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Targeting Signaling Pathways in T-cell acute lymphoblastic leukemia initiating cells

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A B S T R A C T

Leukemia initiating cells (LICs) represent a reservoir that is believed to drive relapse and resistance to chemotherapy in blood malignant disorders. T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive neoplastic disorder of immature hematopoietic precursors committed to the T-cell lineage. T-ALL comprises about 15% of pediatric and 25% of adult ALL cases and is prone to early relapse. Although the prognosis of T-ALL has improved especially in children due to the use of new intensified treatment protocols, the outcome of relapsed T-ALL cases is still poor. Putative LICs have been identified also in T-ALL. LICs are mostly quiescent and for this reason highly resistant to chemotherapy. Therefore, they evade treatment and give rise to disease relapse. At present great interest surrounds the development of targeted therapies against signaling networks aberrantly activated in LICs and important for their survival and drug-resistance. Both the Notch1 pathway and the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) network are involved in T-ALL LIC survival and drug-resistance and could be targeted by small molecules. Thus, Notch1 and PI3K/Akt/mTOR inhibitors are currently being developed for clinical use either as single agents or in combination with

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conventional chemotherapy for T-ALL patient treatment. In this review, we summarize the existing knowledge of the relevance of Notch1 and PI3K/Akt/mTOR signaling in T-ALL LICs and we examine the rationale for targeting these key signal transduction networks by means of selective pharmacological inhibitors.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive blood malignancy deriving from T-cell progenitors in the thymus. T-ALL accounts for 10–15% of pediatric and 25% of adult ALL cases (Ferrando et al., 2002). With the current intensified multi-agent chemotherapy protocols, the 5-year event-free survival (EFS) of children with T-ALL has reached 70–75%, whereas the EFS is 30–40% for adults below 60 years of age, and 10% above this age (Pui and Evans, 2006). However, these therapies are highly toxic. Moreover, relapsed patients often develop resistance to chemotherapy and have an extremely poor prognosis (Bhojwani and Pui, 2013).

It is therefore important to understand the mechanisms that cause relapses and chemoresistancy in T-ALL for identifying novel molecular targets and designing more effective therapies.

The original cancer stem cell (CSC) hypothesis implied that tumors are arranged in a developmental hierarchy reflecting heterogeneity at the cellular level. Within a given neoplasia, only a numerically-restricted subset of cells displays self-renewal capacity through asymmetric division, gives rise to all cell types found in the original tumor, and maintains the tumor long-term (Nguyen et al., 2012). Therefore, the CSC model entailed that CSCs possess features that mirror those of healthy stem cells, although in a deregulated manner. CSC lie dormant in the tumor niche microenvironment for long periods of time and they are therefore protected from cytotoxic agents that target proliferating cell populations (Crews and Jamieson, 2013). Thus, the existence of CSCs could explain why a wide variety of malignancies continue to relapse and develop resistance to standard therapies.

CSCs were first isolated in 1994 by Dick and coworkers (Lapidot et al., 1994) in acute myelogenous leukemia (AML) patients and named leukemia stem cells (LSCs). However, LSCs are now usually referred to as leukemia initiating cells (LICs). In AML, LICs constituted a rare cell subpopulation (on the order of 1 in 2.5×10^5 leukemic cells) that could be serially transplanted into immunocompromised mice and were comprised exclusively in the flow-sorted $CD34^+/CD38^-$ cell subset (Lapidot et al., 1994). By definition, LICs display at least two distinctive characteristics, i.e. the ability to self-renew and generate more LICs and the capacity to differentiate into a progeny with limited self-renewal potential. Putative LICs were subsequently identified in other hematological malignancies, including chronic myeloid leukemia (Eisterer et al., 2005), as well as both B- and T-ALL (Cox et al., 2004, 2007). However, our concept of LICs is rapidly evolving (Magee et al., 2012b). First of all, it has become clear that LIC activity can be endowed in phenotypically different cell subpopulations, even in the same patient (Gerby et al., 2011; Lemoli et al., 2009; Rehe et al., 2013; Taussig et al., 2010). Moreover, it has also become apparent that LICs could be much more numerous than initially thought, in some cases as high as 1 cell in 500 (Ailles et al., 1999). This could depend not only on the strain of immunocompromised mice used for the xenograft assay [nonobese diabetic/severe combined immunodeficiency (NOD/SCID) vs NOD/SCID/γ (NSG) mice, see (Chiu et al., 2010; Rehe et al., 2013)], but on other technical factors, including sample cryopreservation prior to xenoengraftment assay (Greystoke et al., 2013). More importantly, it has been documented that the LIC population displayed genomic heterogeneity in the same patient and evolved during B-ALL progression (Anderson et al., 2011; Notta et al., 2011), thus presenting a formidable challenge for the development of effective therapeutic strategies. Similar findings have been reported also in AML (Klco et al., 2014) and T-ALL (Clappier et al., 2011) patients.

Multiple reports have demonstrated that LIC initiation, propagation, and survival depend on the aberrant activation of self-renewal and pro-survival signaling pathways (Gachet et al., 2013; Luis et al., 2012; McCubrey et al., 2014). Here, we review the evidence which links Notch1 and phosphoinositide

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3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathways, two signaling cascades aberrantly activated in T-ALL patients (Hales et al., 2014), with T-ALL LIC initiation, propagation, and survival. We also highlight how therapeutic targeting of these two signaling networks, could be of benefit to T-ALL patients.

T-ALL LICs

For the scopes of this review, it is useful to briefly recapitulate the phenotypes of T-ALL LICs described so far. Cox et al. (2007) were the first to document the existence of putative LICs in newly diagnosed T-ALL patients. They reported that $CD34^+/CD4^-$ (5–10% of total leukemic cells) or $CD34^+/CD7^-$ cells (4–6% of total leukemic cells) were the only subsets capable of engraftment in NOD/SCID mice. These T-ALL cells successfully repopulated secondary and tertiary recipients with equivalent levels of engraftment, thus demonstrating self-renewal ability. The immunophenotype and genotype of the original leukemic cells were preserved with serial passages in the mice. No engraftment was observed with the $CD34^+/CD7^+$, $CD34^-/CD7^+$, or the $CD34^-/CD7^-$ subfractions, despite the inoculation in mice of a significantly higher numbers of cells (Cox et al., 2007). However, these conclusions were questioned by a subsequent study that could not confirm the existence of a strict correlation between the transplantation potential in NOD/SCID mice and the presence of $CD34^+/CD4^-$ or $CD34^+/CD7^-$ cells in T-ALL patient samples, even early after cell transplantation. These findings suggested that CD34 might not be a reliable and reproducible marker for T-ALL LICs in all patients (Armstrong et al., 2009). Intriguingly, when the phenotype of NOD/SCID–engrafted T-ALL cells was analyzed in secondary and tertiary transplants, it was observed a variable phenotype evolution that further questioned the use of cell surface markers for the identification of LICs (Armstrong et al., 2009).

Subsequently, the capacity to initiate leukemia in xenografts by the $CD7^+/CD1a^-$ subset of primary T-ALL samples was found to be superior to other subsets (Chiu et al., 2010). However, it this study the authors used NSG mice. It should be emphasized here that, besides displaying no activity of T- and B-cells, this strain of mice also lacks NK-cell function and show alterations in dendritic cell functions, due to the absence of interleukin (IL) 2Rγ (Ito et al., 2008). Therefore, NGS mice generate a faster and more efficient leukemic disease compared to NOD/SCID mice (Agliano et al., 2008). Also in the report by Chiu et al. (2010), it was shown that CD34 was not an universal marker for T-ALL LICs, although it was a reliable marker in some samples. Interestingly, $CD7^+/CD1a^-$ cells exhibited in vivo resistance to glucocorticoids, that are widely used for treating T-ALL patients (Inaba and Pui, 2010). It was also documented that $CD7^+/CD1a^+$ T-ALL cells recovered from primary xenografts proliferated in response to Notch1 activation and engrafted in secondary NSG recipients. Nevertheless, changes in leukemia immunophenotype in xenografts of some samples were observed. These results suggested that LIC function and immunophenotype could not be a fixed relationship in T-ALL and that there could be selective outgrowth of distinct cell subclones in the murine microenvironment where the genetic alterations they harbored resulted in a less rigid association between phenotypic markers and LIC activity (Chiu et al., 2010).

The importance of CD34 as a marker of LIC activity in T-ALL was nevertheless emphasized again in another study which documented that LIC activity could be detected in the $CD34^+/CD7^+$ cell subset, whereas $CD34^+/CD7^-$ cells mostly contained normal human progenitors and hematopoietic stem cells that differentiated into T- and B-lymphoid, as well as myeloid cells (Gerby et al., 2011). However, LIC activity was also detected, although to lower levels, in the $CD34^-/CD7^+$ cell subpopulation. In this study, both NOD/SCID and NSG mice were used. Intriguingly, $CD34^+/CD7^+$ cells proliferated more upon Notch1 activation than $CD34^+/CD7^-$ cells and were sensitive to both glucocorticoids and Notch inhibitors (Gerby et al., 2011).

In the most recent report, it was documented that in Notch1-mutated T-ALL samples, $CD34^+$ cells displayed greater engraftment and serial transplantation potential in recombination activating gene 2 $(RAG2)^{-/-}$ $\gamma c^{-/-}$ mice than their $CD34^-$ counterpart (Ma et al., 2012). It is worth mentioning here that, similarly to NSG mice, $RAG2^{-/-}$ $\gamma c^{-/-}$ mice lack B-, T-, and NK-cell functions (Sanchez et al., 2013). In contrast, both $CD34^+$ and $CD34^-$ subsets derived from wild type Notch1 samples harbored roughly equivalent engraftment potential, although at lower levels than Notch1-mutated samples and with lower serial transplantation capacity. Interestingly, a humanized anti-Notch1 monoclonal antibody

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reduced LIC survival and self-renewal in Notch1-mutated T-ALL LIC-engrafted mice and resulted in depletion of $CD34^+/CD2^+/CD7^+$ cells that harbored serial transplantation capacity (Ma et al., 2012).

The above-outlined discrepancies could well reflect differences among distinct molecular T-ALL subtypes. Nevertheless, these studies have disclosed both the complexity of LICs in human T-ALL and the key role played by Notch1 signaling in supporting the engraftment and outgrowth of LICs. In particular, the work by Chiu et al. (2010) was one of the first papers to clearly establish that different subclones of LICs existed in acute leukemias, and that they exhibited different levels of growth advantage when transplanted into xenografts.

Notch1 signaling

Notch signaling is an evolutionary conserved pathway responsible for the direct transduction of signals at the plasma membrane into changes in gene expression in the nucleus. Notch1 is one of a family comprising four types of transmembrane receptor proteins, composed of a N-terminal ligand-accessible Notch extracellular domain (NEC) and a C-terminal Notch transmembrane fragment (NTM) which comprises an extracellular stub, a transmembrane domain, and an intracellular domain (NCID) (Takebe et al., 2014). NEC and NTM are non-covalently linked via the heterodimerization (HD) domain (Koch and Radtke, 2011; Lobry et al., 2011) (Fig. 1). Notch proteins function as ligand-activated transcription factors and are involved in morphogenesis and development (Koch et al., 2013), as well as in cell differentiation and survival (Yashiro-Ohtani et al., 2010). Notch ligands include Delta-like-1, -3, and -4, as well as Jagged-1 and -2. A distinctive feature of Notch signaling is juxtacrine signaling between adjacent cells: That is, the signaling cascade is initiated by binding of a Notch ligand expressed on one cell to a Notch receptor on a neighboring cell (Fig. 1). Upon ligand binding to NEC, Notch receptors are subjected to conformational changes which sequentially trigger two proteolytic steps. The first step is executed by a member of the A Disintegrin And Metalloproteinase (ADAM) family of metalloproteases (Saftig and Reiss, 2011). This cleavage leads to the dissociation of NEC, and generates a membrane-associated Notch extracellular-truncated intermediate (NEXT) (Takebe et al., 2014). The second step, which involves the γ-secretase complex, results in the liberation of active NICD in the cytoplasm (Koch et al., 2013) (Fig. 1). NICD translocates to the nucleus where it engages transcriptional activators such as CBF1/Su(H)/Lag2 (CSL), mastermind-like (MAML), and p300, thereby activating the transcription of various target genes involved in cell cycle progression, cell differentiation, and apoptosis inhibition. These include c-myc, Hairy Enhancer of Split (HES) family proteins, HES-related with YRPW motif (HEY) family proteins, $p21^{cip1/waf1}$, $p27^{kip1}$, cyclin D1 and D3, p19/INK4d, and S-phase kinase-associated protein (SKP) 2 (Takebe et al., 2014) (Fig. 1). Of note, F-box/WD repeat-containing protein (FBXW), an E3 ubiquitin ligase that recognizes the proline/glutamic acid/serine/threonine-rich (PEST) domain of NCID, mediates the polyubiquitination and subsequent proteasomal degradation of NICD, thus terminating Notch1 signaling in the nucleus (Aster et al., 2008; Tosello and Ferrando, 2013).

The possible role of aberrantly activated Notch1 signaling in human malignant disorders was first unraveled in T-ALL, in which the very rare (found in less than 1% of patients) t(7;9)(q34;q34.3) chromosomal translocation that fuses the intracellular form of Notch1 to the T-cell receptor (TCR) β locus, was discovered (Reynolds et al., 1987). It was later found that the locus on chromosome 9 involved in the translocation is a homologue to the Drosophila Notch1 gene (Ellisen et al., 1991).

Subsequently, the key oncogenic role of activated Notch1 signaling in mouse was fully documented when 50% of irradiated mice reconstituted with bone marrow progenitors expressing activated (truncated) forms of Notch1 developed clonal hematopoietic tumors displaying an immature T-cell phenotype (Pear et al., 1996). The pathogenic role of Notch1 in murine T-ALL reflects its physiological functions during normal T-cell development, as Notch1 is a master regulator of T-cell lineage commitment and maturation (Yashiro-Ohtani et al., 2010). Indeed, in the thymus, αβ T-cells require continuous Notch1 signaling activation for their maturation to the DN3 stage of development (Wolfer et al., 2002). During this process, Notch1 controls the expression of several factors that are essential for T-cell development, including pre-TCRα (pre-TCRA), IL7Rα chain (IL7RA) (Ribeiro et al., 2013), and c-myc (Paganin and Ferrando, 2011). Both pre-TCRA signaling and Notch1 activity are required for growth and survival at the β-selection checkpoint (Ciofani and Zuniga-Pflucker, 2006), where Notch1 signaling

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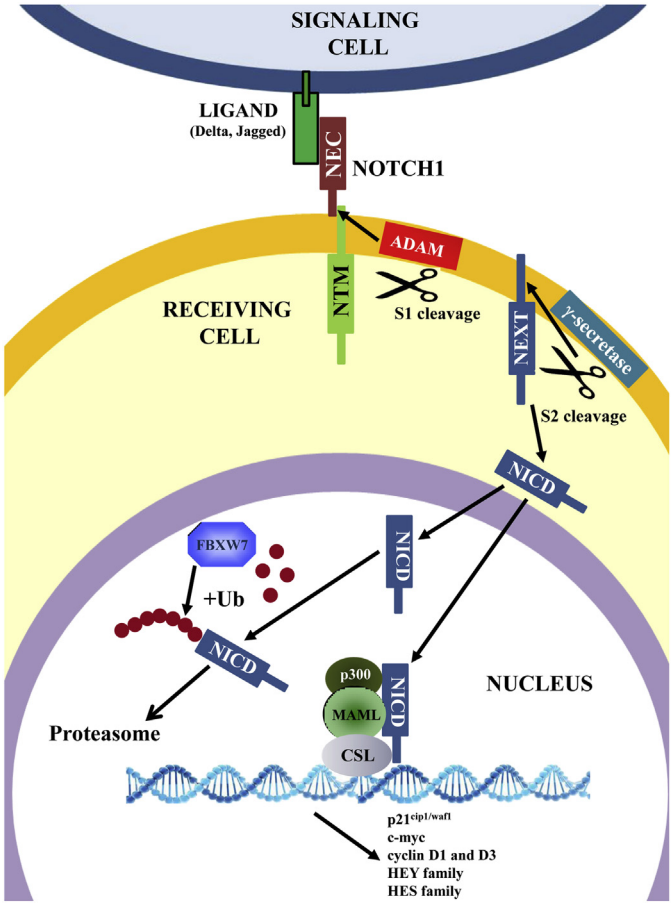

Fig. 1. Overview of Notch1 signaling. Notch1 is composed of a N-terminal ligand-accessible Notch extracellular domain (NEC) and a C-terminal Notch transmembrane fragment (NTM) which comprises an extracellular stub, a transmembrane domain, and an intracellular domain (NCID). Upon ligand binding to NEC, Notch receptors are subjected to conformational changes which sequentially trigger two proteolytic steps. The first step is executed by a member of the A Disintegrin And Metalloproteinase (ADAM) family of metalloproteases. This cleavage leads to NEC dissociation, and generates a membrane-associated Notch extracellular-truncated intermediate (NEXT). The second step, which involves the γ-secretase complex, results in the liberation of active NICD in the cytoplasm. NICD translocates to the nucleus where it engages transcriptional activators such as CBF1/Su(H)/Lag2 (CSL), mastermind-like (MAML), and p300, thereby activating the transcription of various target genes. These include c-myc, Hairy Enhancer of Split (HES) family proteins, HES-related with YRPW motif (HEY) family proteins, $p21^{cip1/waf1}$, cyclin D1 and D3. F-box/WD repeat-containing protein (FBXW) is an E3 ubiquitin ligase which mediates the polyubiquitination and subsequent proteasomal degradation of NICD, thus terminating Notch1 signaling in the nucleus. Ub: ubiquitin.

is critically needed to sustain cell metabolism via up-regulation of the PI3K/Akt/mTOR cascade (Ciofani and Zuniga-Pflucker, 2005).

Notch1 gain-of-function mutations were subsequently found in approximately 50–60% of T-ALL patients (Maillard et al., 2004). These mutations occur in the PEST domain of the NICD and/or in the HD domain to cause Notch1 constitutive activation via stabilization of NICD and/or ligand-independent generation of NICD (Koch and Radtke, 2011).

Moreover, about 15% of T-ALL patients harbor mutations in FBXW7 that alter the substrate recognition function of FBXW7 and impair the degradation of NICD (Gordon et al., 2009). Furthermore, about 20% of T-ALLs display either dual mutations in the PEST and HD domains of Notch1 or both a Notch1 HD domain allele and a FBXW7 mutation (Thompson et al., 2007; Weng et al., 2004). The combined effect

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of these mutations is an extremely high level of Notch1 signaling as a result of Notch1 ligand-independent activation at the plasma membrane plus defective NICD degradation.

However, available evidence suggests that the common Notch1 mutations found in human T-ALL patients are likely to be secondary events contributing to primary oncogenic hits to accelerate leukemia progression (Chiang et al., 2008), as they generally fail to induce leukemia in murine models (Rakowski et al., 2013). Nevertheless, even these weak Notch1 oncogenic signals have additional roles in development, progression, and/or maintenance of T-ALL, as pharmacological inhibitors of γ-secretase reduced tumor growth in vivo in mouse models of xenografted human T-ALL (Palomero et al., 2007). Indeed, Notch1 impacts on both PI3K/Akt/mTOR signaling (Hales et al., 2014) and c-myc levels (Sharma et al., 2006), that potently sustain human T-ALL cell growth. Accordingly, it has been documented that phosphatase and tensin deleted on chromosome 10 (PTEN) loss accelerated T-ALL onset and produced multiclonal tumors, suggesting Notch1 activation and PTEN loss may collaborate in T-ALL induction (Medyouf et al., 2010). The lipid phosphatase PTEN is a major negative regulator of PI3K/Akt/mTOR signaling cascade (see further on), as PTEN removes the 3-phosphate from phosphoinositide 3,4,5 trisphosphate (PIP3), thus down-regulating the pathway (Ortega-Molina and Serrano, 2013).

Another recently identified, possible collaborator of Notch1 in the development of T-ALL is Zinc Finger, MIZ-Type Containing 1 (ZMIZ1), a transcriptional co-activator of the protein inhibitor of activated STAT (PIAS)-like family (Lee et al., 2007), which is co-expressed with activated Notch1 in a subset of human T-ALL patients and cell lines (Rakowski et al., 2013). From a mechanistic point of view, it was documented that ZMIZ1 interacted with Notch1 to promote the transcription/activity of c-myc, but not of other well-established Notch1 target genes, including *HES1*. Importantly, ZMIZ1 inhibition slowed growth and sensitized T-ALL cells to corticosteroids and γ-secretase inhibitors (Rakowski et al., 2013).

Therefore, it has been conjectured that Notch1 works as a key hub enabling cross-talks among several oncogenic pathways (Capaccione and Pine, 2013).

PI3K/Akt/mTOR signaling

This signal transduction cascade controls a wide range of physiological cell processes, including proliferation, survival, differentiation, metabolism, autophagy, angiogenesis, exocytosis, and motility (Shanware et al., 2013). However, constitutively active PI3K/Akt/mTOR signaling is observed in many types of solid and blood tumors, where it portends a poorer prognosis and negatively influences response to therapeutic treatments (Rodon et al., 2013).

Stimulated by growth factors and/or cytokines, class I PI3Ks catalyze the production of the membrane phospholipid, PIP3, thereby activating a number of downstream targets, including the serine/threonine kinase Akt (Franke, 2008). Once activated, Akt phosphorylates multiple targets. Of note, most of the Akt effects depend on its ability to phosphorylate substrates involved in cell cycle progression, apoptosis, mRNA translation, glycolytic metabolism, and angiogenesis (Toker, 2012), thus unlocking most, if not all, of the critical processes involved in tumorigenesis (Hanahan and Weinberg, 2000). A key Akt target that is highly relevant in carcinogenesis and drug-resistance is mTOR. mTOR is a serine/threonine protein kinase that participates in two multi-protein complexes, referred to as mTOR complex 1 (mTORC1) and 2 (mTORC2), both of which phosphorylate several substrates (Laplante and Sabatini, 2012). The Raptor subunit is specific to mTORC1, whereas Rictor is a specific component of mTORC2 (Jhanwar-Uniyal et al., 2013). Akt activates mTORC1 through PRAS40 and tuberous sclerosis 1/2, while the mechanisms of activation of mTORC2 are still unclear, however they are dependent on PI3K/Akt signaling (Laplante and Sabatini, 2013).

mTORC1 is sensitive to rapamycin/rapalogs and supports tumor cells by stimulating the synthesis of proteins necessary for cell growth, survival, angiogenesis, nutrient uptake, and metabolism. Moreover, it represses autophagy (Laplante and Sabatini, 2013). mTORC2 controls cell survival, metabolism, and cytoskeletal organization by phosphorylating several AGC kinases, including Akt, serum/glucocorticoid-regulated kinase 1 (SGK1), and protein kinase C (PKC) α (Zoncu et al., 2011). It should be noted that prolonged (>24 h) exposure to high concentrations of rapamycin/rapalogs also inhibits mTORC2 activity, especially in cells of hematopoietic lineage (Sarbassov et al., 2006).

However, during normal T-cell development, PI3K/Akt/mTOR signaling is involved in thymocyte differentiation (Fayard et al., 2010). In particular, recent findings obtained in mice, have highlighted

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that Raptor deletion led to a developmental failure of DN2 and DN3 thymocytes, whereas when Rictor was deleted, double-positive ($CD4^+/CD8^+$) thymocytes failed to develop, indicating that mTORC2 controls later stages of differentiation than mTORC1 (Hoshii et al., 2014).

PI3K/Akt/mTOR signaling up-regulation is a very common feature in T-ALL, being detectable in 70–85% of pediatric patients (Silva et al., 2008), and portends a poorer prognosis (Jotta et al., 2010). Multiple mechanisms could lead to increased PI3K/Akt/mTOR activity in T-ALL cells, including PTEN gene inactivation through Notch1/HES1 signaling (Palomero et al., 2007), PTEN mRNA down-regulation by either miR-19 (Mavrakis et al., 2010) or c-myc (Gutierrez et al., 2011), mutations in PI3K/Akt/PTEN (Gutierrez et al., 2009; Trinquand et al., 2013), autocrine insulin-like growth factor (IGF)-1/IGF-1R signaling (Medyouf et al., 2011), gain-of-function IL7Rα mutations (Zenatti et al., 2011), and Notch1-dependent decreased activity of PP2A protein phosphatase on phosphorylated Akt (Hales et al., 2013). However, the most common reason for overactive PI3K/Akt/mTOR signaling in pediatric T-ALL patients is PTEN protein inactivation due to phosphorylation by casein kinase 2 (CK2) and/or oxidation by reactive oxygen species (ROS) (Silva et al., 2008).

Evidence for Notch1 and PI3K/Akt/mTOR signaling involvement in T-ALL LIC self-renewal, survival, and proliferation

Several studies, carried out in both human and murine models, have documented the key roles played by Notch1 and PI3K/Akt/mTOR signaling pathways in regulating the survival and proliferation of T-ALL LICs.

Using serially transplanted NOD/SCID mice, it was documented that co-culturing of primary human T-ALL cells with a mouse stromal cell line expressing the Notch1 ligand Delta-like-1 (MS5-DL1 cells), allowed the maintenance of in vivo LIC activity in both wild-type and Notch1-mutated samples (Armstrong et al., 2009). Inhibition of the Notch1 pathway during primary cultures of T-ALL cells by a γ-secretase inhibitor, abolished the in vivo LIC activity. Altogether, these findings were the first to document a major role played by the Notch1 pathway activation in the maintenance of T-ALL LICs.

Similar results were subsequently reported in a murine model of T-cell acute leukemia 1/LIM domain only 2 (Tal1/Lmo2) T-ALL in which 75% of leukemias developed spontaneous mutations in Notch1 (Tatarek et al., 2011). It should be recalled here that both Tal1 and Lmo2 are oncogenic in mice, and are commonly co-activated in human T-ALL patients (Weng et al., 2004).

In the study by Tatarek et al. (2011), transplantation of leukemic cells was performed in syngeneic mice. Also mouse LICs remained dependent on sustained Notch1 signals as LIC activity was significantly reduced upon treatment with γ-secretase inhibitors. An important finding that emerged from this work was that spontaneous Notch1 mutations in pre-leukemic animals corresponded to an expansion of DN3/DN4 thymocyte clones, which then gave rise to T-ALL. This observation has been subsequently confirmed in a zebrafish transgenic model of T-ALL, where Notch1 expression induced a significant expansion of pre-leukemic clones (Blackburn et al., 2012). However, only a minority of these clones were fully transformed and could induce leukemia when transplanted into recipient animals. This suggested that a primary role of Notch1 signaling in T-ALL was the expansion of a population of pre-malignant T-cells, of which only a subset subsequently acquired the necessary mutations to become fully transformed LICs (Blackburn et al., 2012).

Very recently, it has been shown, in a murine model of T-ALL induced by expression of oncogenic Kras (Kras G12D), that activating mutations of Notch1 were detected in 100% of mice that developed T-ALL (Kong et al., 2013). Importantly, pre-leukemic T-cells in which Notch1 mutations could not be detected, did not induce leukemia in secondary recipient mice, whereas T-ALL cells with Notch1 mutations did. Overall, these findings suggested that Notch1 activation played a key role in both leukemia initiation and progression in this model of T-ALL. Interestingly, the work by Kong et al. (2013) highlighted that LICs were enriched in both the $CD8^+/cKit^+$ and $CD8^+/cKit^-$ cell subpopulations. This was at variant with a mouse PTEN-deficient model of T-ALL, where LIC activity was endowed in the $CD3^+/cKit^{mid}/Lin^-$ cell subset (Guo et al., 2008). This observation seems to indicate that the LIC phenotype could be dependent on the genetic hits causing T-ALL.

A Notch1 target gene which is important for T-ALL LIC survival is IGF-1R, as documented by a study in which moderate levels of IGF-1R signaling were sufficient for the expansion of bulk T-ALL cells,

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whereas higher levels were required to support LIC activity (Medyouf et al., 2011). Treatment with γ-secretase inhibitors resulted in down-regulation of IGF-1R mRNA in human T-ALL cell lines and negatively impacted on PI3K/Akt signaling. Perhaps most intriguing, however, was the observation that a moderate decrease in IGF-1R signaling resulted in selective loss of LIC activity, yet the growth and survival of bulk T-ALL cells remained relatively unaffected. These findings suggested that T-ALL LICs may be uniquely sensitive to IGF-1R signaling inhibition (Medyouf et al., 2011).

c-myc is another highly expressed Notch1 target gene involved in the growth/survival of T-ALL LICs. A recent paper has demonstrated that c-myc deletion suppressed established T-ALL by eradicating LICs and that pharmacologic targeting of c-myc induction hampered the growth of both human and murine T-ALLs, including those that carried FBXW7 mutations and were resistant to γ-secretase inhibitors (King et al., 2013). FBXW7 is mutated in approximately 20% of T-ALL patients (Thompson et al., 2007). Of note, these mutations specifically affected the ubiquitination and stability of c-myc protein, leading to an increase in c-myc levels independently from Notch1 (King et al., 2013). The importance of c-myc in T-ALL LIC survival is underscored by the findings documenting how the BRD4 protein inhibitor, JQ1, suppressed c-myc expression and prevented leukemia initiation in mice by eliminating LIC activity (Roderick et al., 2014). BRD4 is one of the four members of the bromodomain and extra-C terminal (BET) family of proteins, which also includes BRD2, BRD3, and BRDT. BET proteins act as "epigenetic readers" and bind to acetyl-lysine residues on the tails of histones H3 and H4 for regulating chromatin structure and gene expression (Garnier et al., 2014).

In this connection, it is very important to highlight that recent findings have documented an important role played by BRD4 by sustaining the survival of a subpopulation of T-ALL cells, resistant to γ-secretase inhibitors, referred to as "persister cells" (Knoechel et al., 2014). "Persister cells" displayed elevated levels of c-myc and Bcl-2, which were not down-regulated by γ-secretase inhibition. Intriguingly, they had elevated levels of mTOR phosphorylated on Ser 2481 (a readout for mTORC2 activity) and were sensitive to both an Akt inhibitor and rapamycin. A combination treatment consisting of JQ1 and a γ-secretase inhibitor was effective in vivo against human T-ALL cells, xenografted in NSG mice, that had developed resistance to γ-secretase inhibition (Knoechel et al., 2014).

As to the relevance of PI3K/Akt/mTOR signaling in T-ALL LICs, Guo et al. (2011) documented, in a murine model of PTEN-deleted T-ALL, that long-term rapamycin treatment of pre-leukemic mice blocked LIC formation and halted T-ALL development. Nevertheless, rapamycin did not inhibit mTOR signaling in the $CD3^{+}$/c-Kit^{mid}/Lin^{-} population already enriched for LICs and did not eradicate this cell subset. These observations indicated that when T-ALL had expanded, LICs had already developed additional genetic anomalies, that prevented the therapeutic efficacy of mTOR inhibition. This observation was in agreement with a previous paper documenting that multi-genetic events collaboratively contributed to LIC formation in PTEN-deleted murine T-ALL (Guo et al., 2008).

Given the important role played by PTEN in T-ALL development, it is important to understand the causes for decreased PTEN activity in this type of leukemia, especially when one considers that Notch1 activation and PTEN loss-of-function cooperate in the leukemogenic transformation process (Medyouf et al., 2010). This might be important in those T-ALL cases in which, despite Notch1 activation, PTEN expression is not repressed by HES1 or no inactivating mutations of PTEN are found.

In human pediatric T-ALL primary samples, PTEN inactivation by high ROS levels is a common event (Silva et al., 2008). The issue of ROS and T-ALL development seems to be controversial, however, as ROS levels were found to be low in a $CD44^{+}$ cell subset of Notch1-dependent mice model of T-ALL. The $CD44^{+}ROS^{low}$ fraction showed substantially enriched LIC activity as compared to the complementary $CD44^{+}ROS^{high}$ and $CD44^{-}$ subsets (Giambra et al., 2012). Consistently with these observations, LIC activity was enriched in the ROS^{low} fraction of human T-ALL human samples. This study also documented that Notch1 induced the expression of runt-related transcription factor 3 (RUNX3), a repressor of RUNX1, and that RUNX1 up-regulated PKCθ. Therefore, Notch1 acted as a PKCθ repressor. Of note, inactivating RUNX1 mutations have been recently described in 4–18% of T-ALL patients (Della Gatta et al., 2012). PKCθ has been implicated in ROS production in normal T-cells (Kaminski et al., 2007). Interestingly, primary mouse T-ALL cells lacking PKCθ displayed an increased LIC activity, whereas enforced PKCθ expression in both mouse and human primary T-ALLs hampered LIC activity (Giambra et al., 2012). Therefore, the findings by Giambra et al. (2012) seem to exclude a link between Notch1 mutations (or RUNX1 mutations) and ROS-dependent PTEN inactivation in human T-ALL.

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As to PI3K down-stream targets, both mTORC1 and mTORC2 play an important role in T-ALL LIC pathophysiology. Regarding mTORC1, it has been shown that loss of mTORC1 activity caused by Raptor deficiency eradicated T-ALL in a murine model of disease, suggesting that mTORC1 played a key role in LIC survival (Hoshii et al., 2014). Of note, rapamycin was not sufficient for the eradication of T-ALL. This could be due to the fact that rapamycin is an incomplete blocker of mTORC1 functions (Kang et al., 2013). Therefore, dual PI3K/mTOR inhibitors or mTORC1/mTORC2 could be more effective agents against T-ALL, as they efficiently targeted rapamycin-resistant mTORC1 outputs in T-ALL cells (Bressanin et al., 2012; Chiarini et al., 2009; Evangelisti et al., 2011). Very recent findings obtained in a zebrafish model of T-ALL have highlighted how increased levels of Ser 473 p-Akt and p-70S6K (an mTORC1 down-stream target) correlated with an increase in LIC frequency (Blackburn et al., 2014). Treatment with the Akt inhibitor, MK-2206, or the dual PI3K/mTOR inhibitor, PI-103, reduced the frequency of LICs. It is intriguing that Akt activation could be down-modulated by epigenetic modifying drugs, including 5-azacytdine. This implied that the regulation of this signaling pathway could be dependent also on epigenetic mechanisms, at least in zebrafish Myc-induced T-ALL.

An important role for mTORC2 in T-ALL development was suggested by the findings of Magee et al. (2012a). It was shown that deletion of the mTORC2 component, Rictor, prevented leukemogenesis and hematopoietic stem cell (HSC) depletion after PTEN deletion in adult mice, implicating mTORC2 activation in these processes. However, Rictor deletion (and hence mTORC2 function) had little effects on the physiology of normal (non PTEN-deleted) HSCs. Moreover, PTEN deletion from neonatal HSCs did not activate PI3K signaling or promote HSC proliferation/depletion, or leukemogenesis. It was therefore concluded that PTEN is required in adult, but not neonatal, HSCs to negatively regulate mTORC2 signaling downstream of PI3K. These observations could explain why B-ALL, where PTEN deletions are rare (Mullighan et al., 2011), is a disease of the early childhood with a peak incidence at 2–5 years of age (Inaba et al., 2013), whereas pediatric T-ALL, in which PTEN deletion/inactivation is more frequently observed (Jotta et al., 2010), displays an older mean age of presentation (~9–10 years) (Karrman et al., 2009).

The findings of Magee et al. (2012a) are in agreement with those of an independent group that also demonstrated an important role played by mTORC2 in promoting Notch1-driven leukemogenesis in mice (Lee et al., 2012). By exploiting Rictor deletion in mouse HSC transduced with activated Notch1, they were able to document that mTORC2 was a key determinant of the well-known capability of Notch1 to induce NF-κB activation (Vilimas et al., 2007) and chemokine receptor CCR7 expression, as well as to accelerate tissue invasion and death in T-ALL. However, they also demonstrated the important contributions of mTORC2 to Notch1-dependent proliferation and differentiation of healthy pre-T cells in the thymus, through both Akt and NF-κB activity (Lee et al., 2012).

Therapeutic targeting of Notch1 and PI3K/Akt/mTOR signaling in T-ALL LSCs

The two major current options for blocking aberrant Notch1 activity are the use of γ-secretase inhibitors (Sarmento and Barata, 2011) or monoclonal antibodies targeting Notch1 (Wu et al., 2010). γ-secretase inhibitors were initially developed to block the activity of the γ-secretase aspartyl protease in Alzheimer's disease (Golde et al., 2013). It has been documented that this class of drugs significantly reduced T-ALL LIC activity (Armstrong et al., 2009; Tatarek et al., 2011). MK0752, PF03084014, and BMS-906024 are γ-secretase inhibitors that either underwent or are undergoing phase I clinical trials in refractory/relapsed T-ALL patients (National Cancer Institute trials: NCT00100152; NCT00878189; NCT01363817). BMS-906024 is being tested in association with dexamethasone. However, it should not be underestimated that γ-secretase has many physiological substrates besides Notch proteins, thus substantial side effects are to be expected when the enzymatic activity is pharmacologically blocked (Lleo, 2008). The most prominent and serious side effects associated with γ-secretase inhibitors are gastrointestinal toxicities such as intractable diarrhea, especially when continuous dosing schedules were employed (Wei et al., 2010). Diarrhea is caused by the rapid differentiation of stem cells into secretory goblet cells in the intestinal crypts (a phenomenon referred to as goblet cell metaplasia) in response to γ-secretase inhibitor administration, as Notch signaling is required to maintain the normal architecture of the intestinal lining epithelium (Fre et al., 2005). Mechanistically, γ-secretase inhibitor treatment resulted in cell cycle arrest and accumulation of goblet cells mediated by up-regulation of

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the gene encoding the transcription factor Krüppel-like factor-4, a negative regulator of the cell cycle required for goblet cell differentiation (Real et al., 2009). However, the use of glucocorticoids could improve the anti-leukemic effects of γ-secretase inhibitor and reduce their intestinal toxicity in vivo in a xenografted model of human T-ALL, as glucocorticoids induced transcriptional up-regulation of cyclin D2 and protected mice from developing the intestinal goblet cell metaplasia (Real et al., 2009).

As to anti-Notch1 monoclonal antibodies, evidence indicated that selective inhibition of either Notch1 or Notch2 alleviated the severe gastrointestinal toxicities caused by simultaneous pan-inhibition of the four Notch receptors by γ-secretase inhibitors (Wei et al., 2010). Humanized anti-Notch1 monoclonal antibodies have proven their efficacy against T-ALL LICs (Ma et al., 2012) and synergized with glucocorticoids in vivo (Agnusdei et al., 2014). OMP-59R5 and OMP52M51 are two anti-Notch monoclonal antibodies (targeting Notch 2/3 and Notch1, respectively) which are being tested in clinical trials in tumor patients (National Cancer Institute trials: NCT01277146; NCT01703572).

It should be highlighted, however, that also Notch3 signaling could play an important role in T-ALL (Masiero et al., 2011). Notch3 is overexpressed in all T-ALL cases studied (Bellavia et al., 2002) and was oncogenic in mouse. No activating mutations of Notch3 have been disclosed so far in T-ALL (Aifantis et al., 2008). Notch3 could be a down-stream target of Notch1 in T-ALL (Palomero et al., 2006), however it has been shown that Notch3 activation by its ligand Delta-like-4 expressed on angiogenic vessels was able to coordinate escape from a quiescent status, leading to progressive tumor growth in a xenograft model of T-ALL (Indraccolo et al., 2009). Hence, it could be envisaged that therapeutic treatment of T-ALL would require, at least in some patients, co-targeting of Notch1 and Notch3, for example using blocking monoclonal antibodies to either receptors or their ligands. In this context, OMP21M18 and REGN421/SAR153192 are humanized anti-Delta-like-4 neutralizing antibodies which are being tested in clinical trials (National Cancer Institute trials: NCT00744562; NCT00871559).

As to PI3K/Akt/inhibitors, the results obtained by Guo et al., 2011) have proven the efficacy of rapamycin in preventing LIC formation and halting T-ALL development.

It should be recalled here that LICs, besides self-renewal, share additional properties with the healthy HSC counterpart, including high expression of ATP-binding cassette (ABC) transporter protein family members, among which ABC subfamily G member 2 (ABCG2) is one of the most important and well characterized (Hadnagy et al., 2006). The high expression of ABCG2 enables a flow cytometric identification of these cells as side population (SP), which is characterized by a low retention of vital dyes (typically Hoechst 33342) (Zhou et al., 2011) and is enriched in CSCs (Duan et al., 2013). We have identified the SP in both T-ALL cell lines and primary lymphoblasts, and we have documented how drugs targeting PI3K/Akt/mTOR signaling were effective against the SP (Chiarini et al., 2010; Martelli et al., 2012). It is possible to identify the SP in T-ALL cell lines also using the Vybrant® DyeCycle. Co-staining with Annexin V-FITC enabled us to identify apoptotic cells. Both BKM120, a pan-PI3K inhibitor (Lonetti et al., 2013), and CX-4945, a CK2 inhibitor which impacts on PTEN function (Buontempo et al., 2014), decreased the SP by inducing apoptosis in this cell subset (Fig. 2A–B). Interestingly, the non-SP of MOLT-4 cells was less sensitive to BKM120 than the SP (Fig. 2C), which may indicate that SP cells strongly rely on up-regulated PI3K/Akt/mTOR signaling for their survival.

Concluding remarks

In summary, several lines of evidence point to key roles played by Notch1 and PI3K/Akt/mTOR signaling pathways in supporting T-ALL LIC activity. It is also clear that the two signaling cascades are inter-twined at different levels and could cooperate in leukemogenic events. Therefore, there is a strong rationale for targeting these two signaling networks in T-ALL patients. Indeed, the development of curative strategies that could eradicate LICs is the ultimate goal of the monumental research efforts that are being devoted to understand these important signaling pathways over the recent years.

However, there are still unresolved issues regarding the real impact of Notch1 and/or PI3K/Akt/mTOR signaling up-regulation on T-ALL patient outcome. For example, increased Notch1 activity has been associated with a better outcome in both pediatric (Fogelstrand et al., 2014; Jenkinson et al., 2013) and adult T-ALL (Van Vlierberghe et al., 2013). In contrast, PTEN mutations in children were significantly associated with increased resistance to induction chemotherapy and a trend towards poor long-

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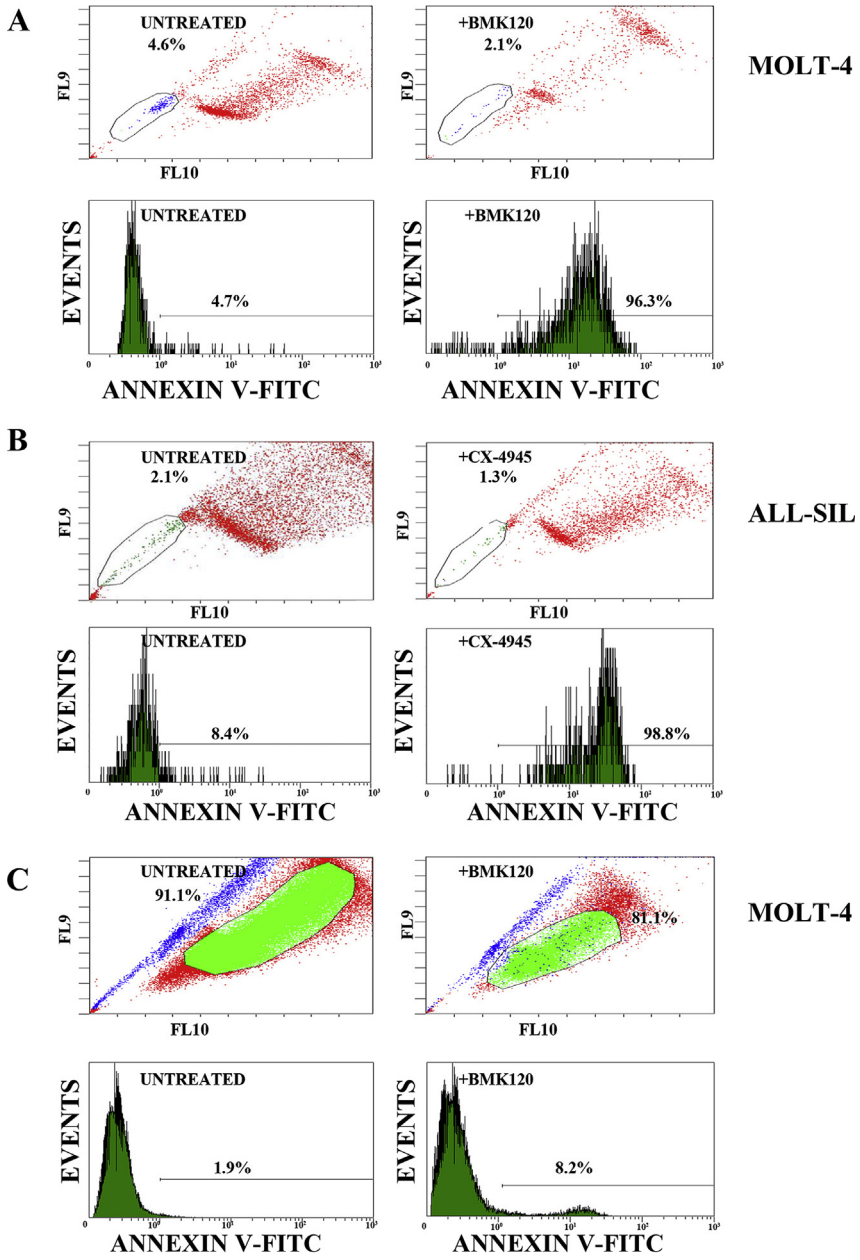

Fig. 2. PI3K or CK2 inhibition targets the SP of T-ALL cell lines (MOLT-4 and ALL-SIL). A–B: Cells were incubated for 24 h with either BKM-120 (1 μM) or CX-4945 (5 μM). Cells were stained with the Vybrant® DyeCycle in the absence or the presence of the ABCG2 inhibitor, verapamil (50 μM) (Lonetti et al., 2013). The SP, which disappeared in the presence of Verapamil, was gated and Annexin V-FITC staining evaluated. In C, the non-SP cells were gated and analyzed for apoptosis using Annexin V-FITC staining.

term outcome (Bandapalli et al., 2013). Nevertheless, patients with both inactivating PTEN and activating Notch1 mutations showed marked sensitivity to induction treatment and excellent long-term outcome, which was similar to patients with Notch1 mutations only, and more favorable than in patients with PTEN mutations only. Also in adult T-ALL, the presence of PTEN mutations could identify a

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subset of patients with a poorer outcome (Trinquand et al., 2013). Taken together, these findings are surprising in light of the data demonstrating a molecular synergism between Notch1 activation and PTEN inactivation in pre-clinical models of T-ALL, as we have highlighted in this review, and pose the intriguing question of how the two signaling networks interact in clinical terms. Although the mechanisms of the unexpected clinical interactions between Notch1 activation and PTEN inactivation remain to be clarified, the findings by Trinquand et al. (2013) seem to suggest that some patients, and notably those with a combination of Notch1 activation and PTEN inactivation, may not benefit from the use of Notch1 inhibitors in multimodal treatment regimens.

In addition, both anti-Notch1 and anti-PI3K/Akt/mTOR therapies may be most effective in the context of combination therapies with chemotherapeutics, particularly with glucocorticoids (Piovan et al., 2013), but possibly also with drugs targeting other important signaling networks involved in T-ALL pathogenesis.

Whatever the case, novel targeted therapies aimed to dampening Notch1 and PI3K/Akt/mTOR signaling activities have the potential to dramatically improve the quality of life of T-ALL patients by shortening their course of treatment, reducing disease relapse, and providing more effective interventions for those patients who are resistant to traditional therapies.

Conflict of interest

The authors declare that there are no conflicts of interest.

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