What are the advantages and disadvantages of mouse models of chronic lymphocytic leukemia in drug discovery?

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1. Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoid malignancy characterized by the proliferation and accumulation of mature $CD5^+$ B-cells in the peripheral blood (PB), bone marrow (BM) and lymphoid tissues. When the number of $CD5^+$ B-cells is lower than $5x10^9$ cells/L, this entity is called monoclonal B lymphocytosis (MBL) and it is asymptomatic monoclonal or oligoclonal proliferation of B-cells. The disease may have a stable course but also become aggressive, with frequent relapses, or even transform into an aggressive lymphoma, typically diffuse large B-cell lymphoma (DLBCL) (Richter transformation). Two major molecular subgroups have been identified: those harboring unmutated immunoglobulin heavy-chain variable region (IGHV) genes (U-CLL, ≥98% identity with the germline) and those with mutated IGHV genes (M-CLL). Genomic and epigenomic studies have elucidated multiple aspects of the pathogenesis of the disease. Nowadays CLL should be considered as a complex disease in which genetic and epigenetic mechanisms cooperate with microenvironmental factors in the malignant transformation and in leukemia progression [1]. In parallel, new targeted therapies and management strategies have been developed. Due to this complexity and

heterogenousity, the generation of adequate pre-clinical mouse model fully reflecting tumor biology has not yet been successfully achieved. Here we describe and discuss the mouse models developed for CLL, the most prevalent type of leukemia in adults in Western countries.

2. Mouse models in CLL

Several murine genetically engineered and human xenograft models for CLL have been reported. The New Zealand Black (NZB) mouse naturally develops a CLL-like disease without the induced of oncogenes or deletion of regulatory regions. These mice have a MBL phenotype at an early age which always progress to an aging-associated clonal $CD5^+$ B-cell disease. Then this model could be useful the study the progression from MBL to CLL [2].

2.1 Genetically engineered

In 2002 was described the first transgenic model for CLL, the T-cell leukemia/lymphoma 1 transgenic (*Eμ*-TCL1) mouse [3] that mimic aggressive CLL. The *Eμ*-TCL1 is based on the exogenous expression of the human *TCL1 g*ene under the control of the *Igh* variable region (V_H) promoter and the IgH intronic enhancer region (*Eμ*) to target *TCL1* gene expression. Older mice (10-18 months), develop a clonal expansion of $CD5^+$ B-cell that progress as a CLL-like clonal disease, resembling aggressive U-CLL with splenomegaly, hepatomegaly and expanded B-cells in PB [4]. Disease progression in *Eμ*-TCL1 mice can be accelerated by adoptive transfers of splenocytes from *Eμ*-TCL1 murine CLL cells into wild-type recipients, being this model very useful for preclinical evaluation of novel therapies and to study the interplay of CLL with tumor microenvironment, being an important tool for studying CLL progression [5]. Nowadays several groups have crossed transgenic *Eμ*-TCL1 mice with

other genetically modified mice by modulating genes related to B-cell receptor (BCR) signaling, leukemia-environment interactions and proliferation and cell survival in order to achieve a better knowledge of CLL biology [6,7,8,9]. **Figure 1** describes all the *Eμ*-TCL1-related mouse models in CLL.

Other strategies have been the generation of transgenic mice carrying some of the most important genetic alterations reported in CLL such as 13q chromosome deletion, overexpression of several survival factors including *BCL2*, *TRAF2*, *BAFF* or *APRIL*, deletions of *IRF4* and ATM or SF3B1 mutations (**Table 1**).

2.2 Xenograft mouse models

In a xenograft model, human tumor cells or cell lines are transplanted subcutaneously (*s.c.*), intravenously (*i.v.*) or orthotopically into the organ type of origin in immunocompromised mice. Given the poor engraftment of primary CLL cells, several efforts have been made in the last decade to improve the engraftment into key organs of mice that mimic the typical spread of CLL in humans.

It has been reported that hematopoietic stem cells (HSCs) purified from patients with CLL displayed lymphoid-lineage gene priming and developed monoclonal or oligoclonal B-cells simulating MBL after xenogeneic transplantation, but other events are needed to develop a CLL [10].

The most used strains are the immunodeficient NOD/SCID model, where the SCID (severe combined immunodeficiency) model has been transferred onto a diabetes-susceptible mouse, the non-obese diabetic (NOD) background. This model exhibits better engraftment of human cells due to dysfunctional NK-cells, lowered cytokine production, and defective T or B-cells. The other mouse strain used is the NSG (NOD scid gamma) mice. In this model, the NOD/SCID strain has been modified with a

mutation in the gene encoding the interleukin-2 receptor common gamma chain (IL2Rγ$^{-/-}$). This model lacks mature B, T and NK-cells, to avoid immune rejection of human cells [11].

The first successful studies have been achieved in 2007 [12] using the NOD/SCID mice, where after 4 weeks, an engraftment and growth of cells was achieved in spleen and peritoneal cavities (~90 to 95% of efficiency), BM (~54% of efficiency) and PB (~36% of efficiency) [12]. Later in 2011, using NSG mice, they demonstrated that the successful xenograft of CLL cells was dependent on autologous T-cells [13]. This model was refined by the addition of autologous T-cells that have been activated previously *in vitro* [5,14]. Other modifications such as transfection of CLL cells with *miR-15a/16-1* microRNAs (miRNAs) has also been tested [15]. Even though there are few number of human CLL cell lines available, most of them have also been useful for xenografting into immunodeficient mice [11,16]. A summary of all these models is represented in **Figure 2**.

A common handicap of primary CLL cells xenografts models is often the incompatibility between the high number cells required and the limited availability of CLL samples. This is avoided by the establishment of patient-derived xenograft (PDX) models, able to exponentially expand a relative few amount of starting primary CLL cells. Most of CLL-PDX models have been established by the initial xenograft of primary CLL cells with activated autologous T-cells into NSG mice, which has been clinically useful to distinguish between spontaneous and treatment-induced clonal selection in CLL [17,18,19].

3. Expert Opinion

CLL is a well-defined lymphoid neoplasm with very heterogeneous biological and clinical behavior. New models that recapitulate the key features of CLL are needed to study CLL biology and to test novel drugs in CLL. Due to the facility to obtain primary CLL cells, a high number of studies to test the efficacy of new drugs have been done, and we have learn that CLL cells die spontaneously *in vitro* and that they need external factors to survive. Thus although new *in vitro* systems to check the efficacy of drugs have been developed (co-culture with stromal cells or cytokines in a long term culture to induce the proliferation of CLL cells), these systems only partially recapitulate the response of CLL patients and probably new 3D models are needed.

The transgenic *Eμ*-TCL1 mouse model has been widely used to study pathophysiology, clonal evolution, the interplay of CLL with tumor microenvironment and drug efficacy in aggressive CLL. Unfortunately exome sequencing of CLL samples from the *Eμ*-TCL1 mice showed that although mutations in mice are frequently subclonal and heterogeneous and upon transplantation a clonal evolution was observed, they lack any of the most recurrent mutations detected in human CLL cells [20]. Then, the development of new genetically engineered mouse models reflecting the different recurrent genetic lesions with clinical impact described in CLL are warranted as they will help to improve the knowledge of CLL complexity and response to new therapies. One of the disadvantages of these transgenic mice is that they cannot be used to test some new immunotherapy drugs that are not expressed in mouse.

On the other hand, the development of more immunodeficient mice and the knowledge that CLL cells are dependent on autologous T-cells for engraftment in mice have improved the methodology to generate xenograft CLL models, without the bias of humanization or irradiation. These models could be considered as an autologous T humanized mice. Nowadays, the main problem of these models is that tumor

development is highly variable and is unable to recapitulate the complex host stroma–tumor interactions, which depend on autocrine and paracrine interaction among cells with the adaptive and innate immune system. In this way these models cannot be used to pre-clinical testing of immunomodulatory compounds. In addition, SCID mice exhibit defects in DNA repair that prevent the testing of some cytotoxic compounds and nude mice demonstrate complete fragility which could hamper their ability to tolerate new therapies. In this way, the establishment of PDXs able to exponentially expand a relative few amount of starting primary CLL cells could be of great interest. These studies might help to study clonal evolution in CLL cells and use these expanded cells to check the efficacy of new drugs using new 3D models that might recapitulate the stroma-tumor interactions that exists in CLL.

Funding

Our group has been supported by the Spanish Ministry of Economy and Competitiveness through the Plan Estatal de Investigación Científica y Técnica y de Innovación (MINECO) [RTI2018-094584-B-I00] and was cofounded by the European Regional Development Fund (ERDF) and the CERCA program from Generalitat de Catalunya, Centro de Investigación Biomédica en Cáncer (CIBERONC) and Generalitat de Catalunya [2017 SGR 1009]. HPA is a recipient of a predoctoral fellowship from the Spanish Ministry of Science, Innovation and Universities [FPU19/03110].

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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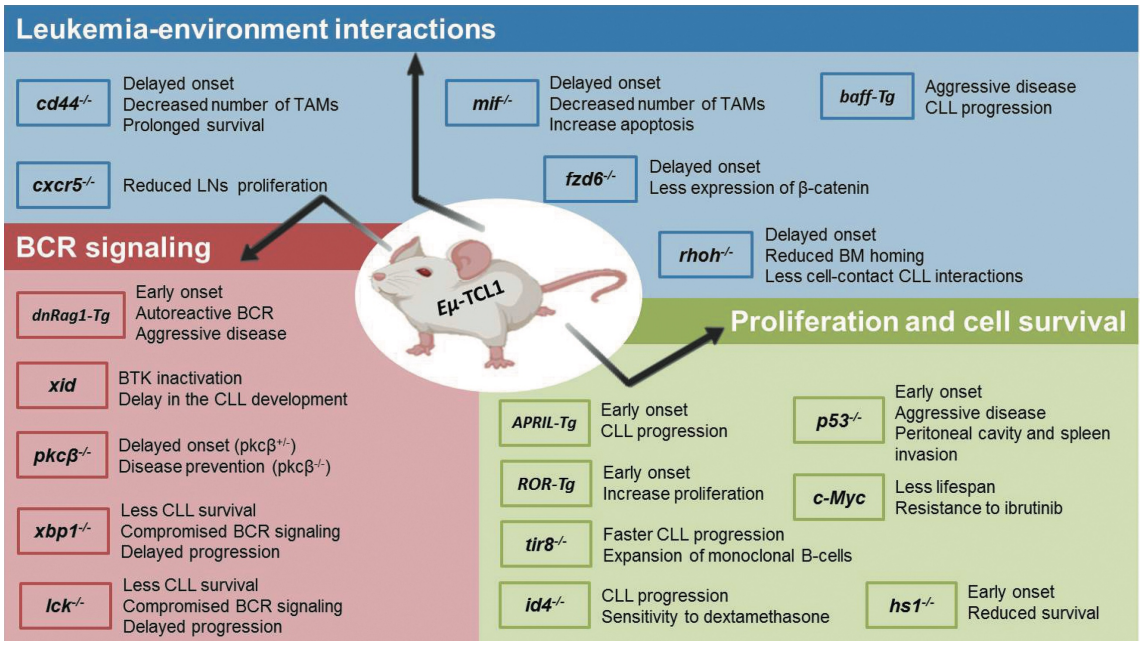

Figure 1. *Eμ*-TCL-1 driven leukemia mouse models as a tool for studying CLL pathogenesis. *Eμ*-TCL-1 mouse model has been crossed with constructs to overexpress or to delete molecules related to leukemia-environment interactions, BCR (B-cell receptor) signaling, proliferation and survival. [6,7,8,9]. *cd44*$^{-/-}$: homing cell adhesion molecule involved in cell-cell interactions; *cxcr5*$^{-/-}$: C-X-C chemokine receptor type 5 involved in B-cell migration to the lymph nodes; *baff*-Tg: B-cell activating factor belonging to the tumor necrosis factor (TNF) family. It is a potent B-cell activator of proliferation and differentiation; *mif*$^{-/-}$: macrophage migration inhibitory factor is related with innate immunity and survival of tumor-associated macrophages; *fzd6*$^{-/-}$: Frizzled 6 is associated with Wnt signaling pathway playing a key role in development, tissue-specific stem-cell maintenance and tumorigenesis; *rhoh*$^{-/-}$: Ras homolog gene family, member H is a small G protein that is involved in CLL BM homing and its engraftment; *dnaRag1*-Tg: recombination activating genes 1 is a protein involved in V(D)J rearrangement, *xid*: X-linked immunodeficiency mouse; *pkcβ*$^{-/-}$ or *pkcβ*$^{+/-}$: protein kinase C beta type, protein involved in BCR signaling and overexpressed in CLL patients; *xbp-1*$^{-/-}$: X-box binding protein 1 is a transcription factor related with the ER-

stress, reduced expression of XBP1 protein compromise the BCR signaling disadvantaging the leukemic cells survival; *APRIL*-Tg: a proliferation-inducing ligand is a protein from the TNF family that mediates for CLL cell survival and leukemogenesis; *ROR*-Tg: receptor tyrosine kinase-like orphan receptor 1 is an oncoembryonic antigen found on CLL cells; *tir8*$^{-/-}$: also known as single Ig IL-1 related receptor (SIGIRR) is involved in the inflammation pathway and the Toll-like receptor (TLR) signaling; *id4*$^{-/-}$: inhibitor of DNA binding protein 4 is a member of the dominant-negative basic helix-loop-helix transcription factor family that lacks DNA binding activity and has tumor suppressor function; *p53*$^{-/-}$: tumor protein p53; *c-Myc*: c-myelocytomatosis oncogene product. (BM: bone marrow; BTK: Bruton's tyrosine kinase; LN: lymph node; TAM: tumor-associated macrophages).

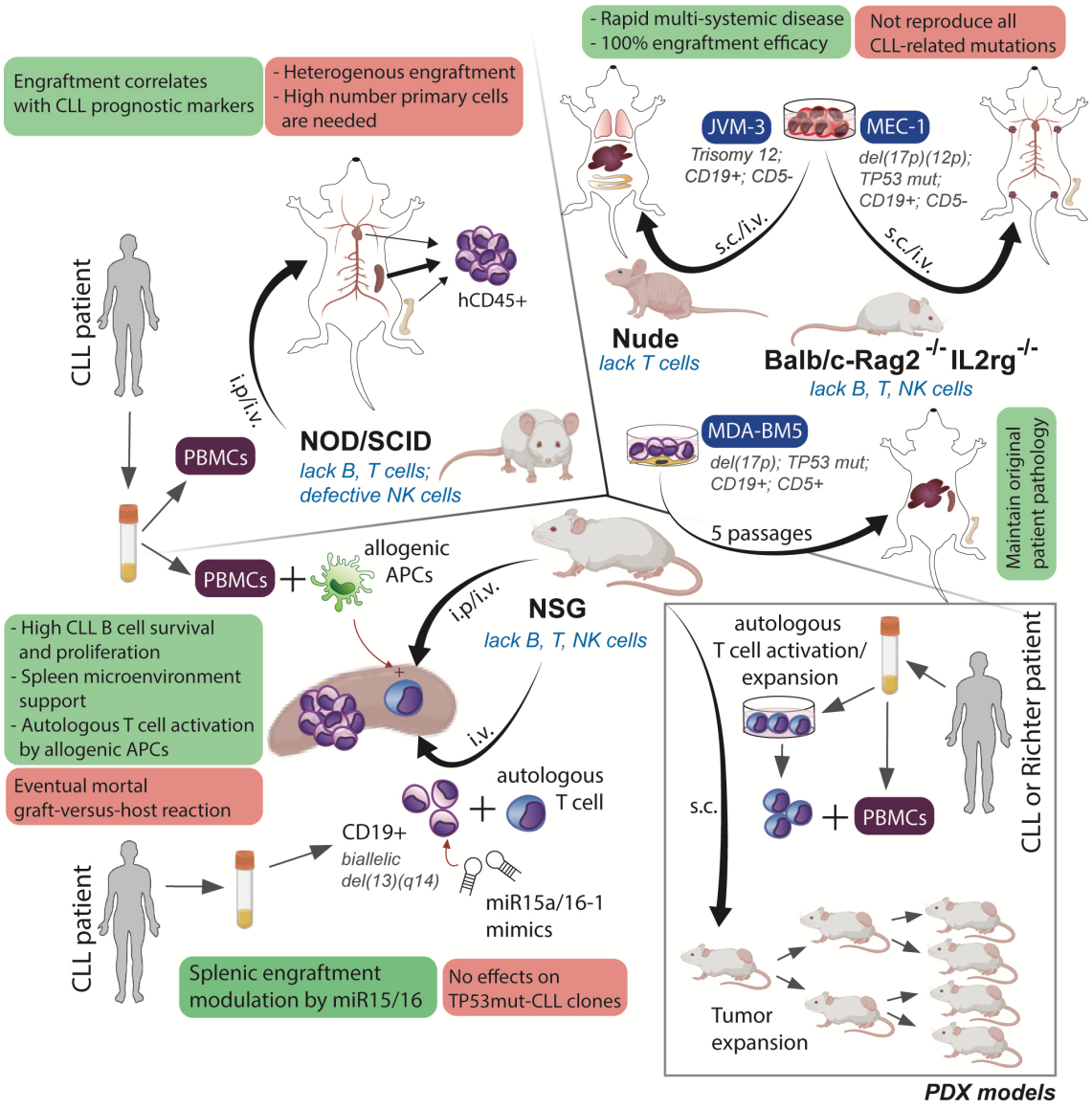

Figure 2: Xenograft models in CLL. The most relevant xenograft strategies in CLL are showed. The findings/advantages or disadvantages of each strategy are highlighted in green or red, respectively. Immunodeficiency of each mouse used is labeled in blue and italics. Models developed in a NSG mice using PBMCs or purified CD19+ cells, has allowed to discover that (i) splenic-hCD45+ cells engraftment correlated with CLL prognosis markers (i.e. U-IGHV genes) [11]; (ii) the murine splenic microenvironment sustained CLL cell proliferation like human lymph nodes, with induction of NF-κB and BCR signaling in the xenografted cells, which served as proof-of-concept to test the efficacy of ibrutinib on tumor-host interactions [21]; (iii) the restoration of miR-15a or

miR-16-1 impairs the capacity of CD19+ CLL cells with biallelic 13q14 deletion for engraftment and growth in the spleen of NSG mice, although without synergy between both miRNAs and no effects in CLL clones harboring TP53 alterations (TP53 mut) [15], evidencing that the CLL cell proliferation capacity into murine splenic microenvironment also depends of non-genetic factors; (iv) co-xenotransplant of CLL PBMCs with allogenic APC (CD14+ or CD19+) promote the activation of autologous T-cells, which is crucial for CLL cells to engraft in secondary lymphoid tissues, survive, and proliferate mimicking key features of human CLL [13]. The engraftment of CLL cell lines have been achieved using JVM-3, MEC-1 [11] and MDA-BM5 [16]. To avoid the limited availability of CLL primary samples, PDX models in CLL and Richter syndrome have been described. [17,18,19], allowing an exponentially expand few amount of primary CLL cells to study of tumor biology, discovery of novel therapeutic targets, and preclinical screening of drugs. (APC: antigen presenting cell; BCR: B-cell receptor; CLL: chronic lymphocytic leukemia; i.p.: intraperitoneal injection; i.v.: intravenous injection; NOD: non-obese diabetic mouse; NSG: NOD scid gamma mouse; PBMCs: peripheral blood mononuclear cells; PDX: patient-derived xenograft; s.c.: subcutaneous injection; SCID: severe combined immunodeficiency).

Table 1. Transgenic mouse models of CLL non TCL-1 related

Gene	Rationale	Mutation	Penetrance	Mouse strain	Reference
13q chromosome deletion	Most frequent deletion in CLL	*mir-15a/16-1*$^{-/-}$ and *mir-15a/16-1*^{floxed}	25-30% development of MBL	NZB background	[22]
		Deletion of Dleu-2 and Dleu-5 (14qC3 minimal deleted region (MDR)$^{-/-}$)	40-45% development of MBL, CLL and CD5^{-} NHLs	129/Sv-C57BL/6 mixed background	[23]
		Deletion of common deleted region (14qC3CDR^{floxed})	40-45% development of CLL	129/Sv-C57BL/6 mixed background	[24]
APRIL-Tg	High levels in serum, associated with proliferation and survival	Overexpression of *APRIL*	Between 9 to 12 months mice developed mild lymphoproliferation of CD5^{+} B1 cells in the peritoneal cavity	C57BL/6 background	[25]
Eμ-mir-29-Tg	*miR-29a/b* is overexpressed in CLL	Overexpression of *APRIL* and microRNA cluster *miR-29a/b*	At 24 to 26 months ~20% of mice developed leukemia and died. Aggressive CLL phenotype	C57BL/6 background	[26]
irf4$^{+/-}$	Regulator of B-cell development and function	Heterozygous mutation of *IRF4*	Accelerated disease	NZB background	[27]
Vh11 x *irf4*$^{-/-}$	*IGHV11* knock-in mice lead to anti-PtC (autoantigen) development.	Crossed *Irf4*-deficient mouse (*irf4*$^{-/-}$) with *IGHV11* knock-in mouse	Spontaneously developed CLL (5-10 months) at 100% penetrance. Aggressive phenotype	*irf4*$^{-/-}$ mouse	[28]
ROR1-Tg	*ROR1* is overexpressed in CLL cells	Overexpression of *ROR1* with the mouse IgH promoter/ enhancer	After 15 months (3% to 65% mice) developed lymphocytosis and splenomegaly	C57BL/6 background	[29]
Sf3b1-K700E$^{fl/+}$ x *Atm*$^{fl/fl}$	*SF3B1* and *ATM* are recurrently mutated in CLL	Heterozygous expression of *Sf3b1*(K700E) (*Sf3b1-K700E*$^{fl/+}$) and *Atm* deletion (*Atm*$^{fl/fl}$)	At 9 to 12 month mice developed lymphocytosis, splenomegaly and BM infiltration of B-cells carrying *SF3B1* and *Atm* mutations.	C57BL/6 background	[30]