previous paper.[6] CLL: chronic lymphocytic leukemia; mRNA; messenger ribonucleic acid.

(PBMCs) from CLL patients were cultured with different doses of SKI-II, we found that SKI-II induced cell death in a dose-dependent way (Figure 2A and *Online Supplementary Figure S3A*). We observed that the percentage of cell death induced by 50 μM of SKI-II inversely correlated with the basal *SK1/S1PL* ratios of each sample (*Online Supplementary Figure S3B*). In line with this, CLL patients with low *SK1/S1PL* ratios were more sensitive to 50 μM of SKI-II compared to patients with high *SK1/S1PL* ratios (Figure 2B). In addition, we found that non-apoptotic doses of SKI-II (15 μM) increased the susceptibility of CLL cells to die by other therapeutic agents, such as fludarabine, bendamustine and ibrutinib (Figure 2C), more markedly in CLL patients with low *SK1/S1PL* ratios compared to CLL patients with high *SK1/S1PL* ratios (*Online Supplementary Figure S3C*). Similar results were recently reported by others showing that SKs inhibition induces apoptosis in primary multiple myeloma,[9] acute myeloid leukemia[10] and natural killer-large granular lymphocyte leukemia cells[11] and also sensitizes CLL cells to other therapeutic agents such as EGCG (-epigallocatechin-O-3-gallate)[12] or lysosome-targeting drugs.[13] SKI-II-induced cell death was associated with a downregulation of BCL-2 expression in CLL cells (*Online Supplementary Figure S3D*), as previously reported in SKI-II-treated gastric cancer cells.[14] Thus, CLL samples with low *SK1/S1PL* ratios, which were more sensitive to 50 μM of SKI-II (Figure 2B), showed a higher BCL-2 downregulation

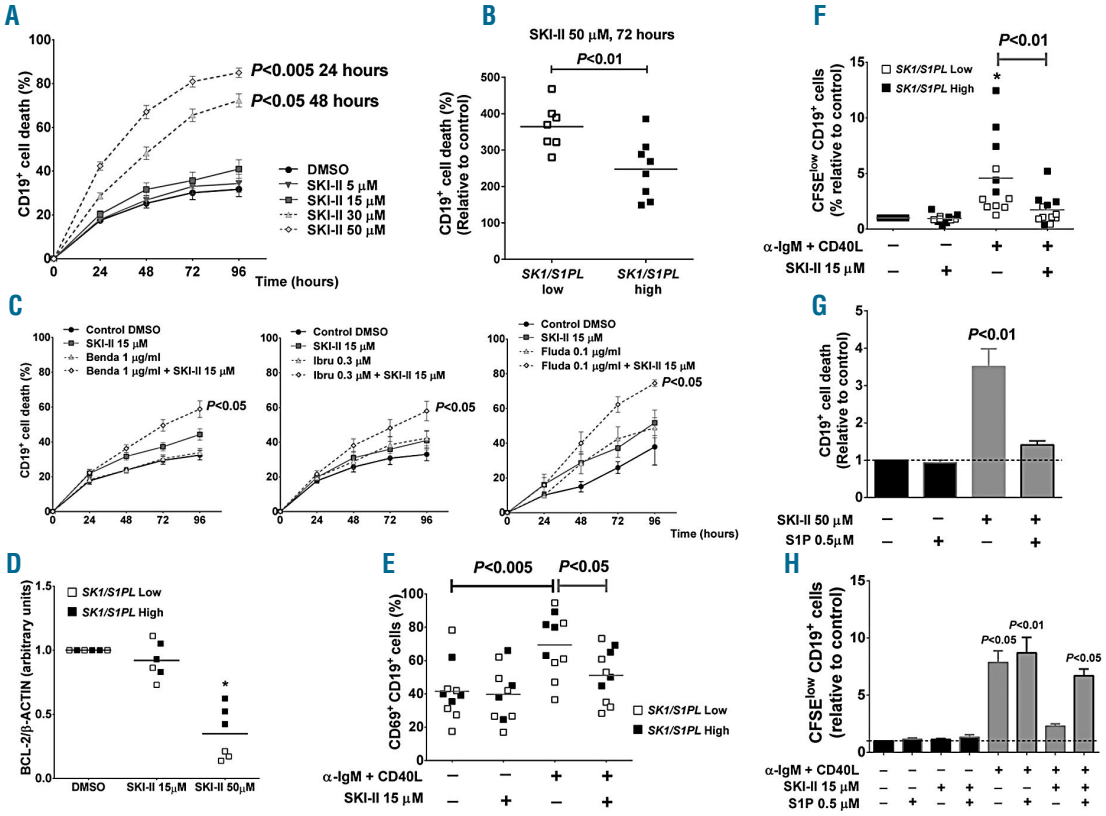

Figure 2. SKI-II regulates the survival, activation and proliferation of CLL cells. (A) PBMCs from CLL patients (>85% leukemic B cells) were cultured with DMSO (vehicle) or different doses of SKI-II. The cell death of CD19+ cells was evaluated daily by flow cytometric alterations of light scattering properties and confirmed by Annexin V (see the *Online Supplementary Figure S3A*). The figure shows the mean ± SEM of the percentage of CD19+cell death in control and SKI-II cultures (n=15). Statistical analysis was performed using the Friedman test followed by Dunn's post test. (B) The figure shows the percentage of CD19+ cell death induced by SKI-II (50 μM) for 72 hours relative to control in CLL patients segregated by low or high *SK1/S1PL* ratios. Statistical analysis was performed using the Mann–Whitney test. (C) PBMCs from CLL patients (>85% leukemic B cells) were pre-treated with DMSO (Vehicle) or SKI-II (15 μM) for 30 minutes, and then non-apoptotic doses of bendamustine (Benda), ibrutinib (Ibru) or fludarabine (Fluda) were added to the cultures. The percentage of CD19+cell death in the cultures was evaluated daily as mentioned above. Statistical analysis was performed using the Friedman test followed by Dunn's post test. (D) PBMCs from CLL patients (>85% leukemic B cells) were cultured with DMSO (vehicle) or SKI-II for 24 hours and BCL-2 expression was evaluated by western blot as detailed in *Online Supplementary Materials and Methods*. The graph shows the quantitative densitometry protein expression of BCL-2 relative to β-ACTIN (arbitrary units relative to DMSO) from CLL patients (n=6) with high or low *SK1/S1PL* ratios in each condition. Statistical analysis was performed using Wilcoxon's signed-rank test (*P<0.05). (E) PBMCs from CLL patients were pre-treated with DMSO (Vehicle) or SKI-II (15 μM) for 30 minutes and then cultured with α-IgM plus CD40L. The expression of the activation marker CD69 on CD19+ cells was evaluated by flow cytometry at 24 hours. The figure shows the percentage of CD69+CD19+ cells in each condition. Statistical analysis was performed using the Friedman test followed by Dunn's post test. (F) CFSE-labeled PBMC from CLL patients were pre-treated with DMSO or SKI-II (15 μM) for 30 minutes and then cultured without (control) or with α-IgM plus CD40L. After 5 days of culture, the proliferation of CLL cells was analyzed using the CFSE dilution assay. The figure shows the percentage of CFSE^{low}CD19+ fraction relative to control culture. Statistical analysis was performed using Wilcoxon's signed-rank test (*P<0.01) and the Friedman test followed by Dunn's post test. (G) PBMCs from CLL patients (>85% leukemic B cells) were cultured with DMSO (vehicle) or SKI-II (50 μM) for 30 minutes and then with or without of S1P 0.5 μM as detailed in *Online Supplementary Materials and Methods*. After 48 hours of culture, cell death was evaluated as mentioned above. Bars represent the mean ± SEM of cell death for each treatment relative to control at 48 hours (n=10). Statistical analysis was performed using Wilcoxon's signed-rank test. (H) CFSE-labeled PBMC from CLL patients were pre-treated with DMSO or SKI-II (15 μM) for 30 minutes, and then without (control) or with α-IgM plus CD40L in the presence of S1P as detailed in *Online Supplementary Materials and Methods*. After 5 days of culture, the proliferation of CLL cells was analyzed using the CFSE dilution assay. The figure shows the percentage of CFSE^{low}CD19+ fraction relative to control culture. Statistical analysis was performed using Wilcoxon's signed-rank test. DMSO: dimethyl sulfoxide; CFSE: carboxyfluorescein succinimidyl ester; BCL-2: B-cell lymphoma 2.

upon SKI-II treatment compared to CLL patients with high *SK1/S1PL* ratios (Figure 2D and *Online Supplementary Figure S3E*).

On the other hand, we found that non-apoptotic doses of SKI-II were able to impair CLL activation induced by anti-IgM + CD40L at 24 hours, measured by both the upregulation of CD69 (Figure 2E) and CLL proliferation evaluated at 5 days (Figure 2F). Remarkably, those samples with high *SK1/S1PL* ratios presented a greater leukemic cell proliferation induced by anti-IgM + CD40L compared to patients with low *SK1/S1PL* (Figure 2F), suggesting that the SK1/S1P/S1PL pathway might regulate this proliferative response in CLL. Representative carboxyfluorescein succinimidyl ester (CFSE) histograms are depicted in the *Online Supplementary Figure S3F*.

Finally, in order to determine whether S1PRs signaling may counteract the effects of SKI-II, we added exogenous S1P to the cultures and found that it rescued CLL cells from the cell death induced by 50 μM of SKI-II (Figure 2G) and from the anti-proliferative effect of 15 μM of SKI-II (Figure 2H). These findings suggest that S1P production by CLL cells may favor leukemic cell survival and proliferation in an "inside-out" signaling manner acting through S1PRs.[1]

Taken together, the results presented herein and our previous data showing that the activation of CLL cells transiently impair the expression of S1PR1,[6] allow us to hypothesize about the role of the SK1/S1P/S1PL axis in CLL. Thus, the possibility exists that microenvironment signals from lymphoid tissues, by increasing *SK1/S1PL* ratios in leukemic CLL cells, favors S1P production. Exported S1P can then bind S1PRs, reduce S1PR1 expression[6] and transduce stimulating signals to the leukemic clone.

In conclusion, our results suggest that the SK/S1P/S1PL pathway supports the survival and proliferation of CLL cells, hence favoring the progression of the disease, thus we encourage the use of SKs inhibitors in combined therapy as a promising treatment option in the future.

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