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A novel class of anti-tumoral compounds, pyrrolonaphtoxazepines (PNOXs), targets the p66Shc/STAT4 axis in CLL prymary cells and reduces tumor burden in Eμ-TCL1 mice

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ABSTRACT

Chronic lymphocytic leukemia is an haematological malignancy characterized by the accumulation of $CD5^+CD19^+$ mature B cell clones in peripheral blood, spleen, lymph nodes and bone marrow (B-CLL). Leukemic cells accumulation is both dependent upon enhanced B cell receptor (BCR)-driven proliferation and defects in the apoptotic machinery. Additionally, alterations of surface chemokine receptors have been accounted for the enhanced accumulation and survival of CLL cells within the secondary lymphoid organs (SLOs). At present, treatment options for CLL patients include drugs targeting proteins participating in the BCR signaling pathway (i.e Ibrutinib, Idelalisib) and in the apoptotic process (i.e Venetoclax). However, relapsed or refractory CLL is still a clinical problem, and prolonged treatment with the above mentioned drugs leads to resistance occurrence as well as severe side effects, indicating that novel therapeutic options are needed.

The adaptor protein p66Shc is a negative regulator of BCR-signaling and a pro-apoptotic protein whose loss in CLL B cells accounts for the extended survival of tumoral cells and poor prognosis. p66Shc has been also shown to control B cells trafficking by altering CXCR4 function, CCR7 and S1PR1 expression. The pathogenetic role of p66Shc in CLL was recently confirmed in the Eμ-TCL1 mouse model of CLL. Eμ-TCL1 mice lacking the *p66Shc* gene (Eμ-TCL1p66Shc$^{-/-}$ mice), showed indeed a more severe disease characterized by earlier onset and higher nodal and extranodal accumulation of leukemic cells compared with the Eμ-TCL1 mice. Hence, identification of compounds that up-regulate p66Shc expression and function in malignant B cells are likely to target cell proliferation, migration and apoptosis and may represent an appealing therapeutic option for CLL.

In this study, we decided to investigate the molecular mechanism underlying the anti tumoral efficacy of a recently developed class of compounds, the pyrrolonaphthoxazepines (PNOXs), which have been demonstrated to be effective *in vitro* in several tumoral cells, including primary human CLL cells and

to test, for the first time, these novel compounds in the murine model of human CLL, the Eμ-TCL1 mouse.

Our results show that PNOXs restore p66Shc expression and activate transcription factor STAT4 in primary human CLL cells without affecting normal B cells. Accordingly, our data show that the recovery of p66Shc expression in human CLL cells directly correlates with the apoptosis rate induced by PNOXs. In agreement with human CLL, we found that PNOXs efficiently upregulate p66Shc and promote apoptosis in murine CLL cells. Moreover, we demonstrated that PNOXs were able to activate pro-apoptotic activity of p66Shc through a JNK dependent phosphorylation of Ser36 residue on p66Shc, suggesting that the anti-tumor effect of PNOXs in human CLL cells relies at least in part on its ability to first promote p66Shc expression and subsequently to foster its apoptotic function. PNOXs treatment of Eμ-TCL1 mice resulted in a significantly longer overall survival and in the reduction in tumor burden in the spleen and in the peritoneum. The powerful antitumor effect of PNOXs *in vivo* correlates with upregulation of S1PR1 and in the mobilization of leukemic cells from the spleen into the blood. Interestingly, treatment with PNOXs compounds on Eμ-TCL1p66Shc$^{-/-}$ mice highlighted that only one compound, PNOX-3, exerts a p66Shc-independent pro-apoptotic activity towards circulating CLL cells, while absence of p66Shc impairs the ability of PNOXs to mobilize CLL cells from the spleen to peripheral blood. Since accumulation of leukemic cells within secondary lymphoid organs is responsible for the enhanced survival of leukemic cells and drug resistance, our data indicate that PNOXs may represent a novel effective treatment for CLL by favouring the mobilization of leukemic cells from the protective tumor microenvironment of the SLOs into the blood and by promoting apoptosis through the upregulation of p66Shc.

RIASSUNTO

La Leucemia Linfatica Cronica (LLC-B) è una neoplasia ematologica caratterizzata dall'accumulo di linfociti B maturi $CD5^+CD19^+$ monoclonali nel sangue, nella milza, nei linfonodi e nel midollo osseo. L'incremento delle cellule leucemiche è in parte dovuto alla proliferazione indotta dalla stimolazione del BCR (recettore dei linfociti B), e in parte dipende da difetti nel macchinario apoptotico delle cellule leucemiche, che risultano apoptosi-resistenti. Inoltre, alterazioni nell'espressione di recettori di *homing* e di *egress* sulla superficie delle cellule leucemiche determinano l'accumularsi di queste cellule negli organi linfoidi secondari, promuovendone la sopravvivenza. Ad oggi, le terapie utilizzate nella cura della LLC includono farmaci di nuova generazione come inibitori di chinasi coinvolte nella segnalazione del BCR (es. Ibrutinib e Idelalisib) oppure antagonisti di proteine anti-apoptotiche, altamente espresse nelle cellule leucemiche (es. Venetoclax). Tuttavia, la LLC è tuttora una neoplasia incurabile, i pazienti possono mostrare recidive o resistenza alle terapie, e le terapie basate sui farmaci menzionati, essendo prolungate nel tempo, sono associate a farmaco-resistenza e a seri effetti collaterali. Per queste motivazioni, è auspicabile trovare nuove opzioni terapeutiche.

L'adattatore molecolare p66Shc è un regolatore negativo della segnalazione intracellulare del BCR ed ha anche una funzione pro-apoptotica. L'assenza di p66Shc nelle cellule B di LLC è infatti correlata con la loro aumentata sopravvivenza ed è stata associata ad una prognosi sfavorevole. Inoltre, p66Shc regola direttamente l'espressione superficiale e la funzionalità di recettori per le chemochine come CXCR4, CCR7 e del recettore S1PR1, acquisendo così un ruolo centrale nel controllo dell'entrata e dell'uscita delle cellule B dagli organi linfoidi secondari. Il ruolo di p66Shc nella patogenesi della LLC è stato ulteriormente confermato grazie al modello murino di LLC umana in cui il gene *p66Shc* è stato deleto, il topo Eμ-TCL1p66Shc$^{-/-}$. Questi animali presentano infatti una malattia più aggressiva, con manifestazione precoce e con maggiore infiltrazione degli organi linfatici e non da parte delle cellule leucemiche. Ristabilire l'espressione di p66Shc nelle cellule B di LLC potrebbe quindi rappresentare un'interessante opzione terapeutica che andrebbe a ridurre la proliferazione, sopravvivenza e migrazione delle cellule leucemiche.

In questo studio abbiamo analizzato il meccanismo molecolare responsabile dell'effetto proapoptotico ed antiproliferativo di una nuova classe di farmaci, i pyrrolonaphthoxazepines (PNOXs) che si sono rivelati efficaci *in vitro* in molteplici linee cellulari di origine tumorale, oltre che nelle cellule B di pazienti con LLC. Inoltre, abbiamo studiato per la prima volta gli effetti di questi composti nel modello murino di LLC umana, utilizzando topi Eμ-TCL1. I nostri risultati mostrano che i PNOXs inducono l'espressione di p66Shc e attivano il fattore di trascrizione STAT4 nelle cellule leucemiche umane, senza alcun effetto su cellule B da donatori sani, e una volta ristabilita l'espressione di p66Shc, questa correla direttamente con il tasso di apoptosi indotto dai PNOXs in queste cellule. L'abilità dei PNOXs di indurre l'espressione di p66Shc nelle cellule leucemiche è stata confermata *in vitro* su cellule isolate da topi Eμ-TCL1. Abbiamo inoltre dimostrato che i composti PNOXs, quando l'espressione di p66Shc è stata ripristinata, sono in grado di promuoverne l'attività pro-apoptotica tramite la fosforilazione del suo residuo di Ser36 da parte della chinasi JNK, suggerendo quindi per i PNOXs una duplice azione sia sull'espressione che sulla funzione pro-apoptotica di p66Shc.

L'analisi degli effetti dei PNOXs *in vivo* ha evidenziato come il trattamento promuova la sopravvivenza dei topi Eμ-TCL1 che hanno sviluppato spontaneamente la malattia e la sua efficacia nel ridurre il tumore nella milza e nel peritoneo.

Inoltre, nei topi Eμ-TCL1 trattati con PNOXs abbiamo dimostrato che la riduzione del peso della milza era accompagnata dall'aumento delle cellule leucemiche nel sangue, attribuendo ai PNOXs un'ulteriore capacità nel mobilizzare le cellule leucemiche dalle nicchie tumorali degli organi linfoidi secondari verso il sangue mediante l'up regolazione di S1PR1. Il trattamento con PNOXs nel modello murino Eμ-TCL1p66Shc$^{-/-}$ ha evidenziato che solo il composto PNOX-3 ha una potente attività anti tumorale anche in assenza di p66Shc, e che p66Shc è necessario ai PNOXs per svolgere la loro funzione di mobilizzazione. Poichè gli organi linfoidi secondari promuovono la sopravvivenza e la chemio-resistenza delle cellule LLC, i composti PNOXs potrebbero essere una valida opzione

terapeutica sia per ridurre l'accumulo delle cellule leucemiche negli organi linfoidi secondari sia per la loro attività pro-apoptotica tramite l'induzione di p66Shc.

INTRODUCTION

1. Chronic lymphocytic leukemia

1.1 Diagnostic and clinical features

B cell chronic lymphocytic leukemia (B-CLL) is the most common lymphoproliferative disease in western countries but at present it is still incurable and, moreover, complex to diagnose due to an heterogeneous clinical course (Kikushige, 2020). For a reliable diagnosis of B-CLL, it is necessary to analyze collectively blood smear, immunophenotype and genetic alterations of leukemic cells, as resumed in Figure 1. Patients diagnosed with CLL present for at least three months $\geq 5x10^9$ mature B lymphocytes/per liter in peripheral blood (Hallek *et al.*, 2018). Circulating leukemic lymphocytes are typically small and mature with a narrow border of cytoplasm and dense nucleus (Oscier *et al.*, 2016). Moreover, immunophenotypic analysis evidences surface coexpression of typical B cell antigens such as CD19, CD20 and CD23 together with the T-cell-associated antigen CD5 on leukemic B cells. In borderline cases, markers such as CD43, CD79b, CD81, CD200, CD10, or ROR1 may help to refine the diagnosis (Hallek *et al.*, 2018). In patients showing evident lymphadenopathy, lymph node (LN) biopsy can be often useful to confirm CLL diagnosis. LNs from CLL patients typically show diffuse infiltration of mature lymphocytes and altered architecture with evident proliferation centres appearing as pseudo follicles with scattered, vaguely nodular, pale haematoxylin and eosin-stained areas. Marrow biopsy instead is not necessary to establish diagnosis, but in case of CLL it presents hypercellularity due to high frequency of mature lymphocytes (Kipps *et al.*, 2017; Hallek *et al.*, 2018). CLL leukemogenesis is a complex and heterogeneous process including multiple steps of genetic alterations, thus rendering patients' clinical course extremely variable and, not less important, therapeutic approaches not always efficient. At present, a main distinction of CLL patients could be done depending upon the mutational status of the immunoglobulin heavy-chain variable region (IGHV) gene, associated with different clinical behaviour, B cell of origin of the leukemia, survival

expectations and response to therapies between the two groups. Patients with CLL cells that express a mutated IGHV (M-CLL) typically have less-aggressive disease and better prognosis than patients with CLL cells expressing the unmutated IGHV (U-CLL) (Parikh, 2018). In physiological conditions, mutations in the IGHV region are necessary for B cells to generate the diversity of antigen–binding of immunoglobulins, and this process, named somatic hypermutation (SHM), occurs when B cells pass through germinal centres (GC) within secondary lymphoid organs. Here, B cells undergo high proliferation rate and consequent SHM of immunoglobulins in the so-called dark zone of GC. Then, B cells move towards the light zone where, thanks to the interaction with both Follicular Dendritic Cells (FDCs) and $CD4^+$ T helper cells, they are selected on the basis of the affinity of their immunoglobulins for antigens (Mesin, Ersching and Victora, 2016). Given these evidences, CLL cells that express a mutated IGHV are supposed to originate from a post-germinal centre B cell, as demonstrated also by the panel of mature B cells antigens expressed on leukemic cells as mentioned above. On the contrary, CLL cells with unmutated IGHV are thought to arise from B cells that have not undergone differentiation in the dark zone of germinal centres (Fabbri and Dalla-Favera, 2016). Nevertheless, it is reported that, independently of the IGHV mutational status, CLL cells always express the memory B cell surface protein CD27 and always produce a functional activation-induced deaminase (AID), the enzyme responsible for SHM in normal B lymphocytes (Rozovski, Keating and Estrov, 2018). A possible explanation is that M-CLL cells originate from a post-GC, T cell-dependent maturated B cell, whereas U-CLL cells arise from a minor fraction of $CD27^+$ memory B cells whose mature phenotype was acquired in a GC- and T cell-independent mechanism. This model is the most accepted one, but it is still difficult to clearly assess the cell of origin in M-CLL and U-CLL respectively (Fabbri and Dalla-Favera, 2016). In addition to the mutational status of the IGHV, the ectopic expression of zeta-chain-associated protein kinase 70 (ZAP70) and the expression of surface molecule CD38 represent other two crucial prognostic factors in CLL since both these molecules support B cells proliferation, survival as well as cell migration. Their overexpression is found more in cells from U-CLL patients compared with M-CLL and as a consequence, it is related to the

unfavourable prognosis (Crespo *et al.*, 2003; Parikh, 2018). The individual prognostic value of each CLL patient results from the combination of the aforementioned prognostic markers as well as additional genetic, molecular and biochemical characteristics which always need to be taken into account in order to promptly identify and properly treat patients.

1.2 Genetic lesions

In comparison with other lymphoid neoplasm, CLL shows overall a lower mutational status (Bosch and Dalla-Favera, 2019). Anyway, genome wide sequencing has enabled deep understanding of CLL genomic landscape evidencing the main genetic alterations associated with the disease. Of note, none of these aberrations taken individually seems to be a disease-driver, although all together drive CLL leukemogenesis in humans (Kikushige, 2020). Deletions of 13q14 (del13q14) are the most frequent genetic lesions in CLL, detected in almost 50-60% of patients but generally associated with a favourable prognosis and a prolonged time to first treatment (TTFT). Results of these deletions are abnormalities in cell cycle regulation and apoptosis. In particular, loss of the microRNA cluster mir15A and MIR16-1, results in modulation of genes involved in G_0/G_1 transition and in downregulation of the *BCL-2* anti-apoptotic gene, thus explaining the two main features of CLL cells: the non proliferative phenotype and the increased resistance to apoptosis (Cimmino *et al.*, 2005; Klein *et al.*, 2010). Deletion of the 17p13 locus determines loss of *TP53* gene which, among others genetic alterations, is the most strongly associated with adverse prognosis, shorter overall survival and refractoriness to genotoxic chemotherapy. Inactivation of *TP53* dramatically affects DNA damage response thereby favouring genomic instability. The frequency of *TP53* mutation is relatively low in newly diagnosed patients, but tends to increase sharply with disease progression, suggesting that this mutation may represent an evolutionary mechanism of resistance (Buccheri *et al.*, 2018). For this reason detection of del(17p) and *TP53* gene mutations has become an integral part in routine diagnostics and should always be performed before deciding about treatment (Malcikova *et al.*, 2018). Trisomy of chromosome 12 is another quite recurrent genomic abnormality in CLL, whose

pathological significance is still not clear but it is considered a negative prognostic factor when associated with NOTCH1 mutation. Whole genome and exome sequencing have identified, besides chromosomal aberrations, recurrent driver mutations of CLL, observed in genes participating in the DNA damage response (for example, *TP53* and *ATM*), mRNA processing (for example, *SF3B1* and *XPO1*), chromatin modification (for example *HIST1H1E*, *CHD2* and *ZMYM3*), WNT signaling, Notch signaling (for example, *NOTCH1*) and inflammatory pathways (for example, *MYD88*). Thus, these deregulated biological pathways coordinately drive CLL leukemogenesis in human: mutation of *MYD88*, trisomy 12 and deletion of 13q were found to be disease drivers, while mutations of *ATM*, *TP53* and *SF3B1* were more frequently sub-clonal (Kipps *et al.*, 2017; Crassini *et al.*, 2019; Kikushige, 2020).

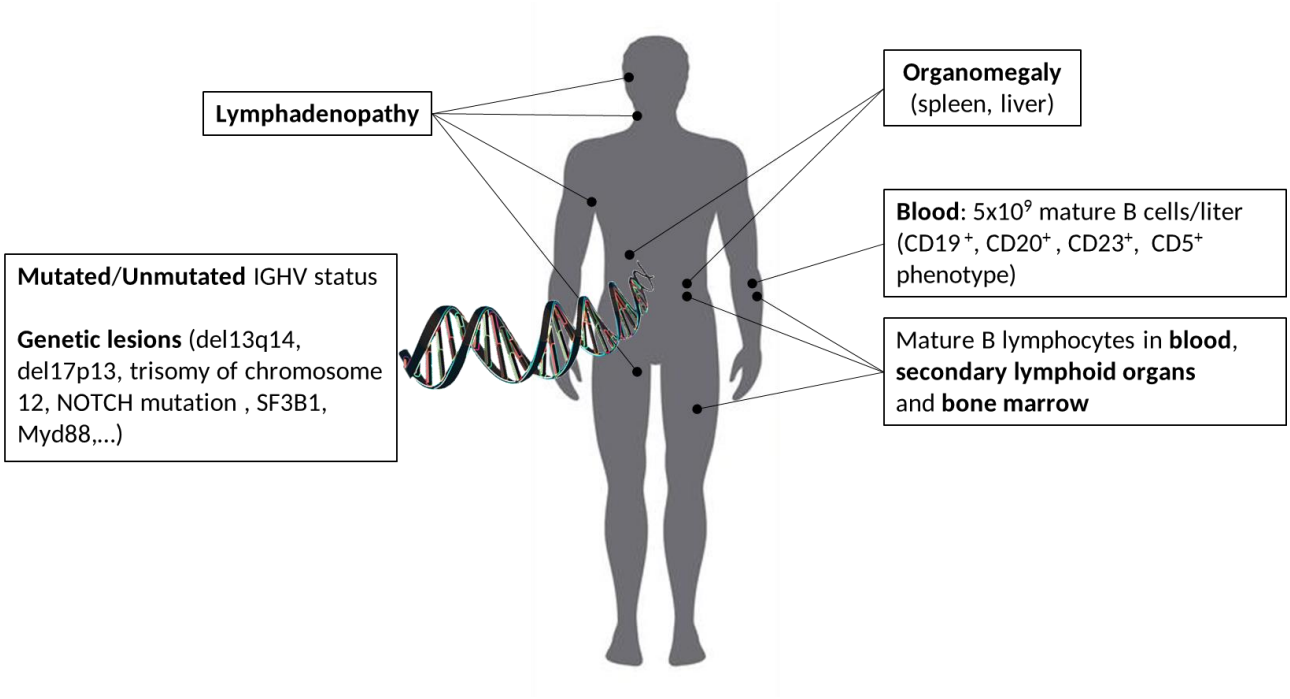

Figure 1. **Pathophysiology and genetics of chronic lymphocytic leukemia**

Chronic lymphocytic leukemia is characterized by the clonal proliferation and accumulation of mature and typically CD5 positive B-cells within the blood, bone marrow, lymph nodes, and spleen. Leukemic transformation in most patients is initiated by the loss or gain of chromosomal material, but additional somatic mutations acquired during the course of the disease make leukemia more aggressive and resistant to treatment.

1.3 Molecular mechanisms of CLL pathogenesis

1.3.1 Impaired apoptosis and role of Bcl-2 family members

Differently from other leukemias or solid tumors CLL B cells only partially proliferate and selectively in certain districts, as deeper described in next paragraphs, but their accumulation mainly accounts on extended survival caused by intrinsic defects in apoptotic machinery (Capitani and Baldari, 2010). Apoptosis, known also as programmed cell death, is an evolutionary highly conserved physiological process involved in tissue homeostasis. Cell shrinkage, nuclear fragmentation, chromatin condensation and plasma membrane blebbing are main events associated with apoptosis, each of them orchestrated by tightly controlled biochemical and molecular steps (Nagata, 2018). From the 1990s onwards, receptors, enzymes and regulatory proteins among the large and complex web of molecules controlling cell death have emerged as key regulators in apoptosis. These regulators, when abnormally expressed or when mutated, have a direct effect on cell death machinery (Czabotar *et al.*, 2013). Among them, for example, is the B cell Lymphoma-2 (Bcl-2) family of enzymes, which regulate apoptotic events connected to development and genotoxic agents, named intrinsic or mitochondrial pathway of apoptosis (Kale, Osterlund and Andrews, 2018). The Bcl-2 family consists of three subfamilies based on the number of Bcl-2 Homology (BH) domains they contain: proapoptotic BH3-only members (Bim, Bid, Puma, Noxa, Hrk, Bmf, and Bad), proapoptotic effector molecules (Bax and Bak) with three BH domains, and antiapoptotic Bcl-2 family proteins (Bcl-2, Bcl-xL, Mcl1, A1, and Bcl-B) which contain all the four BH domains (Nagata, 2018). In healthy cells, the Bax/Bak pro-apoptotic action is arrested by interactions with antiapoptotic Bcl-2 family members. In response to an apoptotic stimulus, BH3-only members are transcriptionally or posttranscriptionally upregulated. The activated BH3-only proteins act on Bak and Bax to promote their dimerization and pore formation on the outer mitochondrial membrane (OMM) thus allowing the release of cytochrome-c from mitochondria. Cytochrome-c release is a fatal event in cell commitment to apoptosis and, together with Apaf, forms the heptametrical complex called apoptosome. This complex recruits monomeric procaspase 9 and helps it to form a dimer, then

undergoing autocatalytic cleavage to form an active heterotetrameric complex. The activated caspase 9 then cleaves and activates caspase 3, the final executor in the caspase cascade responsible for cleavage of more than 1,300 cellular substrates to finally execute apoptosis (Peña-Blanco and García-Sáez, 2018).

In B-CLL the impaired balance between pro- and anti-apoptotic members has been correlated with the extended survival of leukemic B cells: higher levels of Bcl-2, Bcl-xL, Mcl-1 and lower levels of Bax and Bak, compared to B cells from healthy donors, have been indeed detected in B-CLL (Capitani and Baldari, 2010). As previously described, aberrant loss of miR-15/16 expression, dependent upon del13q14, is the most prominent mechanism identified for Bcl-2 overexpression in CLL, but recent evidences demonstrate that also defective DROSHA processing, overexpression of histone deacetylases and hypomethylation of *BCL-2* promoter region could participate to Bcl-2 mysergulation (Pekarsky, Balatti and Croce, 2018). Pro-apoptotic Bax downregulation is instead dependent upon alterations within its promoter sequence such as base pair insertion, with consequent elevated levels both in protein and mRNA (Packham and Stevenson, 2005).

1.3.2 Autoreactivity and chronic activation of BCR signaling

Chronic lymphocytic leukemia arises from mature B cells expressing functional B Cell Receptors (BCRs) mainly of immunoglobulin M (IgM) and D (IgD) isotypes. Considering that survival and activation of mature B cells depend upon signals originated from BCR ligation and the importance of the IGHV mutational status, which encodes part of the BCR, it is evident that BCR may cover an essential role in the development and pathogenesis of CLL (Efremov, Turkalj and Laurenti, 2020).

In addition to a differential degree of somatic hypermutation (SHM) during *IGHV* gene transcription, CLL BCRs also show a recurrent usage in immunoglobulin light chain variable (IGLV) gene which results in a particular pattern of IGHV and IGLV gene combinations. BCRs encoded by these IGHV/IGLV combinations are named stereotyped BCRs and have been identified in approximately one-third of CLL cases, whereas they are rarely seen in normal B lymphocytes. The occurrence of

stereotyped BCRs is unlikely driven by chance, but instead is likely that CLL clones may be selected thanks to particular antigen-binding properties (Ten Hacken *et al.*, 2019). Moreover, soluble immunoglobulins derived from CLL cells, in particular U-CLL immunoglobulins, have been reported to display shared reactivity towards self-antigens. Stereotyping together with autoreactivity could account for the continuous, chronic activation of the BCR signaling pathway observed by gene expression or phospho-protein profiling analysis in CLL cells. In particular, CLL cells from unmutated patients markedly show this activated phenotype, and typically display constitutively activated BCR signaling molecules such as kinases LYN, SYK, BTK and PI3K, which transduce signals to downstream effectors, till transcription factors as NF-κB and NFAT, with consequent B lymphocyte activation and inhibition of spontaneous apoptosis (Packham *et al.*, 2014; Malcikova *et al.*, 2018).

Not only CLL cells show stereotyped BCRs chronically activated, but also aberrant expression of molecules involved in BCR signal transduction, overall supporting survival of CLL cells. An example is represented by the protein kinase ZAP-70 aberrantly expressed in CLL, mostly in U-CLL patients. Both when totally or partially phosphorylated, ZAP-70 enhances BCR signaling by recruiting and activating molecules participating in the BCR signaling pathway (Gobessi *et al.*, 2007; Chen, Moore and Ringshausen, 2020). Concomitantly, in CLL is observed a deficiency of negative regulators of BCR signaling such as phosphatase PHLPP1, inhibitor of AKT and ERK (Suljagic *et al.*, 2010-a-). However, sustained hyperactivation of BCR signaling can lead to negative selection and apoptosis of B cells, including CLL B cells. Phosphatase PTPN22 is overexpressed in CLL cells and thanks to its dual function is one of the molecules that protect them from mechanisms that would eliminate autoreactive B cells. PTPN22 overexpression increases the survival of BCR-stimulated CLL cells by enhancing AKT activation and, at the same time, protect them from activation-induced cell death by inhibiting p38MAPK (Negro *et al.*, 2012).

Secondary lymphoid organs (SLOs) represent the main sites of CLL cells activation and tumor proliferation. In SLOs, shaped by leukemic and non leukemic cells as a pro-leukemic and

immunotolerant milieu, malignant cells encounter antigens and autoantigens that, together with other stimuli provided by bystander non tumoral cells, contribute to a dysregulation of the BCR signaling (Herishanu *et al.*, 2011; Arruga *et al.*, 2020). In addition to the above mentioned features of BCRs signaling in CLL, many recent studies evidenced an additional cell-autonomous reactivity and stimuli-independent BCRs oligomerization in nanoclusters through epitopes recognition between adjacent immunoglobulins on CLL cells (Dühren-von Minden *et al.*, 2012; Minici *et al.*, 2017). These cell- and ligand-autonomous signals might provide a repetitive stimulation of low or intermediate strength, which may act as a continuous survival source for the leukemic cells. On the other hand, BCR stimulation with antigens has been shown to increase the expression of the cell cycle regulators MYC, CCND2, and CDK4 and to increase the percentage of CLL cells in the G_1 phase of the cell cycle, suggesting that interactions with autoantigens may provide the initial stimulus required for leukemic cell proliferation (Guarini *et al.*, 2008; Krysov *et al.*, 2012)

1.3.3 Role of CLL microenvironment in supporting leukemic expansion

Intrinsic genetic and molecular alterations described above empower leukemic cells with extended survival, apoptosis resistance and a slight percentage of proliferation, but recent findings are pointing out the attention on the pathogenetic significance of external factors coming from SLOs where CLL cells tend to accumulate leading to the characteristic lymphadenopathy and splenomegaly reported in CLL patients (Kipps *et al.*, 2017). Studies from the last decade have demonstrated the altered expression of homing and egress receptors on leukemic cells surface (López-Giral *et al.*, 2004; Trentin *et al.*, 2004; Burger and Gribben, 2014; Patrussi, Capitani and Baldari, 2019), together with an active role played by SLOs in recruiting leukemic cells towards a protective and tumor-supporting microenvironment as the main mechanism required for the accumulation and survival of leukemic cells in these organs (Herishanu *et al.*, 2011; Choi, Kashyap and Kumar, 2016). Decades of studies on CLL cells evidenced that when isolated *in vitro*, these cells tend to rapidly undergo apoptosis, while the addition of stromal cells or survival signals found in the tumor microenvironment (i.e.

chemokines) to the culture medium, greatly increased their survival in culture (Kipps *et al.*, 2017; Awan and Byrd, 2020). In lymphoid organs stromal cells secrete chemokines such as CXCL12, CCL19, CCL21, and CXCL13 (BCA1) which not only attract leukemic cells towards SLOs but also exert a pro-survival activity. On the other side, CLL cells themselves show an overexpression of chemokine receptors for homing to nodal and extra-nodal localization, thus supporting their massive infiltration in these sites. In addition, evaluation of homing receptors expression has recently acquired a strong clinical relevance, for example overexpression of the receptor for CXCL12 chemokine, CXCR4, on patients' leukemic cells has been directly correlated to worse prognosis and shorter time to first treatment (Redondo-Muñoz, García-Pardo and Teixidó, 2019). At the same time, also egress receptors expression is affected in CLL B cells. It is reported that CLL B cells from patients with unfavorable prognosis express low levels of S1PR1. This receptor binds to the sphingolipid S1P (sphingosine 1-phosphate) triggering lymphocyte egress from lymphoid organs to peripheral blood. Hence, impaired S1PR1 expression abnormally extends leukemic cell residency in the prosurvival niche of lymphoid organs, thereby favoring their survival (Capitani *et al.*, 2012; Borge *et al.*, 2014).

1.3.4 Role of p66Shc

Four members compose the Src homologus and collagen (Shc) adaptor protein family, namely Shc/ShcA, ShcB/Sli, ShcC/Rai and ShcD/RaLP, all of them participating in many signaling pathways controlling cell proliferation, survival, apoptosis and migration (Finetti, Savino and Baldari, 2009). ShcA protein was first discovered in 1992 as a participant in signaling pathways coupling receptor Protein Tyrosine Kinases (PTKs) to Ras activation. In mammals, ShcA protein is composed by an N-terminal phosphotyrosine-binding (PTB) domain, a central collagen homology (CH1) domain and a C-terminal Src Homology (SH2) domain, with little molecular weight differences among the three isoforms identified: p66, p52 and p46. The two isoforms p52Shc and p66Shc, share the cytochrome-c binding domain (CB), absent in the p46 isoform, while p66Shc show an additional CH2 domain at the N-terminus dependent on an alternative promoter-mediated transcription (Ravichandran, 2001).

The p46 and p52 isoforms are constitutively and ubiquitously expressed through alternative usage of two in frame ATGs, while the p66Shc isoform is tissue restricted and is subjected to extracellular stimuli modulation (Kisielow *et al.*, 2002). p66Shc expression is usually lower if compared to the other two isoforms p52 and p46, nevertheless p66Shc levels are low but detectable in human peripheral blood T and B lymphocytes, and tend to increase following pro-apoptotic stimuli with a mechanism dependent upon an active DNA promoter demethylation on CpG sites (Pezzicoli *et al.*, 2006).

Study of p66Shc role in lymphocytes, identified this molecule both as an attenuator of mitogenic signaling associated to the T and B cell receptor and a promoter of apoptosis. Similar to p52Shc and p46Shc, p66Shc becomes tyrosine-phosphorylated in the CH1 domain upon growth factor receptors stimulation, then binds to activated receptors and forms stable complexes with Grb2. However, unlike p52Shc/p46Shc, p66Shc interaction with receptors is more likely to act as a competitive inhibition for p52 to prevent its binding to activated receptors, while p66Shc complex with Grb2:Sos prevent their participation in functional Ras-activating networks thus inhibiting Ras-MAPK mitogenic signal activation (Migliaccio *et al.*, 1997). p66Shc pro-apoptotic activity mainly consists in positive regulation of Reactive Oxigen Species (ROS). ROS are identified as critical regulators of apoptosis. An external source of ROS or their intracellular accumulation following stress conditions potently impairs mitochondrial integrity, thus priming mitochondrial apoptosis (Kudryavtseva *et al.*, 2016). Phosphorylation on the Serine 36 (S36) residue in the N-terminal CH2 domain is critical for the pro-apoptotic function of p66Shc, as demonstrated by cells lacking S36 which are resistant to apoptosis. Recently, c-Jun N-terminal kinase (JNK) has been demonstrated to phosphorylate p66Shc, specifically, in S36 residue (Khalid *et al.*, 2016). In agreement with the important role played by JNK in S36 phosphorylation on p66Shc function, pharmacological inhibition of JNK results in reduced levels of intracellular ROS and no p66Shc-S36 phosphorylation (Khalid *et al.*, 2016). When phosphorylated on S36, cytosolic p66Shc is enabled to inactivate transcription factors Forkhead box O (FoxO) responsible for antioxidant enzymes expression, thus increasing intracellular ROS

(Migliaccio *et al*., 1999; Nemoto and Finkel, 2002). Moreover, besides p66Shc was constitutively found in mitochondria-associated membranes (MAMs) and in the mitochondrial intermembrane space (IMS), oxidative stress-mediated S36 phosphorylation of p66Shc further facilitates the import of the molecule into the mitochondria, thus increasing mitochondrial p66Shc during oxidative stress (Pinton *et al*., 2007). Here, associated to molecular complex with TIM -TOM import complex and Mitochondrial Heat Shock Protein 70 (mtHSP70), p66Shc is maintained inactive, but following pro-apoptotic signals, p66Shc assumes a catalytically active tetrameric conformation (Orsini *et al.*, 2004; Pellegrini, Pacini and Baldari, 2005; Gertz *et al.*, 2008). Once active, p66Shc acts as a redox enzyme binding and oxidizing cytochrome-c and interrupting the respiratory chain (Giorgio *et al.*, 2005). The resulting ROS production leads to opening of Permeability Transition Pore (PTP) with consequent dissipation of mitochondrial membrane potential and release of pro-apoptotic factors, which in turn are involved in mitochondrial-mediated apoptosis (Pellegrini, Pacini and Baldari, 2005). Recently, p66Shc was identified as a novel regulator of B cell autophagy and mitophagy (Onnis *et al.*, 2018). p66Shc causes the interruption of the mitochondrial respiratory chain, resulting in a decrease in ATP production and consequent activation of the autophagy-promoting kinase AMPK (Onnis *et al.*, 2018). Moreover, p66Shc-dependent production of ROS causes disruption of mitochondrial integrity resulting in ubiquitylation on the outer mitochondrial membrane. p66Shc was found to act as a receptor for LC3-II to promote recruitment of autophagosomes towards damaged mitochondria, evidencing a role for p66Shc in mitophagy (Onnis *et al.*, 2018). These results place p66Shc at the crossroads of multiple pathways that control cell survival through its ability to sense and respond to survival signaling, metabolic status or cellular stress.

In the last decade p66Shc was found implicated in CLL pathogenesis. In the study conducted in 2010 by Capitani (Capitani *et al.*, 2010) an impaired expression of p66Shc was found in B cells from CLL patients compared with B cells from healthy donors. p66Shc expression defect in CLL cells was recently mapped at the transcriptional level. It was demonstrated that the transcription factor Signal Transducer and Activator of Transcription 4 (STAT4), whose expression is severely impaired in CLL

cells (Cattaneo *et al.*, 2016), specifically interacts with several binding sites in the p66Shc promoter, indicating a direct correlation between defective expression of p66Shc and levels of its specific transcription factor STAT4 (Cattaneo *et al.*, 2016). A negative correlation was observed between p66Shc expression and BCR-dependent activation of the Ras/MAPK pathway, and p66Shc presence was found to impair activation of early and late kinases associated to BCR signaling such as Erk, Akt and Syk (Finetti *et al.*, 2008; Capitani *et al.*, 2010). CLL cells protection from apoptosis together with their resistance to chemotherapy is attributed at least in part on an altered balance among anti-apoptotic and pro-apoptotic Bcl-2 family members (Reed *et al.*, 1994), and a link between expression of these molecules and p66Shc was already reported in T cells (Pacini *et al.*, 2004). In B CLL cells p66Shc deficiency was found to be directly correlated to a significant increase in the levels of anti-apoptotic Bcl-2 and Bcl-xL and a concomitant decrease in the levels of Bax and Bak, the pro-apoptotic members of the Bcl-2 family. Indeed, reconstitution of p66Shc in leukemic cells resulted in a significant impact on Bcl-2 family members' expression and a recovered balance towards pro-apoptotic molecules, with consequent sensibilization of CLL cells to undergo apoptosis. In addition, p66Shc impairment was found to be more severe in those patients with worse prognosis like U-CLL (Capitani *et al.*, 2010). Later studies evidenced the contribution of p66Shc in promoting accumulation and infiltration of leukemic cells in lymphoid and non lymphoid organs, a condition that further supports CLL cells survival. In human CLL, the impaired expression of p66Shc is associated to the downregulation of the egress S1P receptor (S1PR1) and the upregulation of the CCR7 receptor which drives homing to lymphoid organs, resulting in massive accumulation of leukemic cells in lymph nodes (Capitani *et al.*, 2012). Accordingly, selective deletion of p66Shc in B lymphocytes of the mouse model of human CLL, the Eμ-TCL1p66Shc$^{-/-}$ mice, resulted in the deregulated expression of homing and egress receptors on leukemic cells and consequent massive accumulation and infiltration of leukemic cells in lymphoid and non lymphoid organs (Patrussi *et al.*, 2019).

2. Therapeutic options targeting CLL altered pathways

2.1 Current treatment of chronic lymphocytic leukemia

Chemoimmunotherapy (CIT) has represented the standard treatment for CLL for many decades combining anti tumoral activity of conventional cytostatic agents as chlorambucil, fludarabine or bendamustine and cyclophosphamide with monoclonal antibodies against CD20 such as rituximab, ofatumumab, obinutuzumab (Kipps *et al.*, 2017). Rituximab and its analogues target the CD20 antigen expressed on B cells promoting their depletion through complement-dependent cytotoxicity, opsonization by macrophages or direct killing (Brown *et al.*, 2018). Chemoimmunotherapy regimen revealed good efficacy in terms of survival, but at the same time severe adverse effects have been reported. Moreover, cytostatic agents lose their effectiveness in patients with deletion of band 13 of the short arm of chromosome 17 (del17p13) because of the p53 protein loss and impaired DNA damage response (Eichhorst *et al.*, 2016). CLL patients with this deletion are indeed resistant to any cytostatic agent (Buccheri *et al.*, 2018). To overcome chemoresistance to CIT therapy a huge amount of work has been done in the last decade to understand CLL biology and molecular pathogenesis. This effort resulted in the identification of many altered pathways associated to the disease onset and progression, such as defective apoptosis, misregulated BCR signaling and, more recently, the pro-tumorigenic role played by SLOs (Bosch and Dalla-Favera, 2019). Moreover, CLL is an extremely heterogeneous disease, and features to take into account in CLL diagnosis, clinical staging and treatment for each patient are multiple, highly variable and not always easy to assess, given the extended landscape of genetic, molecular and biochemical alterations (Crassini *et al.*, 2019; Hallek, 2019; Kipps and Choi, 2019). Therefore, CLL complexity and incurability prompt for the continuous development of new agents that, year by year, are completely substituting traditional CIT regimen (Iovino and Shadman, 2020).

Recently, the inhibitor of Bruton tyrosine kinase (ibrutinib), the inhibitor of phosphoinositide 3-kinases (PI3K) catalytic subunit delta (idelalisib), both targeting BCR signaling pathway, and the antagonist of B cell lymphoma-2 (Bcl-2), venetoclax, entered into the clinic, offering the opportunity

for a selective, chemo-free approach to treat CLL (Andreani *et al.*, 2020). Ibrutinib is a small inhibitor that irreversibly inhibits BTK by covalently binding to cysteine 481 in the ATP-binding pocket, thus blocking downstream signaling with consequent reduction of migration and proliferation of the tumor cell, revealing a good efficacy both as first-line treatment and in relapsed or refractory CLL (Byrd *et al.*, 2013; Burger *et al.*, 2020). Idelalisib is a reversible inhibitor of PI3K approved in combination with rituximab for relapsed/refractory CLL with good results in term of survival even in patients with del17p and worse prognosis but, due to its severe adverse effects, is never selected as first choice except when treating those patients not suitable for other therapeutic options (Scheffold and Stilgenbauer, 2020).

Apoptosis resistance caused by Bcl-2 overexpression in CLL B cells was overcome by recently developed BH3 mimetics such as venetoclax, which through direct binding and inactivation of Bcl-2 exerts an immediate apoptotic effect for tumor cells. Venetoclax was approved in 2016 for relapsed CLL and patients failing BCR inhibitors therapy, but also for patients with p53 deletion and bad prognosis (Delbridge and Strasser, 2015; Roberts *et al.*, 2016).

Besides all the three agents described before show good anti-tumoral efficacy even in CLL patients with worse prognosis, and combinations of target-therapy agents revealed to be more potent than chemoimmunotherapy (Hallek, 2019), they are not free of limitations. All of them require long periods of treatment and tend to show occurrence of resistance or severe adverse effects (Scheffold and Stilgenbauer, 2020). Prolonged treatment with ibrutinib causes occurrence and positive selection of clones carrying a mutation of cysteine 481 to serine in the BTK binding site of ibrutinib, resulting in a loss of inhibition of BTK enzymatic activity, or selection of clones with gain-of-function mutations in PLCγ2 leading to a BTK-autonomous BCR signaling (Kanagal-Shamanna *et al.*, 2019). Expansion of resistant clones leads to disease relapse or, in some cases, to disease progression to Richter Syndrome with intense B cell proliferation rate and growth of lymphadenopathy, extranodal disease, and associated multiorgan dysfunction from invasive or obstructive processes (Allan and Furman, 2019). Adverse events observed following ibrutinib treatment include off target kinase

inhibition, prompting the development of more selective next-generation BTK inhibitors with minimal off target events such as acalabrutinib and zanubrutinib (Tam *et al.*, 2019). Idelalisib-related resistance may occur through different and not recurrent mutations (Scheffold *et al.*, 2019), but its stronger limitation is represented by the severe adverse effects, thus requiring great accuracy in drug administration (Cuneo *et al.*, 2019). Efforts to identify safer next-generation PI3K inhibitors lead to development of duvelisib, which has specificity both for subunit delta and gamma of PI3K, and umbralisib, which is instead highly selective for PI3K delta subunit. Duvelisib is already approved as single agent for relapsed CLL, while umbralisib is still under clinical investigation (Cuneo *et al.*, 2019; Visentin *et al.*, 2020)

Venetoclax is the only cytotoxic drug among the novel targeted-therapies here described and for its potent pro-apoptotic effect, early tumor lysis syndrome is the most dangerous consequence, causing renal failure, cardiac dysrhythmia, neurologic complications, seizures, or death (Cheson *et al.*, 2017). Acquired resistance to venetoclax results from a mutation of glycine 101 to valine in Bcl-2, which reduces the affinity of venetoclax for Bcl-2 (Blombery *et al.*, 2019). A further limitation of venetoclax is that the anti-tumor action does not affect leukemic cells in lymph nodes owing to a *de novo* synthesis of BCL-XL that can counteract its activity thereby reducing the efficacy of venetoclax in clearing disease at nodal sites (Roberts *et al.*, 2016). Resistance to venetoclax is also largely driven by MCL-1 overexpression, thus this molecule is under investigation as a promising therapeutic target in preclinical trials with selective mimetics (Kotschy *et al.*, 2016). Beside adverse effects, the two inhibitors of BCR signaling, ibrutinib and idelalisib, showed an interesting mobilizing activity towards leukemic cells localized in SLOs. Ibrutinib treatment is associated to a non pathological increase of leukemic cells in peripheral blood and a concomitant reduction of size of lymph nodes and spleen (Rossi and Gaidano, 2014). Surprisingly, lymphocytosis is not correlated to disease progression, but more likely it is the result of the egress of leukemic cells from SLOs into the blood trough ibrutinib-dependent downregulation of the homing receptor CXCR4 (Chen *et al.*, 2016). Idelalisib has instead an effect on the egress receptor S1PR1 inducing its upregulation, thus promoting

migration of CLL B cells towards S1P chemokine in peripheral blood (Till, Pettitt and Slupsky, 2015). Early redistribution of SLOs-resident leukemic cells to the blood is a common phenomenon observed after administration of inhibitors of BCR-associated kinases in CLL patients, and studies conducted in CLL animal models confirmed and deepen this evidence (Burger and Montserrat, 2013). Lymphocytosis associated to kinases inhibitors together with the inhibition of BCR-dependent proliferation and survival resulted in the so called "death by neglect", where CLL cells, deprived of BCR stimulation and of their protective tumor microenvironment, are more susceptible to apoptosis (Burger *et al.*, 2009). Combination of the abovementioned agents with pro-apoptotic agents as canonical cytostatic chemotherapy is thus necessary to exert a more efficient removal of CLL B cells from peripheral blood and SLOs.

2.2 Emerging therapies

A number of innovative therapies are now under investigation to overcome limitations of agents currently in use for CLL therapy. Identification of another kinase participating in BCR signaling, SYK, supported the development of new inhibitors at present still under clinical investigation for CLL treatment, fostamatinib and entospletinib, which have been proved to disrupt microenvironmental interactions and cause CLL cells redistribution from SLOs (Friedberg *et al.*, 2010).

Crosstalk between CLL cells and microenvironment appears to be meaningful for leukemic cells survival and disease progression, so therapeutic approaches able to interrupt this crosstalk might have a great potential (Lampson and Brown, 2019). The CXCR4 antagonist plerixaflor inhibited CLL cells trafficking and microenvironment-mediated protective effects when tested preclinically, and recent clinical trials evidenced that plerixaflor promotes mobilization of CLL cells from their protective microenvironment to the blood, becoming accessible to conventional drugs (Stamatopoulos *et al.*, 2012; Andritsos *et al.*, 2019).

Immunodeficiency is a feature developed by CLL patients following disease progression and actively promoted by leukemic cells themselves. Effectors of the innate and the adaptive immune response are dysfunctional and skewed towards an immune-suppressive phenotype which promotes the generation of a tolerant environment that favors disease expansion (Arruga *et al.*, 2020). Immunomodulatory therapies able to enhance immune response against CLL cells are under clinical investigation. Lenalidomide, a thalidomide analogue, enhances immune recognition of CLL cells through upregulation of cell surface ligands, activates T and NK cells, downregulates inhibitory ligands on T and CLL cells and reduces microenvironmental factors that support CLL cells survival, but revealed scarce tolerability and to be unsuccessful as first line treatment (Itchaki and Brown, 2017). T cells from CLL exhibit a pseudo-exhaustion phenotype and overexpress the inhibitory checkpoint PD-1. CLL cells as well express PD-1 together with its ligand PD-L1 (Xu-Monette, Zhou and Young, 2018). In this context, PD-1/PD-L1 axis may represent an interesting target for CLL, and preclinical studies gave promising results. Clinical investigation of PD-1 inhibitors pembrolizumab and nivolumab is still ongoing, revealing to be useful when used in combination regimens but inefficient as monotherapy (Jelinek *et al.*, 2017).

Finally, a promising and most recent treatment for CLL is represented by chimeric antigen receptor (CAR) T cells, genetically modified to target specific antigens on malignant cells (Sermer and Brentjens, 2019), and in CLL the more frequent target antigen is CD19. This therapy revealed to be efficient even in patients with worse prognosis and elder patients, but is associated to cytokine release syndrome, a potentially fatal effect and, not to underscore, costs of such therapies are very high (Frigault and Maus, 2020).

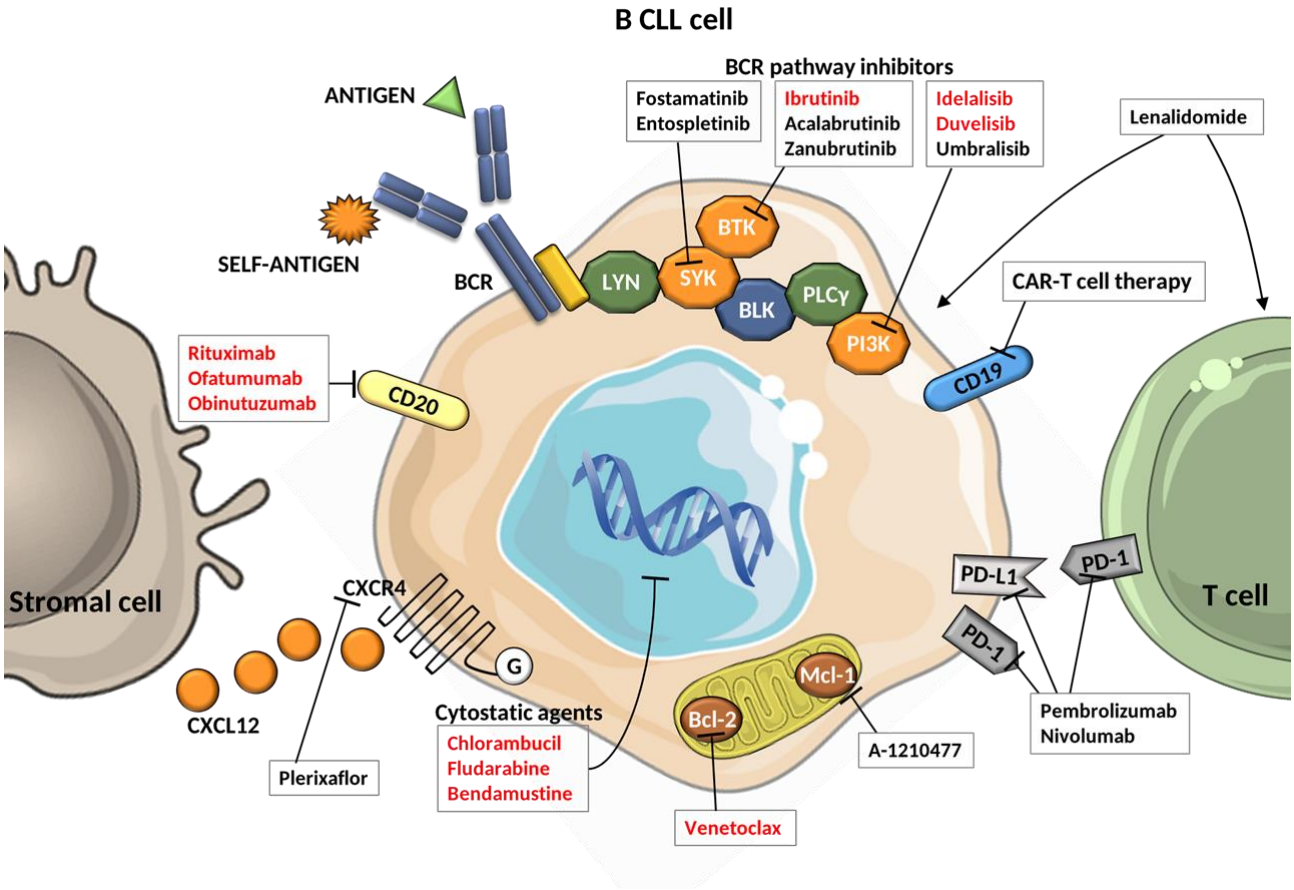

Figure 2. **Schematic representation of the interplay between CLL B cells with other cells within the tumor microenvironment and drugs' molecular targets**

The great majority of survival and proliferating stimuli for CLL B cells come from Secondary Lymphoid Organs (SLOs). Here, stromal cells and Nurse Like Cells produce and release chemokines which, after binding specific receptors on leukemic cells, not only attract leukemic cells within SLOs, but activate survival and proliferation pathways in CLL B cells (Kipps *et al.*, 2017). Figure shows a schematic representation of canonical targets of therapies currently approved for CLL, together with new targets with relative therapies under clinical investigation. Names of drugs with approval for CLL are given in red; drugs in various stages of clinical development are shown in black. The image was modified from Yosifov *et al.*, 2019. BCR= B Cell Receptor; LYN=LCK/YES novel tyrosine kinase; SYK= spleen tyrosine kinase; BTK= Bruton tyrosine kinase; BLK= B lymphocyte kinase; PLCγ= phospholypase C; PI3K= phosphoinositide 3-kinase; Mcl-1= Myeloid cell leukemia 1; Bcl-2= B cell lymphoma-2; PD-1= programmed cell death protein 1; PD-L1= programmed cell death ligand 1; CAR-T cell= chimeric antigen receptor T cell.

3. Models of chronic lymphocytic leukemia: EμTCL1 and EμTCL1p66Shc$^{-/-}$ mice

The T-cell leukemia 1 (*TCL1*) gene was found to be overexpressed in mature T cell leukemias and involved in chromosomal translocations and inversions, but high levels of Tcl1 have also been found in many human tumor-derived B cell lines and in many cases of B cell neoplasias (Narducci *et al.*,

2000; Pekarsky, Hallas and Croce, 2001). In this context the Eμ-TCL1 transgenic mouse was generated. It is a transgenic mouse in which the human oncogene *TCL1* is under the control of the IGHV promoter and IGH enhancer (Eμ), in order to be selectively expressed in B cells (Bichi *et al.*, 2002). Among the many genetically engineered mice generated in the last two decades, Eμ-TCL1 is considered at present the best mouse model recapitulating aggressive human CLL (Pekarsky *et al.*, 2007, 2015). Immunophenotypic analysis in peripheral blood from Eμ-TCL1 mice reveals an increase of $CD5^+$ population of cells between 1 and 9 months of age (≥10%), without any sign of disease (Bichi *et al.*, 2002). Similarly to human CLL (hCLL), disease onset in mice occurs over time and at 8 months of age mice start to present an abnormal expanded population of $CD5^+$ cells in peritoneal cavity (74%), spleen (68%) and bone marrow (43%), while evident hepatomegaly, splenomegaly and lymphadenopathy arise later, around 13-18mo of age, and mice show clear signs of the disease (Bichi *et al.*, 2002). Besides clinical features, Eμ-TCL1 mice additionally share many molecular characteristics with CLL patients with aggressive disease, including Akt abnormal activation, Zap70 expression, stereotyped and autoreactive BCRs (Yan *et al.*, 2006; Efanov *et al.*, 2010; Bresin *et al.*, 2016). Given the striking similarity with aggressive hCLL, Eμ-TCL1 mice has been extensively investigated as a valid tool to study molecular mechanism underlying CLL pathogenesis, and several transgenic and knockout mouse models have been crossed with Eμ-TCL1 mice to elucidate the functional role of specific molecules in the onset and progression of CLL *in vivo* (Simonetti *et al.*, 2014). Among novel mouse models developed for hCLL, the Eμ-TCL1p66$Shc^{-/-}$ mouse was recently generated to uncover the role of p66Shc in CLL pathogenesis. These mice have an accelerated leukemogenesis and enhanced disease aggressiveness, together with massive nodal and extranodal infiltration. Moreover, p66Shc deficiency accounted for the upregulation of leukemic cells' surface expression of receptors for chemokines responsible for nodal and extra-nodal accumulation of CLL cells underscoring p66Shc as a novel relevant therapeutic target (Patrussi *et al.*, 2019).

4. Pyrrolonaphtoxazepines: a novel class of anti-cancer agents

Pyrrolonaphtoxazepines (PNOXs) are a recently developed class of synthetic compounds classified as Mictrotubule Targeting Agents or, more precisely, Microtubule Depolymerizing Agents. Their target is the colchicine binding site on tubulin, so the principal effect of these compounds is the inhibition of dimerization of tubulin (Mulligan *et al.*, 2006; Brindisi *et al.*, 2019). Tubulin is the core component of microtubules, which are involved in a variety of cellular functions ranging from mitosis to intracellular transport of organelles and signaling molecules (Janke and Magiera, 2020). Since microtubule network is implicated in establishing the mitotic spindle and therefore controls cell mitosis, drugs able to disrupt microtubule architecture are largely used in chemotherapy to inhibit the rampant cell proliferation rate of tumoral cells (Kumar *et al.*, 2016). Pyrrolonaphtoxazepines (PNOXs) are pyrrolo-1,5-benzoxazepines (PBOXs) typified by the 4-acetoxy-5-(1-naphthyl)naphtho[2,3-b]pyrrolo[2,1-d] oxazepine whom leading compound, PNOX also known as PBOX-15, together with its analogues, were widely investigated in several haematological malignancies and solid tumors (Brindisi *et al.*, 2016, 2019). PNOX primarily disrupts microtubules dynamics during mitosis, inducing the block of cell cycle following activation of the mitotic spindle checkpoints (Greene *et al.*, 2008). Despite inhibition of microtubule assembly and anti-mitotic efficacy are the main activities exerted by PNOX, many studies evidence that the action of the compound and its analogues is not restricted to tubulin binding but they show instead a wider group of targets including oncogenes and key signaling pathways controlling cancer progression and metastasis (Mc Gee *et al.*, 2002; Greene *et al.*, 2008; Verma *et al.*, 2008; Maginn *et al.*, 2011; Lysaght *et al.*, 2013; Nathwani *et al.*, 2016). Profiling of pro-apoptotic PNOX-induced signaling, identified an early phosphorylation and activation of c-Jun n-terminal kinase (JNK) together with late downregulation of signaling pathways frequently associated with a wide variety of human cancers including Bcr-Abl, c-kit, B cell lymphoma-2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), Myeloid cell leukemia 1 (Mcl-1), phosphoinositide 3-kinase (P13K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) and inhibitors apoptosis proteins (IAPs), further supporting why they

resulted suitable in treating diverse cancer types (Greene *et al.*, 2016). Other studies report that PNOX and its analogues exert an anti tumoral activity as anti-angiogenic (PBOX-6 and PBOX-16) or as inhibitors of migration and integrin-mediated adhesion (PBOX-15) and recently they have been ascribed with autophagy modulation activity (Verma *et al.*, 2008; Lysaght *et al.*, 2013; Brindisi *et al.*, 2019). Considering that tumoral cells account for their survival and proliferation not only on out-of-control mitosis but on more than one altered intracellular pathway, the wide range activity of PNOX and its analogues is of great clinical interest. Moreover, the potent killing activity of these agents is able to overcome drug resistance mediated by the multi-drug efflux pump P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (Nathwani *et al.*, 2010), but still they are associated with a safe profile toward normal cells (McGee *et al.*, 2005; McElligott *et al.*, 2009) and low systemic toxicity was already reported for the compound named PBOX-6, in mouse models of chronic myeloid leukemia (CML) and breast cancer (Greene *et al.*, 2005; Bright *et al.*, 2010). In addition, these compounds can be easily modified to improve efficacy and solubility due to the simple structure of these agents compared to other MTAs (Greene *et al.*, 2016).

The leading compound of the class, PNOX, showed a potent pro-apoptotic effect *in vitro* in B-CLL and B-CML together with a safe profile towards normal B cells, suggesting a possible usage of this compound for the treatment of haematological malignancies (McElligott *et al.*, 2009; Bright *et al.*, 2010). In primary CLL B cells, including cells resistant to fludarabine, PNOX demonstrated a selective pro-apoptotic efficacy suggesting a p53-independent mechanism of action. Furthermore, these data suggested that PNOX-mediated apoptosis involves activation of caspase 8 and perturbation of mitochondria integrity and, similarly to what is observed for other PNOX analogues, PNOX exhibited in CLL B cells a potent JNK-dependent pro-apoptotic activity after long *in vitro* treatment (Mc Gee *et al.*, 2002; McElligott *et al.*, 2009). Considering that CLL B cells are known to be arrested in G_1 phase of the cell cycle, PNOX-dependent apoptosis in these cells is unlikely to result exclusively from a mitotic arrest secondary to microtubule depolymerization, as demonstrated by data collected by McElligott and colleagues. Rather off-target effects may underline the ability of PNOX to restore

the apoptotic defect in CLL B cells. Hence, the precise characterization of the molecular mechanism of action for PNOX in CLL needs to be further elucidated and preclinical studies are required for a future therapeutic use of PNOX in CLL cells.

MATERIALS AND METHODS

1. Mice

Eμ-TCL1 mice in the C57BL/6J background and Eμ-TCL1 mice lacking *p66Shc* gene, Eμ-TCL1p66Shc$^{-/-}$ (Patrussi *et al.*, 2019) were used. Mice were housed in the animal facility of the University of Siena in pathogen-free and climate-controlled conditions (20 ± 2°C, relative humidity 55 ± 10%). Animals experiments were performed in agreement with the 2010/63/EU Directive and approved by the Italian Ministry of Health. Disease progression in Eμ-TCL1 and Eμ-TCL1p66Shc$^{-/-}$ was monitored by peripheral blood sampling followed by immunophenotyping for CD19^{+}CD5^{low} cells. 12 Eμ-TCL1 mice (9 males and 3 females) and 8 Eμ-TCL1p66Shc$^{-/-}$ (5 males and 3 females) which developed spontaneous leukemia with >10mo of age and a CD19^{+}CD5^{low} leukemic cells percentage in peripheral blood ≥20%, were selected for *in vivo* treatment with PNOXs compounds. Mice were intra peritoneally (i.p) injected with 7.5 mg/kg PNOX or PNOX-3 resuspended in 200μl of PBS 1x, a Eμ-TCL1 group receiving PBS was used as control. For all the duration of treatment, mice were daily checked by the operators to assess motility, grooming and sign of sickness as lethargy or labored breathing.

2. Primary CLL samples, healthy controls and cell lines

Peripheral blood samples of CLL patients and healthy donors were provided by the Haematology Unit at the University of Siena Hospital. Informed consent was obtained according to the declaration of Helsinki and the study was approved by the University of Siena Ethical Committee Board. B cells were purified by negative selection using Rosette Sep cocktail (StemCell) then stratified on density gradient medium Fycoll Hipaque-H (Cedarlane Laboratories). CLL murine splenocytes were obtained from spleens of Eμ-TCL1 and Eμ-TCL1p66Shc$^{-/-}$ mice with mechanical disruption using a 70μm cell strainer to obtain single splenocytes suspension. Both human B cells and murine CLL splenocytes were used fresh or frozen till stimulation.

Burkitt lymphoma-derived B cell line (BJAB WT) and the p66Shc$^{-/-}$ clone generated by CRISPR-Cas9 mutagenesis (BJAB KO) were gently provided by *Dott.ssa Anna Kabanova* and *Dott.ssa Vanessa Zurli* from Tumor Immunology Unit at Toscana Life Sciences, Siena, Italy.

3. Cell treatments with PNOXs compounds

PNOXs compounds were synthesized as described previously (Campiani *et al.*, 1996; Brindisi *et al.*, 2019) and provided by *Prof. Giuseppe Campiani* and *Prof. Stefania Butini*. Compounds were resuspended in sterile dimethyl-sulfoxide (DMSO) (Serva) aliquoted and stored at -20°C. Primary human B cells ($3x10^6$ cells/ml) and mouse splenocytes ($3x10^6$ cells/ml) were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% Bovine Calf Serum (BCS) (Hyclone,Thermo Scientific) and 20 U/mL penicillin; BJAB cell lines ($1x10^6$ cells/ml) were resuspended in RPMI 1640 medium supplemented with 10% BCS, 20 U/mL penicillin and 1 mM sodium pyruvate. Cells were treated with 10μM PNOX, PNOX-3 or with equal volumes of vehicle (DMSO) for 4h or 24h. Alternatively, cells were pre-treated for 1h at 37°C with 20μM of the specific JNK inhibitor SP600125 (Sigma-Aldrich) and subsequently treated with PNOXs compounds in presence or absence of SP600125 20μM for 4h or 24h at 37°C.

4. Flow cytometry

Single cell suspensions of mouse spleen, peritoneal wash and peripheral blood were subjected to red blood cell lysis using RBC lysis buffer (0.155 M NH4Cl, 1 mM KHCO3, 0.1 mM EDTA, pH 7.4), formulated with ammonium chloride for optimal lysis of erythrocytes and minimal effect on leukocytes (Dagur and McCoy, 2015). Leukocytes were then incubated with murine FcBlock (Bio Legend) diluted 1:50 in PBS 1% BCS for 15 min on ice. This step is necessary to inhibit non-specific binding of immunoglobulins to the Fc receptors present on many cell types, including granulocytes, B cells, macrophages, and dendritic cells (Andersen *et al.*, 2016). Cells were stained with fluorochrome-conjugated anti mouse CD5 (E-Bioscience) and anti-mouse CD19 (BD Pharmingen) antibodies diluted in PBS 1%BCS and subjected to flow cytometry.

Dissipation of mitochondrial transmembrane potential (Δψm) was assessed in murine and human B cells using the fluorescent dye *tetramethylrhodamine methyl ester perchlorate* (TMRM) (Sigma-Aldrich). This fluorescent lipophilic cationic probe is attracted by the intact membrane potential of mitochondria and is rapidly taken up by living cells. The dye is instead quickly released from cells when mitochondria lose their membrane potential, thus indicating that the cell is engaged in apoptosis (Creed and McKenzie, 2019). Cells were washed in RPMI w/o phenol red (Invitrogen) and loaded with 200 nM TMRM in RPMI w/o phenol red at 37°C for 20 min. Samples were acquired on Guava EasyCyte cytometer (Millipore) and analyzed with FlowJo software (TreeStar Inc., Ashland, OR, USA).

5. Post-nuclear and post-mitochondrial supernatant purification and immunoblots

Primary human and murine cells and BJAB cells were lysed in 1% (v/v) Triton X-100 in 20 mM Tris-HCl (pH 8), 150 mM NaCl with Protease Inhibitor Cocktail Set III (Calbiochem) and 0.2 mg/ml sodium orthovanadate. Post mitochondrial supernatants were isolated as described (Pellegrini *et al.*, 2007). Briefly, 30×10^6 cells/sample were washed twice with PBS 1x, resuspended in 450 µl buffer A (20 mM Hepes pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 250 mM sucrose), incubated 10 min on ice and broken in a Dounce homogenizer (25 strokes). The homogenate was centrifuged at 4°C for 10 min at 600× g, and the supernatant was subjected to a second centrifugation at 4°C for 10 min at 700× g. The latter supernatant was then centrifuged at 4°C for 10 min at 7000× g. The pellet, enriched in mitochondria, was discarded, whereas the post-mitochondrial supernatant was used for immunoblot analysis of cytochrome-c release. Post-nuclear and post-mitochondrial supernatants were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots (antibodies, dilutions and sources are listed in **Table 1**) were carried out using peroxidase-labeled secondary antibodies (GE Healthcare) and a chemiluminescence detection kit (Thermo Fisher). Immunoblots were scanned and quantitated using ImageJ software.

Table 1. Commercial antibodies with dilutions used for immunoblots

Antibody	Host species	Source	Dilutions
Shc-A	Rabbit	Millipore	1:2000
STAT4	Rabbit	Cell Signaling	1:500
Phospho STAT4 (Ser721)	Mouse	Santa Cruz Biotechnology	1:500
Phospho-JNK	Rabbit	Cell Signaling	1:1000
JNK	Rabbit	Cell Signaling	1:1000
Phospho-p38 MAPK (Thr 180/Tyr 182)	Rabbit	Cell Signaling	1:100
p38-MAPK	Rabbit	Cell Signaling	1:1000
Phospho-ERK (Thr202/Tyr204)	Rabbit	Cell Signaling	1:1000
ERK1-2	Rabbit	Santa Cruz Biotechnology	1:1000
Cytochrome-c	Mouse	BD Pharmingen	1:1000
Phospo-Ser36-p66Shc	Mouse	Alexis Biochemicals	1:300
Actin (D11)	Mouse	Millipore	1:10000

6. RNA purification and quantitative PCR

Total RNA was extracted using RNeasy plus mini kit (Qiagen) according to manufacturer instructions. 1µg of RNA from each sample was retro transcribed as complementary single stranded cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). Real Time quantitative PCR (RT-qPCR) was performed using SsoFast™ EvaGreen® supermix kit (Bio-Rad) and 10µM primers. Each cDNA sample was run in triplicate on 96-well optical PCR plates (Sarstedt). First denaturation step was performed for 3 minutes at 95°C, then in subsequent 45 cycles denaturation was performed for 10 seconds at 95°C, followed by primer annealing and elongation at 60°C for 30 seconds and a final elongation step at 65°C for 30 seconds. Analysis of data was conducted using Bio-Rad CFX Manager software (Bio-Rad) to calculate the threshold cycle (Ct). The specificity of the amplified fragments was demonstrated by assessing the melting curve, where a single peak was observed for all the amplified samples. mRNA levels of each analyzed transcript were determined using the comparative ΔΔCT method between Ct of Gene Of Interest (GOI) and Ct of housekeeping genes (HG), GAPDH for mouse-derived samples and 18S ribosomal RNA for human samples. Sequences of primers used are listed in **Table 2**.

Table 2. Human (H) and murine (M) primers sequences used in RT-qPCR

Gene	**Fw**	**Rev**
H-p66Shc	CCA CTA CCC TGT GCT CCT TC	ATC ATC AGC TGC CCT TCC T
H-STAT4	TGC TGG CAG AGA AGC TTA CA	TAG CAA CAG CCG TTC CTT CT
H-Bcl-2	GGA GGC TGG GAT GCC TTT	CCA GAT AGG CAC CCA GGG T
H-18s	CGC CGC TAG AGG TGA AAA TT	CTT GGC AAA TGC TTT CGC
M-p66Shc	TGA GTT GGG AGA GCA GAG GT	CTC ATT CCG AAG TGG GTT GT
M-S1PR1	TTC CGC AAG AAC ATC TCC AAG G	CAG CCC ACA TCT AAC AGT AGT
M-CXCR4	TCC TCC TGA CTA TAC CTG ACT	GAC GAG ACC CAC CAT TAT ATG
M-GADPH	AAC GAC CCC TTC ATT GAC	TCC ACG ACA TAC TCA GCA G

7. Statistical Analysis

One-way ANOVA or 2-way ANOVA with *post-hoc* Bonferroni's test were used for experiments in which multiple groups were compared. Paired t-test was used to determine the statistical significance of differences between two groups. Survival curves were calculated with the Kaplan-Meier method. A log-rank test was used to compare differences between estimated survival curves. GraphPad Prism Software (Version 8.4.2) was used for statistical analyses. A $p < 0.05$ was considered as statistically significant.

RESULTS

1. PNOX restores expression of p66Shc in B cells from CLL patients

Longer survival and defects in apoptotic machinery are hallmarks of B cells from CLL patients which at least in part have been recently ascribed to the defective expression of the molecular adaptor p66Shc (Capitani *et al.*, 2010). In T and B cells p66Shc exists as two pools, one cytosolic and the other mitochondrial (Finetti, Savino and Baldari, 2009). The cytosolic fraction of p66Shc inhibits the activation of the Ras/MAP kinase signaling pathway in response to antigen-receptor engagement on lymphocytes, thereby preventing antigen-dependent activation and proliferation of T and B cells (Pacini *et al.*, 2004; Finetti, Savino and Baldari, 2009). Accordingly, lack of p66Shc in CLL B cells is associated with enhanced BCR signaling and hyperactivation of downstream kinases including Akt, Erk and Syk (Capitani *et al.*, 2010).On the other side, p66Shc has been shown to promote apoptosis in T and B cells by affecting mitochondrial integrity, as its upregulation is associated to a reduction of intracellular ATP, dissipation of mitochondrial membrane potential and cytochrome-c release together with an impairment of the Bcl-2 family members balance towards the pro-apoptotic members (Capitani *et al.*, 2010; Ulivieri, 2010). Moreover, p66Shc pro-apoptotic function was found to be dependent upon an additional redox activity with production of intracellular oxidants (Giorgio *et al.*, 2005), indicating the key role played by this molecular adaptor in the control of mitochondrial dependent apoptosis.

The pro-apoptotic activity reported for PNOX on CLL cells has been ascribed to its ability to activate caspase 8, which is mainly involved in the mitochondrial-independent apoptosis, but also to its ability to affect mitochondrial integrity. Moreover, PNOX induced apoptosis in CLL B cells starts from 24h of *in vitro* treatment but not at earlier time points (McElligott *et al.*, 2009). These data lead us to assess whether the anti-leukemic activity of PNOX in CLL B cells was dependent on its ability to restore p66Shc expression. We treated primary B cells from both CLL patients and healthy donors

with PNOX for 24h and measured p66Shc expression. Interestingly, we found that PNOX specifically induces p66Shc expression, mRNA and protein, in CLL cells but not in normal B cells (Fig 3A and B). Moreover, our results showed that PNOX was able to upregulate p66Shc expression in CLL B cells independently of the IgHV mutational status (Fig 3C). As many others tubulin depolymerizers including nocodazole, PNOX is endowed with a highly selective killing activity towards tumoral cells over normal B cells which has been shown to be independent of mitotic arrest (McElligott *et al.*, 2009; Brindisi *et al.*, 2019). Cell cycle arrest and subsequent cell death is indeed just one of the effects associated with microtubules disruption by MTAs in tumoral cells, where other mitosis-independent functions such as the impairment of vesicular trafficking, migration and cell signaling have been reported (Čermák *et al.*, 2020). Additionally, alteration in gene expression has been reported in response to microtubule depolymerizing agents as vinblastine, nocodazole and vincristine which can upregulate pro-apoptotic proteins as Bax, Bak, Bad, Noxa and Puma (Bates and Eastman, 2017). In CLL B cells, nocodazole, induced downregulation of Bcl-2 (Beswick, Ambrose and Wagner, 2006). In agreement with a specific effect of MTA on gene expression our data show that PNOX activity in CLL is dependent upon transcriptional regulation of p66Shc.

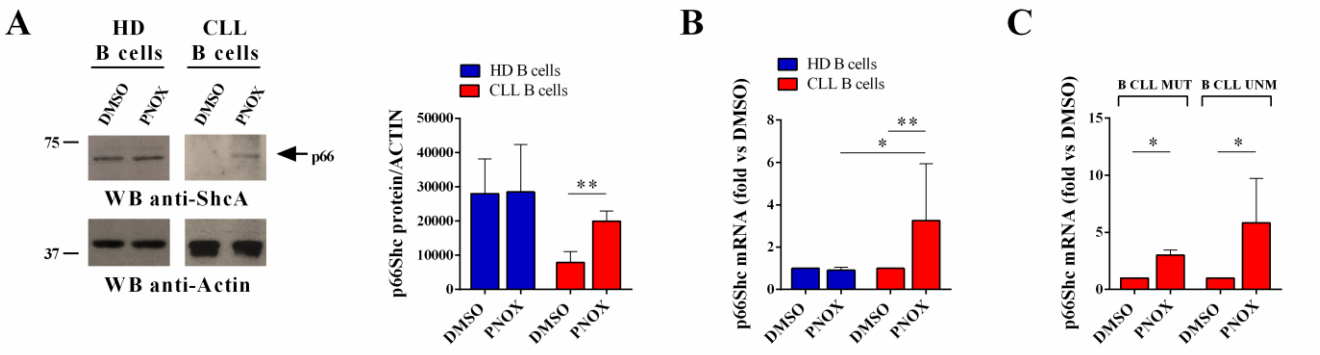

Figure 3. **PNOX induces p66Shc expression in leukemic B cells**

B-lymphocytes were isolated from peripheral blood of Healthy Donors (HD) and chronic lymphocytic leukemia (CLL) patients and were treated with PNOX (10μM) or vehicle (DMSO) for 24h. (A) Immunoblot analysis of p66Shc expression in post-nuclear supernatants of B cells from HD or CLL patients. An actin control blot of the same filter is shown. Quantification of the levels of p66Shc relative to actin by densitometric

analysis is shown in the histogram, bars represent mean values ±SD (HD B cells=4; B CLL cells=4). Quantification by qRT-PCR of p66Shc mRNA levels in B cells from HD and CLL patients (B) and on B cells from CLL patients with mutated (B CLL MUT) or unmutated (B CLL UNM) IGHV region (C). The levels of the transcript were determined using the ΔΔCt method and normalized to the levels of the housekeeping gene 18s. Data are presented as mean values ±SD with DMSO sample taken as 1 (HD B cells=5; CLL B=10). Paired t test (A) and two way ANOVA with post-hoc Sidak's test (B). *p≤0.05, **p≤0.01

2. PNOX promotes JNK-dependent phosphorylation of STAT4 and its transcriptional activation

In order to deepen the molecular mechanism governing p66Shc upregulation in CLL B cells following PNOX treatment, we evaluated its effect on STAT4 activation, which was recently identified as the specific transcription factor for p66Shc in B cells (Cattaneo *et al.*, 2016). To this end we performed a time course analysis of PNOX-dependent phosphorylation of STAT4 on serine 721, which is required for its full transcriptional activity and nuclear translocation (Visconti *et al.*, 2000), in CLL B cells. Results showed that PNOX induced phosphorylation of STAT4 starting from 4 hours of *in vitro* treatment of CLL B cells (Fig 4A); moreover, in agreement with the selective effect of PNOX on p66Shc expression in CLL B cells, STAT4 phosphorylation was not found in B cells from healthy donors following PNOX treatment (Fig 4B).

Tubulin destabilizers have been shown to target ERK, p38 and JNK signaling pathways within 4-6 hours after the treatment in several tumors, even if contrasting results have been also reported (Bates and Eastman, 2017). Since PNOX-dependent JNK activation has been demonstrated in low risk CLL cells following *in vitro* treatment with PNOX for 24h (McElligott *et al.*, 2009) and JNK, p38 and ERK1/2 have been implicated in STAT4 phosphorylation on Ser721 (Decker and Kovarik, 2000; Horvath, 2000; Visconti *et al.*, 2000), we decided to analyze whether PNOX promotes STAT4 phosphorylation by inducing early activation of the abovementioned kinases.

To this end we treated B cells from both HD and CLL patients with PNOX for 4h and analyzed by immunoblot the phosphorylation status of JNK, p38 and ERK1/2. Results showed that rather than a broad activation of stress kinases, PNOX specifically activates JNK and, moreover, JNK activation was observed exclusively in B CLL cells and not in B cells from healthy donors, further supporting

PNOX exclusive activity towards tumoral cells (Fig 4C, D and E). Furthermore, to clearly demonstrate the relationship between PNOX-induced JNK activation and STAT4 phosphorylation, we pre-treated CLL cells with the specific JNK inhibitor SP600125 prior to PNOX treatment and assessed STAT4 phosphorylation on Ser721. JNK-specific inhibition completely abrogated STAT4 phosphorylation induced by PNOX in CLL B cells (Fig 4F). Hence, our data identify STAT4 as a novel target of PNOX in CLL B cells and show that JNK, but not p38 or ERK1/2, acts upstream of microtubule destabilization induced by PNOX. Performing longer treatment with PNOX, 24h, we also found an upregulation of the STAT4 mRNA levels in CLL B cells (Fig 4G), indicating that on one hand PNOX rescues p66Shc expression in CLL B cells by activating the residual levels of STAT4, whose impaired expression accounts, at least in part, for the p66Shc defect (Cattaneo *et al.*, 2016), and on the other hand it maintains p66Shc expression high by restoring also STAT4 expression. Collectively, our results suggest that PNOX, by upregulating both p66Shc and STAT4 supports the circuitry of reciprocal upregulation between the two molecules.

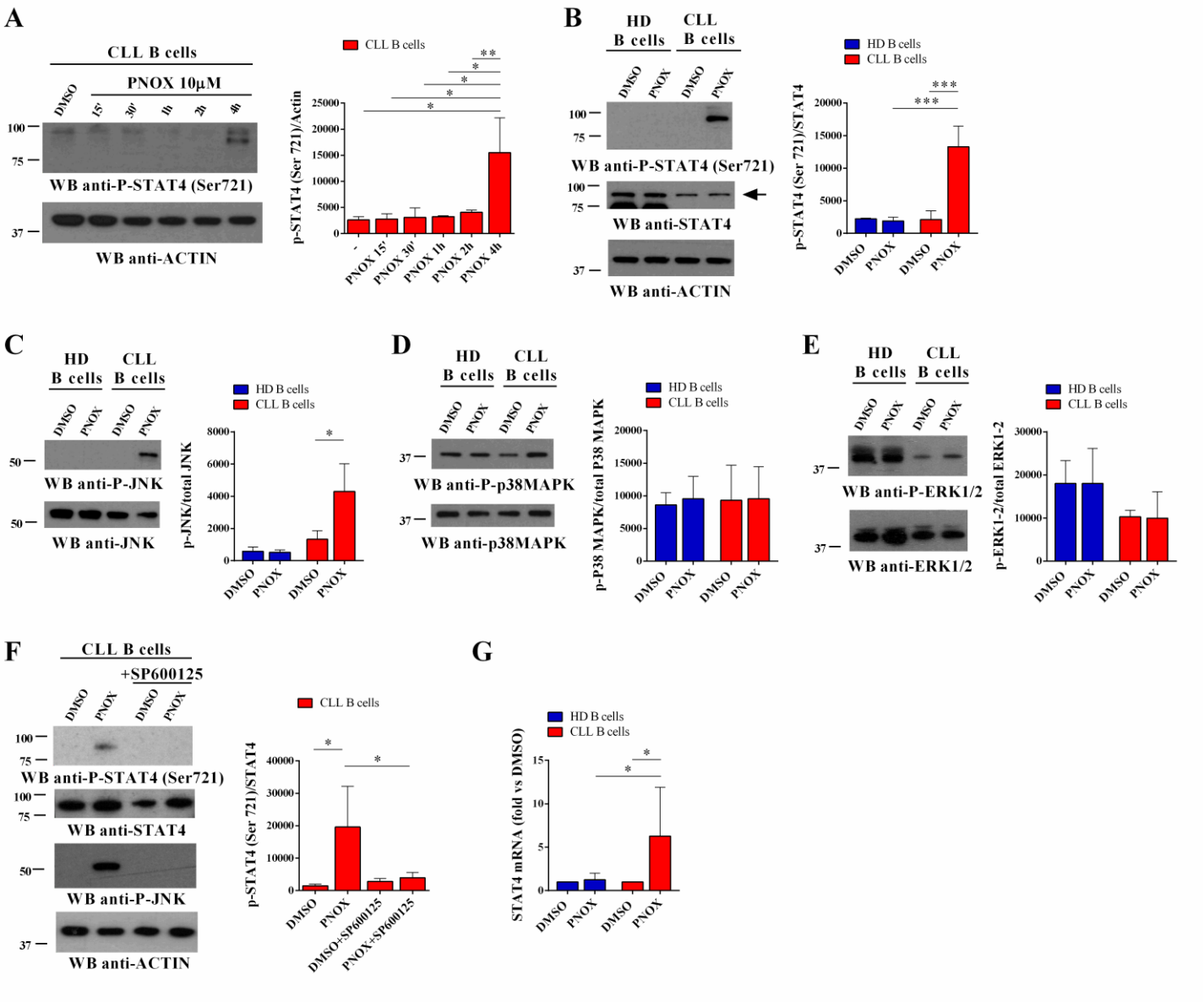

Figure 4. **The transcription factor STAT4 is phosphorylated by JNK in leukemic B cells, but not in normal B cells, following PNOX treatment**

(A) Immunoblot analysis of STAT4 phosphorylation on Ser721 residue of post-nuclear supernatants from B cells of chronic lymphocytic leukemia patients (CLL B cells) treated with vehicle (DMSO) or PNOX (10μM) for 15', 30', 1h, 2h or 4h. A representative blot is shown together with control blot anti-actin. Quantification by densitometric analysis of phospho-STAT4 levels relative to actin is represented in the histogram, where bars show mean values ±SD (n=3). (B) Immunoblot analysis of phospho-STAT4 (Ser721) on post-nuclear supernatants of B cells from healthy donor (HD B cells) and CLL patients (CLL B cells) treated with DMSO or PNOX (10μM) for 4h. The control blot anti-STAT4 and anti-actin are shown. STAT4 phosphorylation was quantified by densitometric analysis and related to STAT4. Values are shown in the histogram as bars representing mean values ±SD (HD B cells=3; B CLL cells=4). Immunoblot analysis with anti-phospho JNK (Thr183/Tyr185) (C), phospho-p38 (Thr180/Tyr182) (D), phospho-ERK1/2 (Thr202/Tyr204) (E) antibodies of post-nuclear supernatants of B cells from healthy donors (HD B cells) and chronic lymphocytic leukemia patients (CLL B cells) treated as in panel B. Control blots anti-JNK, anti-p38 and anti-ERK1/2 of the stripped

filter are shown below. Histograms show the quantification by densitometric analysis of the levels of the phosphorylated form of JNK, p38 and ERK1/2 relative to the total protein. Histogram bars show mean values ±SD (HD B cells=3; B CLL cells=4). (F) B cells from chronic lymphocytic leukemia patients (CLL B cells) were pre-treated for 30 min with SP600125 20 μM (+ SP600125) or not, then PNOX (10μM) or DMSO were added for 4h. Immunoblot analysis was performed on post-nuclear supernatants to assess phospho-STAT4 (Ser 721) levels relative to control blot anti-STAT4. Quantification by densitometric analysis is shown in histogram bars where data are represented as mean values ±SD (n=3). (G) Quantification by qRT-PCR of STAT4 mRNA levels in B cells from HD and CLL patients treated for 24h with DMSO or PNOX (10μM). The levels of the transcript were determined using the ΔΔCt method and normalized to the levels of the housekeeping gene 18s. ΔΔCt values are represented in the histogram, bars show mean values ±SD with DMSO sample taken as 1 (HD B cells=5; CLL B cells=5). Two way ANOVA with post-hoc Sidak's test (B, C, D, E, G) and One way ANOVA with Neuman-Keuls multiple comparisons test (A, F). *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$

3. The pro-apoptotic activity of PNOX in CLL B cells is dependent upon the recovery of p66Shc expression

Our data showed that PNOX activates JNK following 4h of treatment. Given that JNK activation has been linked to the phosphorylation of Bim and the induction of BAX-dependent apoptosis (Lei and Davis, 2003) and the microtubule destabilizer nocodazole directly targets the mitochondria (Rovini *et al.*, 2011), we assessed whether PNOX promotes mitochondrial-dependent apoptosis following 4h of treatment. Data showed that 4h of PNOX treatment is not sufficient to induce mitochondrial membrane depolarization and, consequently, apoptosis in CLL B cells (Fig 5A). In contrast, analysis of the apoptosis rate measured both as mitochondrial membrane potential loss and increase in the percentage of Annexin V^+/PI^+ cells after 24h of treatment, showed that PNOX induced a significant increase of the frequency of $TMRM^{low}$ cells in CLL B cells, while no effect was observed in HD B cells (Fig 5B). The selective pro-apoptotic effect of PNOX in CLL B cells over normal B cells was further confirmed with the AnnexinV/PI assay where apoptosis is detected by specific binding of Annexin V to exposed phosphatidylserine on the outer membrane of the cell and the concomitant loss of plasma membrane integrity which allows PI to enter into the cell (Kabakov and Gabai, 2018) (Fig 5C).

In vitro reconstitution of p66Shc in B cells from CLL patients is able to restore the balance among Bcl-2 family members through the upregulation of Bax and Bak mRNA expression and the reduction

of Bcl-2 and Bcl-Xl mRNA thereby increasing the susceptibility of leukemic cells to undergo apoptosis (Capitani *et al.*, 2010). To address whether the pro-apoptotic activity of PNOX reported in CLL B cells (McElligott *et al.*, 2009 and Fig 5B and C) after 24h of treatment relies on its ability to rescue p66Shc expression, we decided to evaluate if any correlation exists between p66Shc levels and apoptotic rate induced by PNOX. We found a direct correlation between p66Shc mRNA levels and the percentage of $TMRM^{low}$ in B cells of CLL patients. Moreover, a significant upregulation of Bcl-2 mRNA was observed in B cells from CLL patients at this time point, indicating that the pro-apoptotic activity of PNOX in CLL cells relies on its ability to restore p66Shc expression which in turn reduces Bcl-2 expression levels (Fig 5D and E). Accordingly, PNOX did not promote apoptosis in CLL B cells following shorter treatment (4h) when p66Shc expression is not rescued suggesting that PNOX-dependent JNK activation *per se* is not sufficient to promote apoptosis opposite to what has been previously shown (Lei *et al.*, 2002; Lei and Davis, 2003).

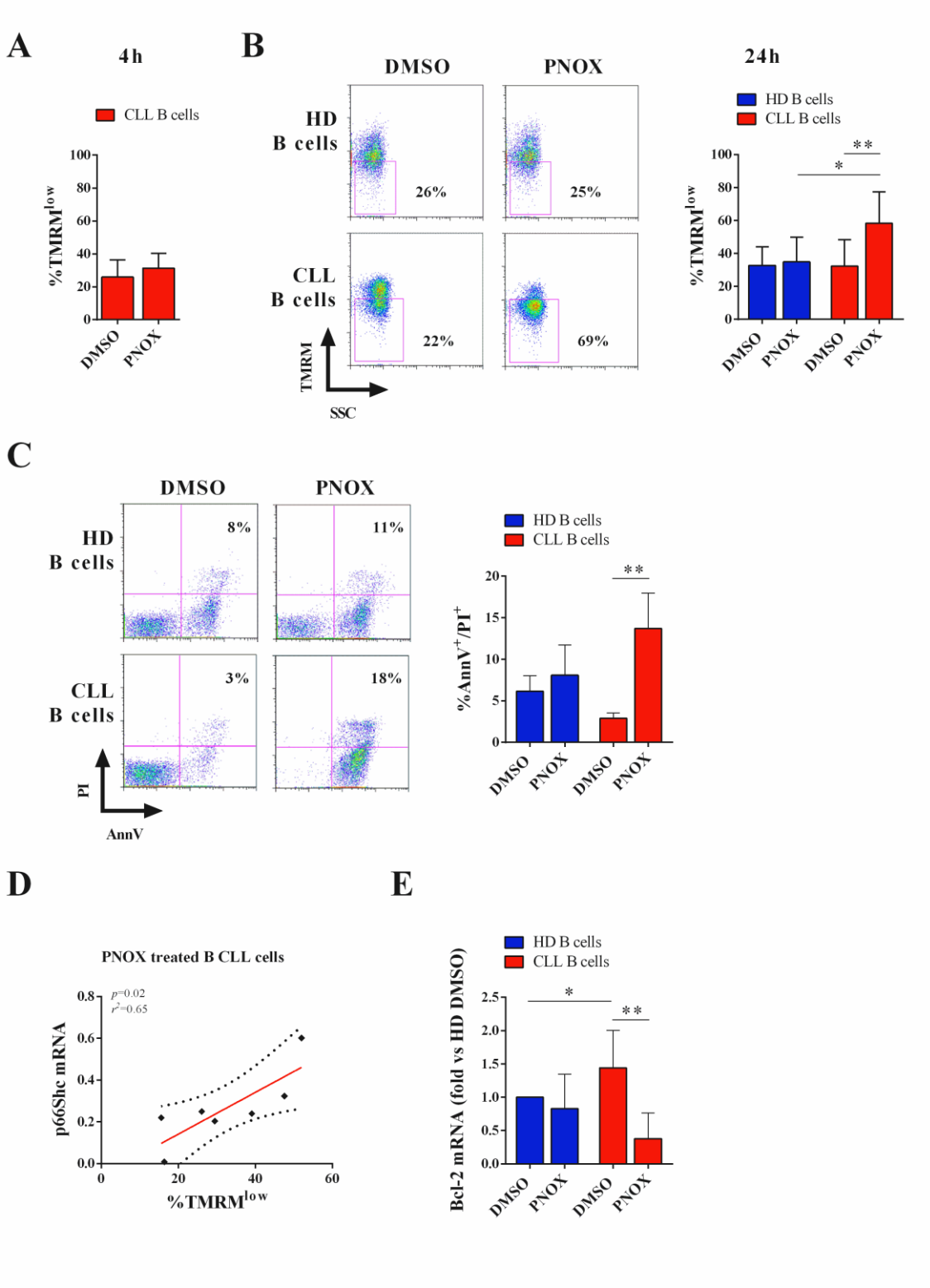

Figure 5. **PNOX-dependent loss of mitochondrial membrane potential correlates with enhanced p66Shc expression in CLL B cells**

Flow cytometric analysis of mitochondrial membrane potential in TMRM-loaded HD B cells or CLL B cells treated for 4h (A) or 24h (B) with DMSO or PNOX (10μM). The histograms show the percentage of cells with depolarized mitochondrial membrane ($TMRM^{low}$) with mean values ±SD represented in bars (B CLL cells (4h) =4; HD B cells (24h) =4; B CLL cells (24h) =12). (C) HD B cells or CLL B cells were treated for 24h with

DMSO or PNOX (10μM) and stained for Annexin V and Propidium Iodide. The histogram represents the percentage of $AnnV^+/PI^+$ late apoptotic cells and bars show mean values ±SD. (D) Correlation between mRNA levels of p66Shc and frequency of $TMRM^{low}$ (%$TMRM^{low}$) in B cells from CLL patients treated with PNOX (10μM) for 24h. (E) Quantification by qRT-PCR analysis of Bcl-2 mRNA expression in HD and CLL B cells treated with DMSO or PNOX (10μM) for 24h. The levels of the transcript were determined using the ΔΔCt method and normalized to the levels of the housekeeping gene 18s. Mean values ±SD with a DMSO sample taken as 1 is shown in bars of the histogram (HD B cells=3; CLL B cells=5). Two way ANOVA with post-hoc Sidak's test (B, C, D) and linear regression (C). *$p \leq 0.05$, **$p \leq 0.01$.

To unambiguously establish the importance of p66Shc on PNOX-dependent apoptosis in human B cells we generated a Burkitt lymphoma-derived B cell line (BJAB) (Menezes *et al.*, 1975), where p66Shc gene was silenced by CRISPR/Cas9 system (BJAB KO) (Fig 6A). Dissipation of mitochondrial membrane potential and cytochrome-c release were measured in these cells following treatment with PNOX and compared with control BJAB. p66Shc deficiency in B cells significantly abrogated the ability of PNOX to promote mitochondria-mediated apoptosis as witnessed by the reduced frequency of cells with depolarized mitochondria and reduced release of cytochrome-c in BJAB KO cells treated with PNOX compared with control BJAB (Fig 6B and C).

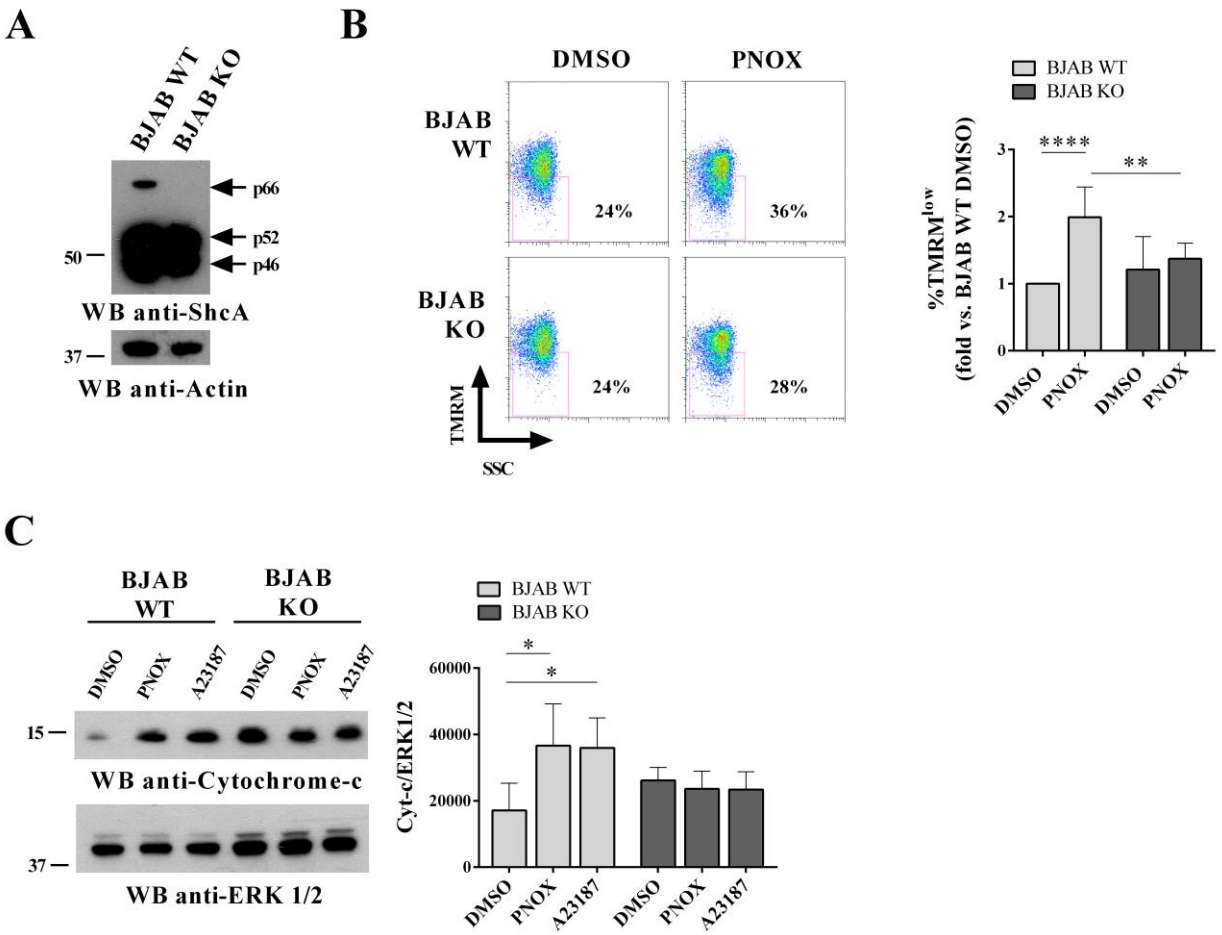

Figure 6. **PNOX requires p66Shc to induce a mitochondrial-mediated apoptosis in B cells**

(A) Representative immunoblot anti-ShcA of post-nuclear supernatants from Burkitt lymphoma-derived B cell line (BJAB) expressing endogenous p66Shc (BJAB WT), and from a BJAB clone where p66Shc gene was silenced with CRISPR-Cas9 mutagenesis (BJAB KO). (B) Mitochondrial membrane potential was assessed by flow cytometry in TMRM-loaded BJAB WT and BJAB KO treated with DMSO or PNOX (10μM) for 24h. The histogram shows the percentage of cells with depolarized mitochondria ($TMRM^{low}$) with BJAB WT DMSO taken as 1. Values are represented with bars showing mean values ±SD (n=5). (C) Immunoblot analysis of cytochrome-c performed on post-mitochondrial supernatants of lysates from BJAB WT and BJAB KO treated for 4h with DMSO or PNOX (10μM). Ionophore A23187 (500 ng/ml) was used as positive control. A control blot of ERK1/2 was shown. The histogram shows the quantification by densitometric analysis of the levels of cytochrome-c relative to ERK1/2. Data are presented as mean values ±SD (n=4). Two way ANOVA with post-hoc Sidak test (B, C). *p≤0.05, **p≤0.01, ***p≤0.001

4. JNK-dependent Serine 36 phosphorylation of p66Shc is required for the pro-apoptotic effects of PNOX

The pro-apoptotic activity of p66Shc has been ascribed to its ability to elevate mitochondrial ROS in response to oxidative stress conditions which results in the impairment of mitochondrial integrity and

cytochrome-c release (Giorgio *et al.*, 2005). This function was mapped on the Serine 36 (S36) residue in the CH2 domain of p66Shc, whose phosphorylation promote p66Shc transportation to the mitochondria (Migliaccio *et al.*, 1999; Pinton *et al.*, 2007). Khalid and colleagues (Khalid *et al.*, 2016) identified in c-Jun N-terminal kinase (JNK) the kinase responsible for the phosphorylation of S36 residue on p66Shc. Absence or chemical inhibition of JNK results indeed in a protective effect toward oxidative stress and in impaired phosphorylation of S36 on p66Shc. Here we found that the ability of PNOX to induce mitochondrial-mediated apoptosis in CLL B cells correlates with its ability to elevate p66Shc expression through the activation of STAT4 transcription factor supporting the notion that p66Shc is required for the pro-apoptotic effect of PNOX. Moreover, we demonstrated that PNOX specifically induced JNK activation in CLL B cells (Fig 4C). We therefore asked whether PNOX, beside promoting p66Shc expression, might directly impact p66Shc function through JNK-mediated phosphorylation of the S36 residue. To this end we used as a model of human B cells expressing high levels of p66Shc, the BJAB cell line (Menezes *et al.*, 1975), and quantitated the specific phosphorylation of the S36 residue on p66Shc. Immunoblot analysis with antibody specific for the phosphorylated S36 residue of p66Shc was performed on total cell lysates of BJAB cells treated with PNOX and compared with cells treated with vehicle. Our results showed that PNOX treatment induced a specific phosphorylation of S36 on p66Shc (Fig 7A). Pre-treatment of BJAB cells with the JNK specific inhibitor, SP600125, prior to PNOX treatment resulted in the inhibition of S36 phosphorylation on p66Shc and in the abrogation of its pro-apoptotic activity (fig 7B and C).

Collectively these data suggest that the pro-apoptotic effects of PNOX on leukemic B cells are associated with its STAT4/p66Shc elevating activity and with its ability to foster p66Shc pro-apoptotic function once p66Shc expression was rescued. Moreover, our data demonstrated that both these effects were dependent on PNOX-induced JNK activation in leukemic cells but not in normal cells.

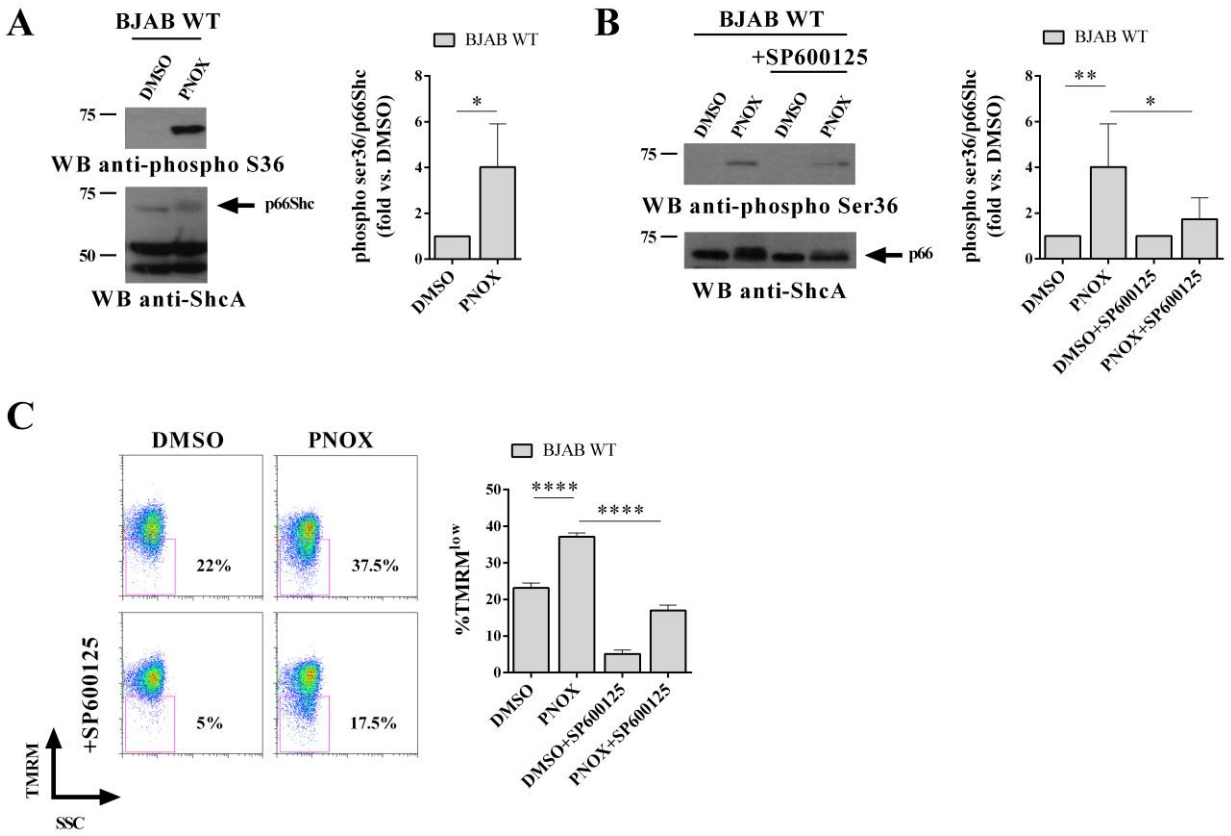

Figure 7. **PNOX promotes p66Shc pro-apoptotic function through a JNK-dependent Ser36 phosphorylation**

(A) 30x10^6 BJAB WT were treated for 24h with DMSO or PNOX (10μM) and total cell lysates resolved by SDS-PAGE and transferred on nitrocellulose blotting membrane. Immunoblot was performed using an antibody specific for the phosphorylated S36 residue of p66Shc. The levels of phosphorylated p66Shc on S36 residue relative to p66Shc were quantitated by densitometric analysis. Data are presented as mean values ±SD (n=4) in the histogram. (B) Representative blot of S36 phosphorylation on p66Shc of total cell lysates of BJAB WT cells treated with DMSO or PNOX (10μM) for 24h in presence or absence of the JNK specific inhibitor SP600125 20 μM. The histogram shows the quantification by densitometric analysis of the levels of S36 phosphorylation on p66Shc relative to p66Shc. Data are presented as mean values ±SD (n=4). (C) Mitochondrial membrane potential measured by flow cytometry in TMRM-loaded BJAB WT cells. The histogram shows the percentage of cells with depolarized mitochondria (TMRM^{low}). Data are presented as mean values ±SD (n=3). Unpaired t test (A) and one way ANOVA with Holm-Sidak's multiple comparison test (B, C). *p≤0.05, **p≤0.01, ****p≤0.0001

5. PNOXs improve survival and reduce tumor burden in Eμ-TCL1 mice

Similar to human B-CLL cells, leukemic cells isolated from Eμ-TCL1 mice with overt leukemia have a reduced expression of p66Shc compared with control mice or with Eμ-TCL1 mice with milder disease (Patrussi *et al.*, 2019). We therefore assessed whether PNOXs compounds, PNOX and a novel analogue, PNOX-3, restored p66Shc expression and promoted apoptosis in leukemic Eμ-TCL1 cells.

In agreement with the data obtained in human CLL B cells, *in vitro* treatment of leukemic Eμ-TCL1 cells with PNOX and PNOX-3 for 24h resulted in a significant increase in p66Shc expression and in enhanced mitochondria-mediated apoptosis compared to vehicle-treated samples (Fig 8A and B). No differences were found between the proapoptotic activity of PNOX and PNOX-3 in leukemic Eμ-TCL1 cells.

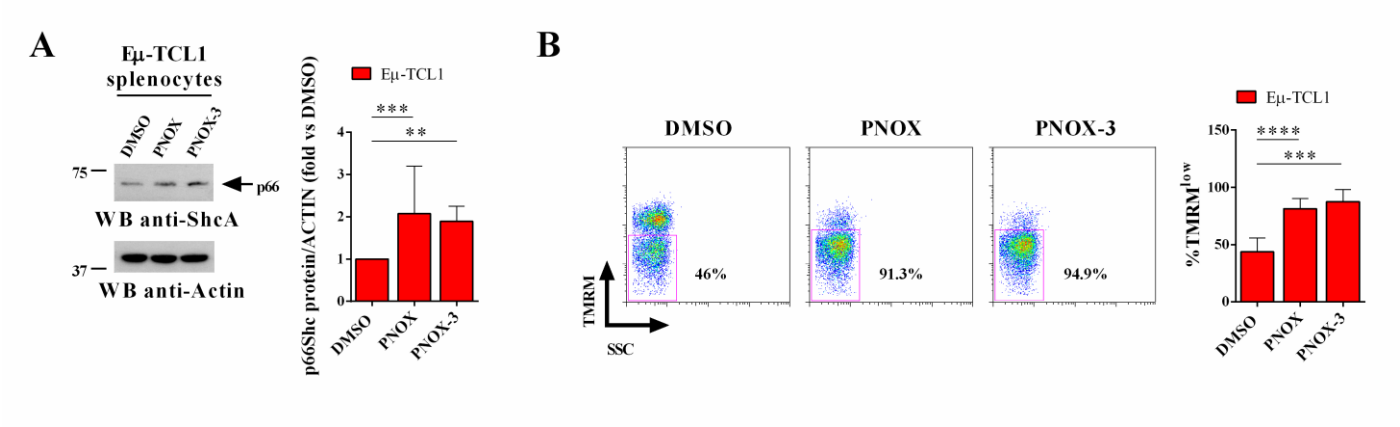

Figure 8. **PNOX and PNOX-3 recover p66Shc expression and promote mitochondrial-mediated apoptosis in leukemic cells from Eμ-TCL1 mice with overt leukemia**
Splenocytes were isolated from Eμ-TCL1 mice with overt leukemia and treated with DMSO, PNOX (10μM) or PNOX-3 (10μM) for 24h. (A) p66Shc expression was assessed by immunoblot analysis with anti-ShcA antibodies. Actin was used as loading control. Histogram shows the quantification by densitometric analysis of the levels of p66Shc relative to actin. Data are presented as mean values ±SD where DMSO is taken as 1 (n=5). (B) Quantification of TMRM fluorescence by flow cytometry in TMRM-loaded Eμ-TCL1 splenocytes treated as above. A representative dot plot is shown. Histogram shows the percentage of cells with depolarized mitochondria ($TMRM^{low}$) as mean values ±SD (n=5). One way ANOVA with post-hoc Bonferroni's test (A, B). **p≤0.01 ***p≤0.001 ****p≤0.0001

Encouraging results obtained on murine leukemic cells *in vitro* lead us to pre-clinically validate PNOXs anti tumoral activity *in vivo* using the Eμ-TCL1 mouse model for human CLL. It has been shown that the response of Eμ-TCL1 mice to treatment with compounds targeting key molecular processes involved in CLL pathogenesis resemble the response that is observed in human patients, and this inevitably includes off-target effects, relapse of disease or development of chemoresistance, as reported for fludarabine, inhibitors of BCR signaling and combinational therapies. Moreover,

similarly to human patients, treatment periods with those agents need to be prolonged and treatment doses are high, ranging from 25 mg/kg to 100 mg/kg per mice (Johnson *et al.*, 2006; Suljagic *et al.*, 2010 -b-; Ponader *et al.*, 2012; Niemann *et al.*, 2017; Patel *et al.*, 2018). Therefore, identification of novel compounds which effectively and safely reduce disease of Eμ-TCL1 mice, make them good candidates for CLL therapy in human patients (Bresin *et al.*, 2016; Jamroziak, Puła and Walewski, 2017).

In this context, results obtained *in vitro* on both human and murine CLL cells evidenced for PNOXs a selective and potent cytotoxic activity towards leukemic cells over normal B cells, even at lower concentration compared with other MTA. Hence, we decided to assess their therapeutic efficacy in Eμ-TCL1 mice. Mice ≥10mo of age that had developed spontaneous leukemia (≥20% $CD19^{+}CD5^{low}$ in peripheral blood cells) were selected (Suljagic *et al.*, 2010 -b-) and divided into three groups (Fig 9A). Mice were administered intraperitoneally with 7.5 mg/Kg of PNOX (4 mice), PNOX-3 (4 mice) or with PBS (4 mice) for three days consecutively as previously described (Bright *et al.*, 2010) and followed for a total of 30 days according to the scheme shown in figure 9B. Mice were checked daily by the operators to ensure that no mouse showed any form of distress such as failing to groom, lethargy, labored breathing, or evident signs of progressive disease. No evident side effect associated to PNOXs administration was observed, demonstrating a good tolerability of treatment. After 30 days of mice follow up, the first result that we obtained was the significant extended survival of those mice treated with PNOX and PNOX-3 compared to the PBS group (Fig 9C). Three out of the four mice from the PBS group were euthanized at day 14, 20 and 21 as they showed evident signs of the disease, while only one mouse of the PNOX group was euthanized at day 21.

Disease progression in mice was monitored by assessing $CD19^{+}CD5^{low}$ cell percentage in blood, before PNOX and PNOX-3 administration on day 10 (pre-treatment), on day 20 and 30, 8 and 15 days after the first treatment respectively. A significant reduction of leukemic cells frequency was found in blood of mice treated with PNOX and PNOX-3 on day 20 compared with the same mice

before the treatment, indicating that both compounds strongly reduced leukemic cells in peripheral blood following 8 days of treatment (Fig 9D).

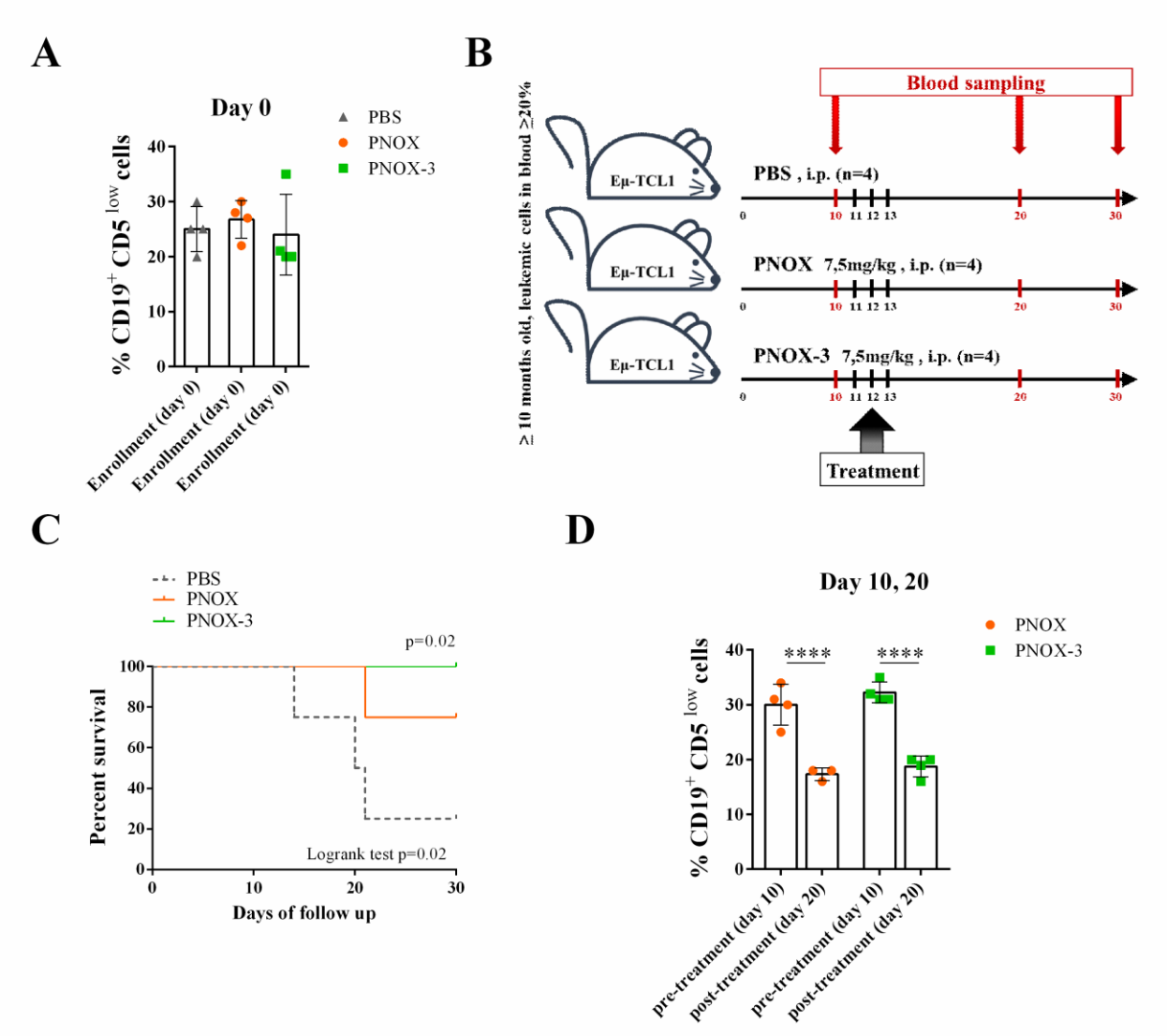

Figure 9. **PNOXs treatment *in vivo* promotes Eμ-TCL1 mice survival and modulates leukemic cells percentage in peripheral blood**

(A) Frequency of $CD19^{+}CD5^{low}$ cells in peripheral blood of mice at day 0 selected for the *in vivo* treatment according to the scheme shown in panel (B). Mice were injected i.p. with PNOX (7.5mg/kg), PNOX-3 (7.5mg/kg) or PBS on day 11, 12 and 13. On day 0, 10, 20 and 30 peripheral blood samples were collected and mice were sacrificed on day 30. (C) Log-rank survival analysis of the Eμ-TCL1 mice treated as in B. (D) Frequency of leukemic cells ($CD19^{+}CD5^{low}$) measured by flow cytometry on day 10 and 20 respectively before the treatment and 8 days post-treatment. Data are presented as mean values ±SD in the scatter plot. Logrank (Mantel-Cox) test (C), two way ANOVA with post-hoc Bonferroni's test (D). ****$p \leq 0.0001$

On day 30 (15 days after the first treatment) mice were euthanized and peripheral blood, spleen and peritoneal wash were collected and analyzed to evaluate CLL progression. PNOXs treatment resulted

in a macroscopical reduction of the spleen size corresponding to a significant reduction in its weight (Fig 10A). Moreover, when compared to PBS-treated mice, spleens from PNOX and PNOX-3 mice showed reduced expansion of $CD19^{+}CD5^{low}$ leukemic cells population (Fig 10B). On the contrary, at day 30, leukemic cells frequency in peripheral blood of mice treated with PNOX and PNOX-3 was found increased when compared to the same mice at day 20, suggesting a non-lasting effect of PNOXs treatment or even a disease relapse over time (Fig 10C). Considering that early development of CLL starts from the peritoneum (Simonetti *et al.*, 2014), we decided to evaluate whether the expansion of leukemic cells in blood was concomitant with an enhanced frequency of leukemic cells in the peritoneum. Analysis of $CD19^{+}CD5^{low}$ frequency in peritoneum revealed a significant decrease of leukemic cells in mice treated with both PNOX and PNOX-3 compared with mice of the control group suggesting that PNOXs effectively counteract the development of the disease (Fig 10D). Leukemic cell accumulation within secondary lymphoid organs (SLOs) is one of the main clinical features of both human CLL and its mouse model, the Eμ-TCL1 mice. Within secondary lymphoid organs the microenvironment not only promotes enhanced survival and proliferation of leukemic cells, but also provides a protective microenvironment towards chemotherapy intervention (Efanov *et al.*, 2010; Bresin *et al.*, 2016). Massive migration of leukemic cells towards SLOs, depends upon an altered expression of chemokine receptors on cells surface, shifted towards an overexpression of homing receptors as CCR7, CXCR4, CXCR3, CXCR2 and CXCR5 and concomitantly a reduced expression of egress ones such as S1PR1 (López-Giral *et al.*, 2004). For this reason, we asked whether the increased leukemic cells percentage observed at day 30 in blood, concurrent with the strong reduction observed at the same time in the spleen, was dependent upon a PNOX-dependent restoration in the balance of homing and egress receptors on leukemic cells, thereby driving the egress of lymphocytes from the spleen. S1PR1 is an egress receptor whose presence in human CLL cells was found to be impaired both in peripheral blood and in SLOs and directly correlated with p66Shc expression (Capitani *et al.*, 2012; Patrussi *et al.*, 2015, 2019). Hence, given the positive effect of PNOX and PNOX-3 on p66Shc restoration in CLL B cells, we decided to evaluate the impact of

PNOX and PNOX-3 on S1PR1 expression. We found that splenocytes isolated from PNOXs treated mice showed a significant upregulation of S1PR1 mRNA levels compared with splenocytes isolated from PBS-treated mice (Fig 10E) indicating that the reduced spleen weight and enhanced lymphocytosis observed in PNOXs-treated mice at day 30 depends at least in part on the ability of both compounds to mobilize CLL cells from the spleen into the blood by modulating S1PR1 expression.

CXCR4 is a surface receptor abundantly expressed on CLL B cells which promotes leukemic cells chemotaxis towards its specific ligands CXCL12 and SDF-1α, produced by stromal cells in bone marrow (Burger, Burger and Kipps, 1999). p66Shc expression *in vivo* does not affect CXCR4 splenocytes levels, but is more likely to affect functionality of this homing receptor by negatively regulating its signaling and recycling (Patrussi *et al.*, 2014, 2018, 2019). In agreement with these evidences, we did not found any effect on CXCR4 mRNA levels in splenocytes from mice undergoing PNOXs treatment (Fig 10E)

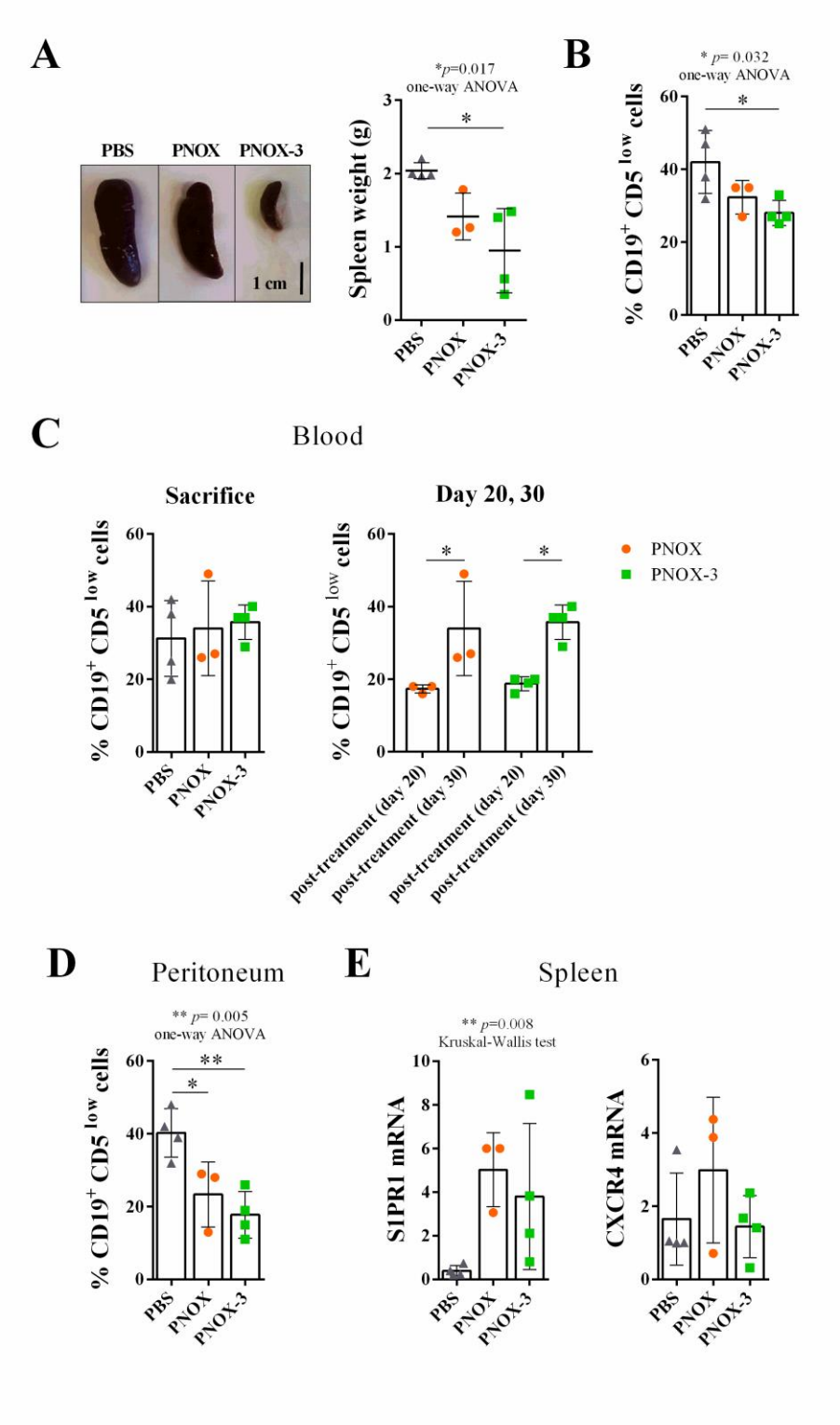

Figure 10. **PNOXs reduce tumor burden in spleen and peritoneal cavity of Eμ-TCL1 mice and promotes mobilization of leukemic cells from the spleen into the blood**

Analysis of spleen weight (A) and of the frequency of leukemic cells (% $CD19^{+}CD5^{low}$) in the spleen (B), blood (C) and peritoneal cavity (D) of Eμ-TCL1 mice at sacrifice. Data are presented as scatter plot of the single data and mean values ±SD within each group. (E) Quantification by qRT-PCR analysis of S1PR1 and CXCR4 mRNA levels in total splenocytes isolated from treated Eμ-TCL1 mice. The levels of S1PR1 and CXCR4 transcripts were determined using the ΔΔCt method and normalized to the levels of GADPH. Graph

shows scatter plot of single data and bar representing mean ± SD for each group. One way ANOVA with post-hoc Bonferroni's test (A, B, C, D), Kruskal-Wallis test (E). *p≤0.05, **p≤0.01

6. PNOX-3, but not PNOX, promotes apoptosis independently of p66Shc and inhibits expansion of leukemic cells in blood in the aggressive CLL Eμ-TCL1p66Shc$^{-/-}$ mouse model

Results obtained from *in vivo* testing of PNOX and PNOX-3 in Eμ-TCL1 mice showed a p66Shc elevating activity (Fig 8A) and a promising anti leukemic effect *in vivo* for both compounds, but a slightly more potent activity was observed for PNOX-3 especially in promoting survival and reducing weight and leukemic cell accumulation in the spleen (Fig 9C, 10A and 10B). To further assess the potential of PNOX and PNOX-3 in efficiently abrogating the expansion of leukemic cells *in vivo* we analyzed their effects in the Eμ-TCL1p66Shc$^{-/-}$ mice, a mouse model recapitulating a more aggressive phenotype of human CLL. Eμ-TCL1p66Shc$^{-/-}$ mice (≥20% CD19^{+}CD5^{low} in peripheral blood cells) were selected. Mice were treated by i.p. injection with PNOX (n=4) or PNOX-3 (n=4) according to the scheme shown in figure 11A and followed for a total of 10 days. At the end of the treatment schedule, mice were euthanized and analysis of disease progression was conducted on peripheral blood, spleen and peritoneal cavity. Opposite to the mice treated with PNOX, a significant reduction of CD19^{+}CD5^{low} cells in peripheral blood of mice treated with PNOX-3 was found (Fig 11B). Accordingly, a significant reduction of the percentage of CD19^{+}CD5^{low} cells was observed in the spleen and in the peritoneum of PNOX-3 treated group compared with the mice treated with PNOX (Fig 11C) suggesting that PNOX-3 is strongly cytotoxic even against leukemic cells lacking p66Shc. Opposite to the results obtained in circulating leukemic cells, no significant differences were found in the spleen weight and in the S1PR1 mRNA levels in the spleen of Eμ-TCL1p66Shc$^{-/-}$ treated *in vivo* with PNOX-3 and compared with PNOX-treated mice (Fig 11D). Of note, our hypothesis that PNOX-3 might promote apoptosis in leukemic cells independently of its ability to rescue p66Shc expression is supported by the results obtained *in vitro* in splenocytes from Eμ-TCL1 and Eμ-TCL1p66Shc$^{-/-}$ mice treated for 24h with PNOX or PNOX-3. These results showed indeed that while

both compounds induced a comparable and significant increase of the percentage of leukemic Eμ-TCL1 cells with damaged mitochondria compared with vehicle treated control, only PNOX-3 significantly enhanced the percentage of Eμ-TCL1p66Shc$^{-/-}$ apoptotic cells compared with vehicle treated control (Fig 12).

Considering that S1PR1 expression is transcriptionally controlled by the p66Shc pro-oxidant activity (Capitani *et al.*, 2012) and that in the Eμ-TCL1 mice both compounds were able to upregulate p66Shc and S1PR1, the lack of effect found for both compounds on S1PR1 mRNA levels in Eμ-TCL1p66Shc$^{-/-}$ may be the result of their inability to rescue p66Shc because of the deletion of the gene in these mice. Taken together, these data indicate that while the PNOXs-dependent reduction of the tumor burden in the spleen relies on the ability of these compounds to restore the p66Shc/S1PR1 axis, the cytotoxic activity is p66Shc-dependent for PNOX and p66Shc-independent for PNOX-3.

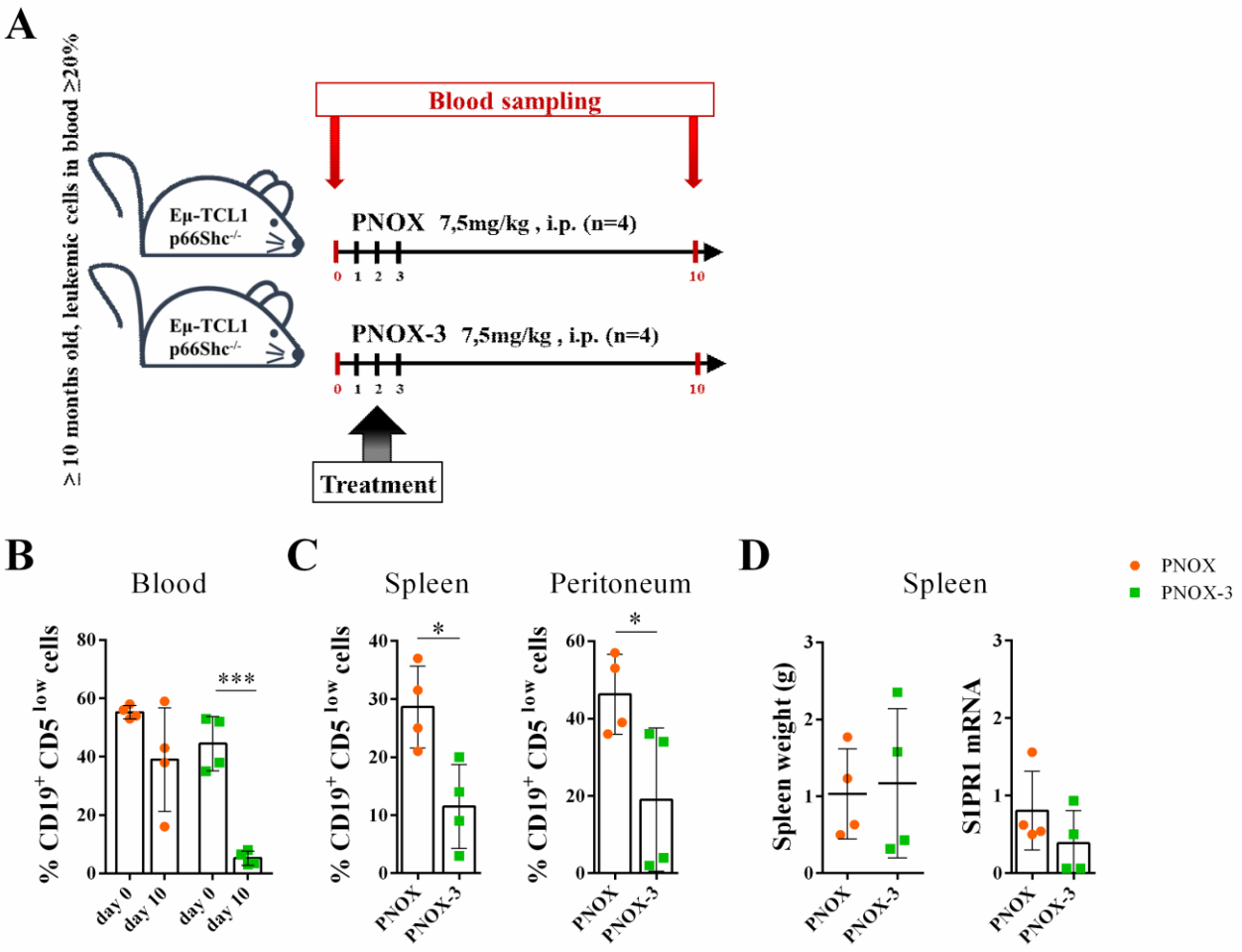

Figure 11. **The anti-leukemic activity of PNOX-3 is independent from p66Shc presence**
(A) Scheme of the experiment. Eμ-TCL1p66Shc$^{-/-}$ mice (≥ 10 months of age with a percentage of leukemic cells in blood ≥ 20%) were divided in two groups on day 0 and treated i.p. with PNOX (7.5mg/kg) or PNOX-3 (7.5mg/kg) on day 1, 2 and 3. On day 0 and 10 peripheral blood samples were collected and mice were sacrificed on day 10. At sacrifice leukemic cells frequency (CD19^{+}CD5^{low}) was measured by flow cytometry in peripheral blood and compared with day 0 (B), in the spleen and in the peritoneal cavity (C). Analysis of spleen weight and of S1PR1 mRNA levels in splenocytes are shown (D). The levels of S1PR1 transcripts was determined using the ΔΔCt method and normalized to the levels of GADPH. Data are presented as scatter plot of the single data and mean values ±SD within each group. Two way ANOVA with post-hoc Sidak's test (B, C), unpaired t test (D). *p≤0.05, ***p≤0.001

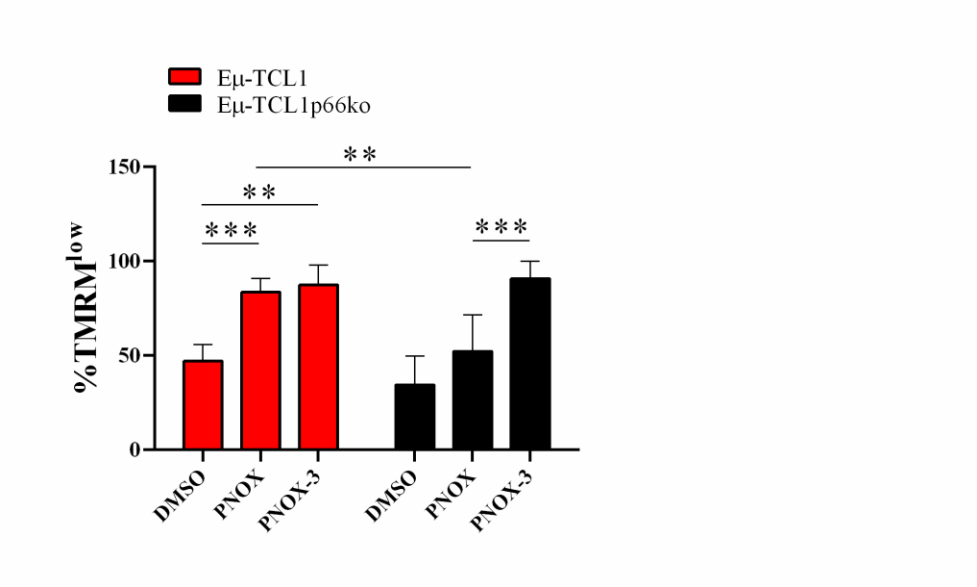

Figure 12. **PNOX-3 promotes apoptosis in splenocytes purified from Eμ-TCL1p66Shc$^{-/-}$ mice**
Quantification of TMRM fluorescence by flow cytometry in TMRM-loaded Eμ-TCL1 and Eμ-TCL1p66Shc$^{-/-}$ splenocytes treated with PNOX (10μM), PNOX-3 (10μM) or DMSO for 24h. Histogram shows the percentage of cells with depolarized mitochondria (TMRM^{low}) as mean values ±SD (Eμ-TCL1=6; Eμ-TCL1p66Shc$^{-/-}$=8). Two way ANOVA with post-hoc Sidak's test. **p≤0.01, ***p≤0.001.

DISCUSSION

Chronic lymphocytic leukemia onset and development account mainly on dysfunctions in the apoptotic mechanism within B lymphocytes which are arrested in G_0/G_1 phase of the cell cycle and accumulate as mature, monoclonal $CD5^+CD19^+$ B cells in peripheral blood, bone marrow and lymphoid organs (Awan and Byrd, 2020; Pekarsky and Croce, 2015). A small percentage of leukemic cells can undergo active proliferation in "pseudofollicles" within the SLOs through stimuli received from bystander cells (Ten Hacken and Burger, 2016). Stimuli coming from the protective microenvironment of SLOs together with the intrinsic defects in apoptotic machinery and constitutive BCR signaling are moreover responsible for the extended survival and retention of leukemic cells within SLOs (Capitani and Baldari, 2010; Ten Hacken and Burger, 2016).

Coordination of these intrinsic and extrinsic features that protect CLL cells from apoptosis and prolong their lifespan has not been attributed to any master regulator by now, but the molecular adaptor protein p66Shc could represent a good candidate. As many adaptor proteins, p66Shc was found to finely tune many cellular processes including antigen-dependent activation and proliferation of T and B lymphocytes, apoptotic responses to oxidative stress and ROS production as well as cell migration and adhesion (Ravichandran, 2001; Finetti, Savino and Baldari, 2009; Bhat, Anand and Khanday, 2015). In B lymphocytes, p66Shc negatively regulates chemotactic responses associated to CXCR4 and CXCR5 receptors by interfering with the reorganization of the actin cytoskeleton (Patrussi *et al.*, 2014, 2018) and negatively regulates the signaling initiated by B cell receptor triggering. On the other hand p66Shc positively regulates B lymphocytes apoptosis through its pro-oxidant activity and by altering the balance between the pro-apoptotic and the anti-apoptotic members of the Bcl-2 family of proteins (Finetti *et al.*, 2008; Capitani *et al.*, 2010). Accordingly, p66Shc expression in CLL B cells was found to be impaired compared with normal B cells and this deficiency was linked to the upregulation of the homing receptor CCR7 and the concomitant downregulation of the egress receptor S1PR1 thereby accounting for the accumulation of leukemic cells within the

tumor-supporting niches located in the SLOs and for chemoresistance of CLL cells (Capitani *et al.*, 2012). Moreover p66Shc modulates CXCR4 and CCR7 signaling and surface recycling (Patrussi *et al.*, 2014). The pathological impact of p66Shc deficiency in CLL progression was recently confirmed in the mouse model Eμ-TCL1p66Shc$^{-/-}$, where deletion of *p66Shc* gene in B cells results in accelerated leukemogenesis and enhanced disease aggressivity (Patrussi *et al.*, 2019).

The above mentioned evidences together with the demonstration that reconstitution of p66Shc in human CLL B cells *in vitro* is able to revert some of their abnormalities (Capitani *et al.*, 2010) underscore therapeutic potential of p66Shc reconstitution. Moreover, differently from other molecules altered in CLL and currently target of novel pharmacological therapies (Bcl-2, BTK), whose altered levels or activation are not common to all patients, p66Shc levels were severely impaired in B cells from all CLL patients compared with normal B cells, even if at different extent in B cells from CLL patients depending on indolent or aggressive phenotype of the disease (Capitani *et al.*, 2010; Kikushige, 2020; Patrussi, Capitani and Baldari, 2020). Histone deacetylation and cytosine methylation in CpG rich region within the p66Shc promoter is the principal mechanism governing low p66Shc expression in lymphocytes (Ventura *et al.*, 2002; Pezzicoli *et al.*, 2006). However, in CLL B cells, promoter methylation does not account for p66Shc impaired expression but rather it is the result of the defective expression of the transcription factor STAT4, which has been recently identified as the specific transcription factor for p66Shc (Cattaneo *et al.*, 2016). Recover of STAT4 expression has been shown to have a positive impact on p66Shc expression, evidencing a loop of reciprocal induction between the two molecules (Cattaneo *et al.*, 2016). Therapies aimed at upregulating p66Shc should therefore reconstitute also STAT4 expression and activation to establish a positive feedback loop. At present, ibrutinib treatment of CLL cells *in vitro* has been shown to promote STAT4 expression, leading to partial recovery of p66Shc expression with a still unknown molecular mechanism (Patrussi *et al.*, 2019).

In the present study we have deepen the mechanism responsible for the anti tumoral efficacy of PNOXs compounds on CLL cells and discovered that their ability to induce apoptosis in CLL cells

relies, at least in part, on a positive effect on the JNK/p66Shc/STAT4 axis. The compounds tested *in vitro* were able in 4h to activate residual STAT4 in CLL cells, with consequent increase in p66Shc levels observed after 24h of treatment. At the same time, prolonged treatment with the compounds positively affects also STAT4 levels, thus establishing the positive circuitry of reciprocal induction between p66Shc and STAT4, as previously suggested (Cattaneo *et al.*, 2016). Of note, our data demonstrated that the activation of this circuitry is operational exclusively in B lymphocytes from CLL patients and not in normal B cells, supporting a high specificity of PNOXs action in tumor cells. It has been shown that prolonged PNOX treatment in CLL for 24h causes microtubule disruption leading to JNK activation (McElligott *et al.*, 2009). Here we found that PNOX-dependent JNK activation occurs early in CLL B cells prior to cell cycle arrest and has two important consequences. First, the activation of STAT4 transcriptional activity by Ser721 phosphorylation resulting in the p66Shc recovery, second, once the expression of p66Shc is rescued, PNOX-dependent JNK activation impinges the pro-apoptotic activity of p66Shc through the phosphorylation of its S36 residue. In agreement with a key role played by p66Shc in the PNOX-dependent apoptosis in B cells, our data demonstrated that the apoptotic rate induced by PNOX treatment in leukemic B cells directly correlates with p66Shc expression levels and most importantly is abrogated when p66Shc is genetically deleted.

Treatment of the Eμ-TCL1 mice with PNOXs reduces the tumor burden in peritoneal cavity and spleen and exert a strong cytotoxic activity towards circulating leukemic cells suggesting that PNOXs are able to reach CLL cells in SLOs and overcome both their protective microenvironment, both chemoresistance of leukemic cells located here (Burger *et al.*, 2009; Kipps *et al.*, 2017). In addition, upregulation of the egress receptor S1PR1 observed in splenic leukemic cells of PNOXs treated mice, drives mobilization of CLL cells from SLOs to peripheral blood, depriving leukemic cells from their supportive tumor microenvironment and making them more susceptible to therapeutic intervention (Lampson and Brown, 2019). Lymphocytosis caused by redistribution of CLL cells from SLOs to peripheral blood is an early consequence of therapy with inhibitors of the BCR signaling pathway

too. However, nor BTK or SYK inhibitors have any consequence on S1PR1 receptor expression nut rather on CXCR4 surface expression and intracellular signaling (Chen *et al.*, 2016), while PI3K inhibitor idelalisib specifically upregulates S1PR1 thus suggesting a direct but still not elucidated role of PI3K in upregulating S1PR1 (Till, Pettitt and Slupsky, 2015). Here we show that the reduction of tumor burden in the spleen and the lymphocytosis observed after PNOXs treatment of Eμ-TCL1 mice is dependent upon transcriptional S1PR1 upregulation driven by p66Shc restoration. Accordingly, PNOXs treatment was unable to upregulate S1PR1 in Eμ-TCL1p66Shc$^{-/-}$ mice where p66Shc was genetically deleted.

Results obtained *in vitro* and *in vivo* lead us to hypothesize a possible mechanism of action for PNOXs in CLL B cells resumed in Figure 13. PNOXs-dependent JNK activation selectively in CLL cells leads to the activation of residual levels of STAT4, which in turn restores p66Shc expression in circulating leukemic cells thereby re-establishing the STAT4/p66Shc circuitry and promoting apoptosis. On the other side, our *in vivo* data demonstrated that PNOXs are able to reach and target CLL cells accumulated in the spleen and in the peritoneal cavity driving their mobilization into peripheral blood through the upregulation of S1PR1, where they can be forced to undergo apoptosis by PNOXs-mediated killing. Even though our data strongly suggest a beneficial effect of PNOXs in removal of leukemic cells from the peritoneal cavity and spleen, it would be interesting to test PNOXs efficacy after a cyclic administation, in order to trigger a virtuous circle of continuous mobilization and killing of circulating CLL cells and, not less important, to evaluate any long term treatment-associated toxic effects or occurrence of resistance.

Key point emerging from our study is the great therapeutic potential of PNOXs in CLL. Enclosed in a single agent, we evidenced two beneficial effects for CLL intervention: rescue of apoptosis in leukemic cells and mobilization from SLOs, both of them accounting for the positive effect of PNOXs on the JNK/p66Shc/STAT4 axis. Current agents approved for CLL therapy do not own both cytotoxic activity together with mobilization one, and frequently need to be combined together to obtain efficient therapies (Hallek, 2019; Kipps and Choi, 2019; Yosifov *et al.*, 2019). Moreover, it is reported

that the multifactorial pathogenesis of CLL makes difficult to identify correct agents to cure every patient, and presence of certain genetic lesions or mutations is responsible for the failure of therapies, together with occurrence of resistance over prolonged treatment (Parikh, 2018; Bosch and Dalla-Favera, 2019; Scheffold and Stilgenbauer, 2020). For this reason, identification of new therapeutic compounds for CLL is of great importance. Of note, PNOXs are efficient also in poor-prognosis subgroups of CLL patients. PNOXs has been proved to be efficient *in vitro* in promoting apoptosis of leukemic cells from patients with p53 mutations that cannot benefit from canonical cytostatic agents (McElligott *et al.*, 2009), and here we have provided evidence that PNOXs exert their pro-apoptotic activity and upregulate p66Shc independently from IGHV mutational status, suggesting that it would be a valid therapeutic option even in poor prognosis patients. The demonstration that treatment of Eμ-TCL1p66Shc$^{-/-}$ mice with the compound PNOX-3, which was developed to improve the ability of PNOX to bind tubulin (Brindisi *et al.*, 2019), efficiently reduced the leukemic cells in blood, spleen and peritoneal cavity but fails to upregulate S1PR1 levels in leukemic cells and to mobilize them from the spleen to peripheral blood further support the notion that PNOXs require p66Shc for their effective anti-tumor activity.

At present, despite the undeniable benefits of recently approved target therapies, mainly the BCR inhibitors ibrutinib and idelalisib and Bcl-2 inhibitor venetoclax, the clinical trials have identified great limitations in terms of acquired resistance and progression or, worse still, severe adverse effects, all of them associated to the long duration of these therapies (Scheffold and Stilgenbauer, 2020).

Off-target effects of BCR inhibition such as reduced homing or chemokine production in B leukemic cells have represented an additional advantage for these therapies, anyway, off-target effects of BCR inhibitors also impair T lymphocytes functionality (Seda and Mraz, 2015). In addition, normal B cells taken from ibrutinib-treated patients showed a weaker response to antigens, contributing to impair the already compromised immune response which characterizes the disease (Arruga *et al.*, 2020). On the contrary, PNOX demonstrated to have a safe profile on hematopoietic cells survival (McElligott *et al.*, 2009). At the same time, on T lymphocytes PNOX-dependent microtubule disruption is

correlated to inhibition of migration (Verma *et al.*, 2008), suggesting an interesting anti-inflammatory property of the compound whose impact in tumor context and in CLL could be positive as inflammation is involved in the initiation and progression of several chronic lymphoid malignancies of B-cell type (Caligaris-Cappio, 2011). Further studies addressing the off-target effect of PNOX in the Eμ-TCL1 mice, including that on T cells will be of extremely importance.

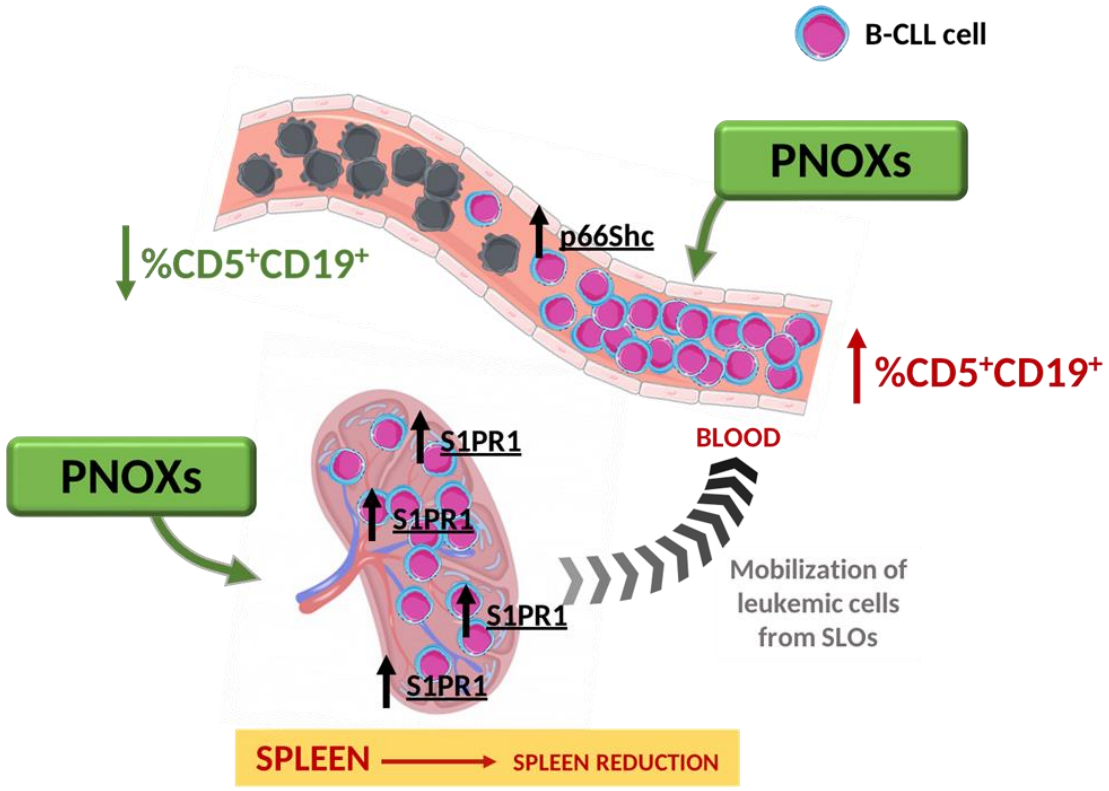

Figure 13. ***In vivo* mechanism of action of PNOXs**

Upregulation of p66Shc by PNOXs treatment causes apoptosis of circulating CLL cell, resulting in an early reduction of $CD5^+CD19^+$ leukemic cells percentage in PB. In spleen, PNOXs-dependent p66Shc recovery is associated to upregulation of the egress receptor S1PR1 in CLL cells, which are consequently forced to leave the spleen towards increasing gradients of S1P, the specific chemokine for S1PR1, which is localized in PB. This mechanism would explain the increased percentage of $CD5^+CD19^+$ leukemic cells observed in PB of Eμ-TCL1 mice *in vivo* treated with PNOXs. PB= peripheral blood.

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