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Pre-clinical modeling of novel therapeutics in chronic lymphocytic leukemia: the tools of the trade

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Abstract

In the last decade our understanding of chronic lymphocytic leukemia (CLL) biology and pathogenesis has increased substantially. These insights have led to the development of several new agents with novel mechanisms of action prompting a change in therapeutic approaches from chemotherapy based treatments to targeted therapies. Multiple pre-clinical models for drug development in CLL are available; however, with the advent of these targeted agents, it is becoming clear that not all models and surrogate readouts of efficacy are appropriate for all drugs. In this review we discuss *in vitro* and *in vivo* pre-clinical models, with a particular focus on the benefits and possible pitfalls of different model systems in the evaluation of novel therapeutics for the treatment of CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the western world¹. It is characterized by the expansion of monoclonal, auto-reactive B-cells that display decreased cell death and increased proliferation rates^{1, 2}. Standard treatment for physically fit symptomatic patients is chemoimmunotherapy (such as FCR – fludarabine, cyclophosphamide and rituximab); however these agents are not well tolerated by elderly patients and do not perform well in patients with adverse cytogenetic profiles (such as deletion of the short arm of chromosome 17)^{3, 4}. In the past few years treatment of CLL has started to undergo dramatic changes; moving away from traditional chemotherapeutics towards targeted agents. While there are multiple preclinical models for drug development, evaluation of therapeutics for the treatment of CLL is typically done using *in vitro* cultures of either cell lines or primary CLL cells collected from the peripheral blood, utilizing cell death as the preferred readout. With the advent of targeted agents, it is becoming clear that not all models and measures of activity are appropriate for the evaluation of all drugs.

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In the context of this review we define novel therapeutics as kinase inhibitors (such as ibrutinib and idelalisib), immunomodulatory agents (such as antibodies against PD-1, PD-L1 or CTLA4), and BH3-mimetics (such as ABT-199). Kinase inhibitors are at the forefront of investigation for the treatment of CLL; with ibrutinib and idelalisib demonstrating impressive clinical activity as single agents⁵⁻⁷ as well as in combination with anti-CD20 monoclonal antibodies^{8, 9}. These agents work by inhibiting both intrinsic signaling pathways as well as disrupting tumor-microenvironmental interactions¹⁰⁻¹⁵. Because of this latter mechanism, these drugs differ greatly from traditional chemotherapy which works primarily through the direct induction of cell death, suggesting that changes in cell viability may not be the most appropriate readout to evaluate these agents. Similarly, immunomodulating agents are used to enhance immunity, for example by blocking PD-1 signaling in T-cells leading to a reversal of T-cell anergy^{16, 17}. Unlike many agents currently used in the treatment of CLL, this latter class of agents does not necessarily target the CLL cell, but rather accessory cells such as T-cells. Lastly, BH3-mimetics, target anti-apoptotic proteins key to CLL cell survival^{18, 19}. Although these agents act directly on the CLL cells and are cytotoxic, pre-clinical evaluation of these agents requires a culture system that mimics the up-regulation of anti-apoptotic proteins observed *in vivo*²⁰.

Herein we discuss *in vitro* and *in vivo* pre-clinical models, with a particular focus on the benefits and potential pitfalls of different model systems to evaluate novel therapeutics for the treatment of CLL.

PRE-CLINICAL MODELING *IN VITRO*

Most pre-clinical modeling of CLL is performed *in vitro* utilizing either primary CLL cells or tumorigenic cell lines mimicking the biological properties of CLL. In recent years, the importance of the microenvironment in the pathogenesis of CLL has become more evident²¹⁻²⁵. Consequently, many novel therapeutic agents currently under development for CLL target not only intrinsic CLL signaling pathways, but also disrupt key tumor-microenvironment interactions. Because of this, traditional read-outs, such as cell death *in vitro*, may be less useful to evaluate these agents. For example, the low rate of apoptosis (<20%) induced by ibrutinib *in vitro* would not have identified this agent as a leading therapeutic¹¹. To better model the activity of such novel therapeutics, microenvironmental conditions need to be recapitulated *in vitro* (Figure 1).

Mimicking the microenvironment

It has been shown that CLL cells depend on signaling from the microenvironment. This is evident by the fact that CLL cells undergo apoptosis *in vitro* unless substitutes of survival signals found in the tumor microenvironment are provided. To this end multiple systems to recreate the microenvironment *in vitro* have been developed as model systems for CLL. Among the most widely utilized and probably most relevant to the evaluation of novel therapeutics are the stromal cell and the nurse-like cell (NLC) co-culture systems. The primary benefits and potential drawbacks of these co-culture systems are summarized in Table 1.

Stromal co-culture systems were first described by Panayiotidis et al. in 1996. They demonstrated that culturing CLL cells on top of bone marrow derived stromal cells (BMSCs) could increase the percent of viable cells after 10 days in culture by more than 30% compared to control²⁶. Additionally, they demonstrated that BMSCs could maintain CLL cells for up to 30 days in 70% of patients²⁶. The protection afforded by these co-culture systems was shown to be mediated by cell-cell contact. This was determined using a transwell system where CLL cells were separated by a porous membrane from stromal cell cultures – preventing any direct cell-cell contact. Under these conditions, no survival advantage was observed²⁶. Further, conditioned media from stromal cells did not induce pro-survival signals in CLL cells²⁷. More recently, it has been demonstrated that in addition to BMSCs, stromal cell lines (such as the murine bone marrow cell lines: M210B4, SUM4 and KUSA-H1 and the human stromal cell lines: Hs5 and StromaNKTert) can protect CLL cells from drug-induced apoptosis²⁸. In addition to providing a survival benefit stromal co-culture systems have been demonstrated to induce pro-survival signaling and up-regulate anti-apoptotic proteins. Utilizing the M210B4 stromal cell line Edelmann et al., demonstrated that co-cultured CLL cells display increased PI3K and NF- κ B signaling as well as signs of a pro-angiogenic switch²⁹. Additionally, an increase in the expression of the oncogene TCL-1, a key protein in CLL pathogenesis, was shown to be up-regulated in these co-culture systems^{30, 31}. Kay et al. developed a unique stromal co-culture composed of four stromal elements: epithelial fibroblast like cells, endothelial cells, phagocytic cells and large adipocytes. Using this system CLL cells were able to be maintained *in vitro* for more than 12 months³². The survival advantage afforded by co-culturing CLL cells on a stromal cell layer occurred concurrently with increases in expression of key anti-apoptotic proteins, including XIAP, MCL-1, BCL-2 and Survivin in the CLL cells³². Additionally a shift towards pro-angiogenesis was also observed in this model. Interestingly, stereotypic CLL B-cell receptors recognize stromal cell antigens suggesting that stromal cell co-culture systems can induce BCR-mediated survival signals³³. This concept has been validated by multiple groups demonstrating that treatment with BCR-directed kinase inhibitors can overcome stromal-mediated protection of CLL cells^{10, 11, 34}. To further increase the utility of stromal cell co-cultures, stromal cell lines over expressing important signaling molecules (such as CD40L) have been developed³⁵.

The NLC co-culture system was first described by Burger et al. in 2000. While investigating the stromal co-culture system, Burger and colleagues noted the outgrowth of adherent cells that overtime grew larger; with a 2-fold increase in nuclear diameter, and gained an oblong appearance (sometimes appearing as fibroblasts)³⁶. CLL cells cultured in the presence of these cells remained viable compared to those that were removed from the adherent cell layer. Because of their ability to support CLL cell growth these cells were named “nurse-like cells”. Conditioned media from the co-cultures did not enhance the viability of CLL cells removed from the NLC co-cultures, demonstrating that a direct cell-cell contact is required; similar to what was previously shown with stromal cells. NLCs were found to have many qualities consistent with stromal cells, such as positive staining for vimentin and weak staining of STRO-1; however, unlike stromal cells, these cells expressed high levels of the CD68 surface antigen and CXCL12 mRNA³⁶. Further characterizing these cells, Tsukada et al. demonstrated that PBMCs depleted of CD14+ cells could not form NLCs suggesting that

these cells derived from monocytes that differentiate upon *in vitro* co-culture with CLL cells³⁷. To this end they found that splenic CD14+ cells from CLL patients expressed similarities with NLCs developed *in vitro*, suggesting these cells are similar to tumor-associated macrophages³⁷. In recent years, further characterization of NLC has revealed that these cells have features of both M1 and M2 macrophages. Characteristic of M1 macrophages, NLCs express the gene for CD14 and lack significant expression of VEGF; however, NLCs also express IL10 and IL8 as well as genes for CCL2 and CD11b which are more common characteristics of M2 macrophages³⁸. Although the survival benefit endowed by NLCs is contact dependent, the secretion of IL-8; a cytokine known to increase CLL cell survival *ex vivo*³⁹ may play a role – especially in the observed pro-angiogenic switch induced in CLL cells by NLC co-cultures³⁸. To better understand the role NLCs play in creating a protective microenvironment, changes in co-cultured CLL cells were evaluated. It was demonstrated that CXCL12 on NLCs can engage the CXCR4 receptor on CLL cells leading to activation of the p44/42 MAPK survival pathway^{36, 40}. Additionally, increases in anti-apoptotic proteins such as BCL2, BCL2A1, Survivin and XIAP, as well decreases in pro-apoptotic proteins such as BAD and FasR were observed⁴¹. To date, CLL co-cultures utilizing NLCs have been maintained up to 14 weeks⁴¹. Hence, NLCs and stromal co-culture systems mimic the CLL microenvironment by up-regulating key anti-apoptotic proteins and increasing BCR and NF-κB signaling, providing an environment that can support long term survival and proliferation of CLL cells.

Conditioned media to replicate microenvironmental-induced signaling

In addition to replicating cell-cell interactions using co-culture systems, microenvironmental-induced signaling can be reproduced using soluble factors. Although many soluble factors can provide a survival advantage to *in vitro* cultured CLL cells, the most widely studied are ligation of the BCR (via anti-IgM), activation of toll-like receptor signaling (via CpG-ODN, a TLR9 agonist) and stimulation with various chemokines or cytokines. Table 1 summarizes the major advantages of adding soluble factors to *in vitro* cultures.

Reports on the effect of ligation of the BCR on CLL cells using anti-IgM have been conflicting. Initial reports suggested that ligation of the BCR did not convey a survival advantage but rather induced apoptosis, even in patients that demonstrated high levels of CD38 where signal transduction in response to anti-IgM was clearly established⁴². In contrast, studies by Bernal et al. and Longo et al. demonstrated that in a majority of CLL patients, ligation of the BCR with 10µg/mL of anti-IgM lead to a significant reduction in the basal apoptotic rate^{43, 44}. This survival advantage was found to coincide with an increase in BCR signal transduction leading to increased NF-κB activity⁴³. Further, increases in NF-κB regulated anti-apoptotic proteins such as BCL-2, MCL-1 and BFL-1 were observed within 24 hours of anti-IgM treatment. More recently, anti-IgM stimulation was found to increase genes related to metabolism, cell cycle regulation, signal transduction, transcriptional regulation and cytoskeleton organization; although this occurred predominantly in the unmutated subset of CLL⁴⁵. Additionally, an increase in proliferation and a reduction in apoptosis was observed after treatment with anti-IgM *in vitro* in patients with an unmutated *IGHV* gene⁴⁵. In addition to the changes in apoptotic proteins, anti-IgM treatment has been

demonstrated to increase the production of CCL3 and CCL4 by CLL cells; consistent with increased plasma levels of these chemokines in patients with CLL⁴⁶. Although there is variability in response to anti-IgM stimulation among different subgroups of CLL, *in vitro* activation of the BCR signaling pathway generally recapitulates the biology of CLL cells isolated from the lymph node⁴⁷.

CpG Oligodeoxynucleotides (CpG-ODN) are short single stranded DNA molecules composed of cytosine and guanine triphosphate deoxynucleotides linked with a phosphodiester backbone. CpG-ODNs stimulate B-cells (including CLL cells), macrophages and dendritic cells via Toll-Like Receptor 9 (TLR9). CpG-ODNs have been demonstrated to induce proliferation of CLL cells^{48, 49}. Additionally, stimulation with CpG-ODNs leads to an induction of pro-survival cytokine production; specifically TNF- α and IL-6, and increases in surface activation molecules, such as CD40, CD80 and CD86⁴⁹. The effect on CLL cell survival, similar to the experience with anti-IgM, is quite variable, with cells from some patients demonstrating increased apoptosis while cells from others gain a survival advantage. Again a link to mutational status was noted with apoptosis induction more prominent in the mutated *IGHV* subgroup^{50, 51}. Similarly to anti-IgM stimulation, activation of CLL cells with CpG-ODN is more pronounced in patients with an *IGHV* unmutated gene compared to those with an *IGHV* mutated gene. In addition to increases in proliferation, survival and cellular activation, stimulation with CpG-ODN reproduces many other aspects of the tumor-microenvironment, including the induction of interactions between CLL cells and their T-cell counterparts⁵².

In addition to activation of the BCR or TLR signaling pathways, cytokines can also recapitulate aspects of microenvironment-induced CLL cell activation. Among the many cytokines used *in vitro* are CD40L and IL-4. Similar to the addition of CpG-ODN and anti-IgM, the addition of cytokines induces the expression of pro-survival proteins and activates signaling pathways such as NF- κ B⁵³. In response to one or more cytokines found in the tumor microenvironment, CLL cells regain their activated phenotype, exhibiting drug resistance and increased proliferation^{53–55}. In addition to reducing cell death promoting and cell division, supplementation with cytokines can also induce increased cellular adhesion to stromal cells⁵⁶. As each cytokine initiates its own specific signaling cascade upon ligation of the CLL cell, the addition of multiple cytokines can amplify the pro-survival effect^{57, 58}. For example, it has been shown that CLL cells respond to IL-4 stimulation leading to an increase in STAT6 signaling, while CD40L stimulation results in enhanced activation of NF- κ B; together these agents synergize to re-create microenvironmental signaling^{21, 59}.

Choosing the appropriate read-outs

The traditional readout for an active therapeutic agent is drug induced cell death in either lymphoma cell lines or primary CLL cells. Novel therapeutics for the treatment of CLL; however, act through multiple mechanisms including disruption of tumor-microenvironment signaling. Notably, many novel therapeutics have minimal effects on cell viability *in vitro*. Importantly, CLL is characterized not only as a cancer of disrupted apoptosis but also of deregulated proliferation and hyper-activation; therefore, inhibition of proliferation and cell activation are valuable readouts predicting potential therapeutic benefit^{47, 60}. Although the

bulk of CLL cell proliferation is thought to occur in the microenvironment, in the lymph node or bone marrow compartments, CLL cells in the peripheral blood express low levels of the proliferation marker Ki67, probably reflecting the release of recently divided cells from the tissue compartments into the peripheral blood⁴⁷. Although proliferation *in vitro* occurs at almost undetectable levels, it can be increased using the co-culture or soluble microenvironmental factors already discussed. Inhibition of proliferation is a valuable readout for novel therapeutics as the prevention of cell division results in a gradual reduction of tumor burden. Similarly, inhibition in cellular activation is a relevant readout of on-target effect and conceivably predicts for a reduction in tumor cells by changing the balance between proliferation and cell viability. In CLL, inhibition of cellular activation measures the diminished ability of the tumor to respond to its environment due to a disruption in cell signaling. Readouts of cellular activation include evaluation for reductions in cellular metabolism, activation upon stimulation, and signal transduction. Just as choosing the appropriate culture system to evaluate a novel therapeutic agent is important, choosing a readout that will be pertinent to the mechanism of action is equally crucial to successful pre-clinical modeling.

PRE-CLINICAL MODELING *IN VIVO*

In addition to the ample methods to evaluate drugs *in vitro*, *in vivo* modeling is often necessary to fully understand how new therapeutics will work in patients. Animal models are helpful in identifying unforeseen issues with metabolism (first pass effect), bio-distribution and off-target effects. In addition, such models are of specific interest given the fact that activated, proliferating CLL cells from the tissue compartments are not readily obtainable. This is especially important in the age of targeted agents where the critical targets in a given signaling pathway are often less active in the circulating cells compared to their activated tissue counterparts.

Transgenic mouse models

There are a number of mouse models that mimic CLL by deregulation of oncogenes or the deletion or overexpression of chromosomal regions. Of the currently available CLL like transgenic models, the most widely used, with the potential for pre-clinical modeling of novel therapeutics, are the T-cell Leukemia-1 transgenic (TCL1) mouse (mimicking aggressive CLL)⁶¹, the BCL-2:Traf2DN double transgenic mouse (mimicking refractory CLL)⁶², the DLEU2/miR15a/16-1 cluster-deleted mouse (mimicking indolent CLL)⁶³ and most recently the IRF^{-/-}Vh11 mouse; which has a more heterogeneous phenotype⁶⁴. Table 2 reviews the advantages and disadvantages of these transgenic models as tools for drug development in the age of targeted therapy.

The first described, most widely utilized and most researched model is the TCL1 mouse, which expresses the human TCL1 gene under the control of the immunoglobulin heavy chain variable region promoter and the IgH-E μ enhancer; thereby limiting the expression of the transgene to the B-cell population⁶¹. These mice start to show an expanded Igk clonal⁶⁵ CD5+/IgM+ population in the peritoneal cavity as early as 2 months of age⁶¹. By 4 months the expanded CD5+/IgM+population is apparent in the spleen, by 6 months it is detectable

in the peripheral blood and by 8 months in the bone marrow⁶¹. Also around 8 months, the mice begin to show signs of enlarging spleens with increased numbers of cells in the marginal zone areas that express high levels of the transgene. Between one and 1.5 years of age, most mice exhibit visible illness marked by a palpable spleen and high white blood cell counts⁶¹. The type of disease progression observed in the TCL1 mouse closely mirrors that of patients with CLL; most specifically those with aggressive IgVH-unmutated CLL, making it a good model of the natural history of the disease in this subtype⁶⁵. In addition to reflecting the disease progression of its human counterpart, tumor cells in the TCL1 model expresses many of the important signaling proteins that are of therapeutic interest for the treatment of CLL. These include BCR signaling proteins such as BTK and ERK1/2, Phosphoinositide 3-kinase (PI3K) signaling proteins such as AKT and PDK1 and anti-apoptotic proteins such as BCL-2 and MCL-1^{13, 65, 66}. Additionally, it has been demonstrated that increased TCL1 expression in human CLL corresponds to increased BCR responsiveness to antigen stimulation, again suggesting it as a useful model for the evaluation of targeted agents⁶⁷. Mimicking the natural history of human leukemia requires a substantial length of time. Recent modifications of the TCL1 model have addressed this limitation with two key adoptive transfer models. The first utilizes the injection of leukemic cells from a TCL1 mouse into SCID mice as proposed in Johnson et al., allowing for one leukemic mouse to seed multiple experimental animals⁶⁵. More recently, Chen et al. developed a leukemic TCL1 B-cell clone that reacts strongly to auto-antigens (TCL1-192) and causes an aggressive BCR dependent disease in immunodeficient mice⁶⁸. Both of these TCL1 model adaptations have been used as preclinical tools for the evaluation of novel therapeutics for the treatment of CLL^{13, 66}.

Both mice that overexpress BCL-2 (an anti-apoptotic protein up-regulated in CLL) and TRAF2DN mice (a TNF-receptor associated factor that amplifies pathway activation) develop polyclonal expansions of B-cells; however, these strains rarely develop leukemia and display normal life spans. In contrast to the single transgenic strains, the recently developed BCL-2:Traf2DN double transgenic mouse displays increased B-cell counts with severe splenomegaly and lymphadenopathy by 6 months of age⁶². The majority of these mice develop leukemia consisting of B220^{int}IgM^{high}CD5+CD11b+ cells with low proliferation rates⁶². Additionally, these leukemic cells highly expressed adhesion molecules similar to human CLL cells. By 14 months of age, 80% of the mice succumb to their disease⁶². *In vitro* leukemic cells from these mice are resistant to chemotherapeutic drugs; including dexamethasone and fludarabine, similar to relapsed refractory human CLL⁶². More recently, the leukemic cells from these mice as well as the TRAF2DN single transgenic mouse were shown to have constitutive activation of the non-canonical NF-κB pathway, suggesting their usefulness in studying highly pre-treated/refractory CLL⁶⁹.

The chromosomal deletion at 13q14 occurs in 55% of CLL cases and is even more common in indolent forms of human CLL⁷⁰. The minimal deleted region (MDR) associated with loss of 13q14 contains a cluster composed of Dleu-2/miR15a/16-1 under the control of the DLEU-2 promoter. Based on this knowledge, two mouse models were developed, one with a deletion of the conditional MDR allele (containing both Dleu-2 and miR151/16-1) and the other with deletion of only the miR15a/16-1 cluster⁶³. These mice appear normal at birth, but by one year of age display an increased frequency of CD5+B220^{low} cells in the

peritoneal cavity⁶³. By 15–18 months of age, mice from both transgenic subsets developed an outgrowth of clonal CD5+B220^{low} B-cells in the peripheral blood appearing first as monoclonal B cell lymphocytosis (MBL) in around 5% of mice and then progressing to a CLL like disease in 27% of the conditional MDR knockout and 21% of the miR deleted mice⁶³. Interestingly, a small percentage of mice from both groups (9% vs. 2%, MDR and miR deleted, respectively) developed a CD5-negative non-Hodgkin lymphoma (NHL)⁶³. When the common deleted region (CDR; deletion of a 0.9 megabase region telomeric to the DLEU-2 cluster in addition to the MDR) was deleted as opposed to just the MDR, the disease penetrance increased and the mice developed mostly CLL as opposed to MBL or NHL⁷¹. Similar to the 13q14 deletion in human CLL both the MDR knockout and the miR deleted mice displayed an indolent disease course, with only the MDR knockout mice displaying a significant reduction in overall survival compared to littermate controls. Further, CD5+ cells from both mouse strains were shown to predominantly express unmutated *IGHV* genes with stereotypic antigen binding regions⁶³. In contrast to both the TCL1 and the BCL-2:TRAF2DN mice mimicking 13q14 deletions display increased proliferation rates rather than an inherent survival benefit.

More recently, the Vh11 knock-in allele was bred into the IRF4 knockout mouse, creating a mouse deficient in IRF4 with an expanded B1 population⁶⁴. These mice develop an outgrowth of IgM+CD5+ cells around the age of 5 months, with 60% of mice developing CLL (defined as >20% of PBMCs being IgM+CD5+) and the remaining 40% MBL (defined as <20% of PBMCs being IgM+CD5+)⁶⁴. By 10 months all mice had progressed to a CLL like disease⁶⁴. These cells begin to appear in the peripheral blood between 2–4 months of age and by later time points are apparent not only in the peripheral blood but also in the spleen, bone marrow and lymph nodes; with mice presenting with massive splenomegaly and lymphadenopathy⁶⁴. The leukemic cells localized to the spleen were found to be proliferative and resistant to apoptosis *in vitro*. Although resistant to apoptosis, the leukemic cells did not display dysregulation of BCL-2 or TCL-1 but did show an increase in the expression of the anti-apoptotic protein MCL-1⁶⁴. In contrast to other transgenic models, these mice developed either indolent CLL (70%), which presented with no signs of disease, or aggressive CLL (30%) presenting with infiltration of non-lymphoid organs and tissues and enlarged livers; indicating that these mice can develop a broad spectrum of lymphoproliferative diseases ranging from MBL to aggressive CLL⁶⁴. Expression of the Vh11 allele in these mice forms a well-defined BCR (against phosphatidylcholine; PtC) promoting chronic BCR signaling leading to a shortened disease latency⁶⁴. Additionally, the development of disease in these mice has recently been linked to the expression of Notch2, providing a potential model for studying the importance of NOTCH signaling in the pathogenesis of CLL⁷².

The use of transgenic mice as a tool for pre-clinical modeling of therapeutics for the treatment of CLL was validated by Johnson et al. in the TCL1 model utilizing the traditional chemotherapeutic fludarabine, the CDK inhibitor flavopiridol and the PDK-1 inhibitor OSU03012 (AR-12). Interesting, not only did the TCL1 mouse model reproduce the type of responses seen in patients with CLL but they displayed a similar pattern of resistance to fludarabine⁶⁵. Additionally, Woyach et al. and Ponader et al. demonstrated that ibrutinib, a BTK inhibitor acting downstream of BCR activation, significantly extended survival times in

adoptive transfer models using either TCL1 leukemic cells or the TCL1-192 clone^{13, 66}. To date, only the TCL-1 transgenic mouse model has been evaluated as a pre-clinical tool for evaluation of traditional or novel therapeutic agents; however the aforementioned mouse strains all offer potential as pre-clinical models.

Transgenic mice offer many advantages for use in evaluation of targeted agents; however, there are a few key limitations to their use in pre-clinical modeling. First, transgenic mouse models lack both human target cells and microenvironmental accessory cells making them unsuitable for certain therapeutics such as antibodies against immune check point inhibitors (such as anti-PD-L1 and anti-PD1). Secondly, as described, many transgenic models inadequately model the heterogeneity of CLL. Lastly, transgenic models are biased towards signaling regulated by the altered oncogene or regulatory region and thus mimic only select mechanisms of CLL pathogenesis. For example, although the TCL1 model mimics aggressive CLL it displays wild type p53⁶⁵. While this is in accordance with a majority of human CLL, it prevents the evaluation of novel therapeutics in the setting of TP53 deletion or mutation which occurs in patients harboring a deletion of the short arm of chromosome 17; a hard to treat, high risk population. Similarly, while the IRF4(–/–)Vh11 mouse model has the advantage of mimicking the heterogeneity of CLL pathogenesis it does not reflect the dysregulation of BCL-2 observed in human CLL; a key target of novel therapeutics such as ABT-199⁷³. Mouse models mimicking deletion of 13q14 develop only indolent CLL with no clear evidence of increased expression of pro-survival or anti-apoptotic factors, potentially limiting their usefulness in evaluation of targeted agents⁶³. The BCL2:TRAFDN double transgenic mouse offers a unique insight into a more refractory disease and does present with increased BCL-2 and NF-κB signaling; however along with the traditional TCL1 model the tumor cells don't display an increased proliferation rate^{61, 62}. For these reasons, matching a transgenic model to the scientific question and the therapeutic agent under investigation is paramount.

Although too numerous to discuss in detail, other transgenic models are available that could be used as pre-clinical models to study drug development in CLL. These have been recently reviewed by Chen et al. and Simonetti et al.^{74, 75}. The understanding of CLL biology has increased dramatically in the past decade, as has the ability to engineer mice expressing key players in CLL pathogenesis. This will potentially allow for the generation of novel transgenic mice in the future that may assist in furthering the understanding of the biological differences between distinct subsets of CLL as well as in the evaluation of novel therapeutics in a more individualized way.

Xenograft mouse models

A complementary approach to transgenic mouse models has been the xenograftment of cell lines or primary CLL cells into immune-compromised mice. Until recently, establishment of xenograft models was hampered by the lack of mouse strains sufficiently immunosuppressed to sustain the expansion of many human cell lines and especially of primary cells. In the last two decades this picture has changed due to the availability of severely immunodeficient mice; most notably the NOD/SCID and the NOD-scid IL2R γnull (NSG) mice^{76, 77}. Although both of these models lack mature B- and T-cells and display decreased innate

immunity, the NOD/SCID mice display only muted NK cell activity while the NSG mice are characterized by the absence of NK cells^{76, 77}. Of the available models, the three most widely utilized primary xenograft models are the NOD/SCID primary xenograft model first described by Dürig et al., 2007, the NSG primary xenograft model first described by Bagnara et al., 2011 and the adapted NSG primary xenograft model described by Herman et al., 2012^{78–80}. The key features of these models are summarized in Table 3.

In the model proposed by Dürig et al. 100×10^6 primary CLL PBMCs were injected both intraperitoneally (i.p) and intravenously (i.v) into irradiated NOD/SCID mice. In this model human CD19+CD5+CD23+ CLL cells were found in minimal numbers in the peripheral blood while significant engraftment was observed in the spleen and peritoneal cavity, and to a lesser extent the bone marrow⁷⁸. CLL cell numbers were found to be maintained up to 8 weeks. In the spleen, focal aggregates of CLL cells were observed which stained positive for the proliferation marker Ki67; suggesting that the spleen microenvironment can support CLL cell proliferation⁷⁸. Perhaps most importantly, engraftment in this model was shown to correlate with increased disease activity in the patient from whom the cells were obtained. Most notably, higher Binet stage, LDH serum activity and shorter lymphocyte doubling time predicted for superior engraftment, suggesting that the model is capable of reproducing the clinical heterogeneity observed in human CLL⁷⁸.

Building on this work, Bagnara et al. described a primary CLL xenograft model in which 10^5 normal human cord blood-derived hCD34+ cells were injected i.v. or by intrabone injection prior to the injection of 100×10^6 CLL PBMCs, via the same injection route, into irradiated NSG mice⁷⁹. Alternatively, co-injection of 20×10^6 normal mature allogeneic APCs (CD14+ or CD19+ cells) with patient PBMCs was also utilized with similar results⁷⁹. Strikingly, growth of CLL cells in this model was found to be dependent on CD4+ autologous T-cells⁷⁹. This is supported by the fact that proliferation of the CLL cell population was not observed until after expansion of patient-derived T-cells. Further, administration of either anti-CD3 or anti-CD4 monoclonal antibodies (mAbs), but not anti-CD8 mAbs, resulted in the termination of CLL cell expansion⁷⁹. Again, CLL PBMCs were found to predominantly localize to the spleen, bone marrow and peritoneal cavity, with proliferation occurring to a greater extent in the spleen in follicular structures of CLL cells surrounded by T-cells⁷⁹. Of note was the correlation of CD38 expression between the xenografted mice and the donor patient. It has been well established that CD38 expression has both biologic (via signal transduction) and prognostic significance; with patients expressing CD38 in more than 30% of their CLL clone presenting with a more rapid progression of disease⁸¹. In this model, expression of CD38 was increased in secondary tissue compartments (such as spleen and bone marrow) compared to the circulating cells, mirroring what has been observed in human CLL^{79, 82}. Surprisingly, the level of CD38 expression in the mice and the donor patient's peripheral blood were concordant, suggesting that this model can maintain clinical parameters important to the clinical course of CLL⁷⁹.

Expanding on this work, our group developed a simplified xenografting method⁸⁰. In Herman et al., PBMCs collected from CLL patients were injected without the adoptive transfer of other hematopoietic cells (such as allogeneic APCs or hCD34+ cells) in contrast to the model presented by Bagnara et al., 2011. Additionally, 25mg/kg busulfan was used

instead of irradiation to condition the mice prior to the injection of PBMCs. As expected, CLL cells localized predominantly to the murine spleen, with only a minor fraction detectable in the bone marrow⁸⁰. CLL cell infiltration of the spleen occurred in a nodular pattern, near sites of vascularization, surrounded by CD3+ T-cells⁸⁰. The CLL cells in the murine spleen displayed elevated proliferation compared to those in circulation, mirroring the compartmentalization of proliferation observed in patients^{47, 80}. Further, CLL cells localized to the murine spleen displayed more cellular activation as defined by increased expression of CD38 and CD69 and decreased expression of CXCR4 compared to those in circulation, again emulating CLL cells from the human lymph node compartment^{47, 80}. In addition to mimicking the activation and proliferation of CLL cells localized to the tissue microenvironment, CLL cells harvested from the murine spleen displayed increased BCR and NF- κ B signaling. This suggests that the partially humanized microenvironment created in xenograft models can induce signaling pathways important in the maintenance of CLL; many of which are currently being targeted for treatment. Evidence of the overlap in microenvironmental stimulation between the murine spleen and the human lymph node is displayed in Figure 2.

Primary xenograft models for studying CLL may be particularly useful in the pre-clinical modeling of novel targeted agents. These models are advantageous for a variety of reasons. First, they utilize human cells and thus express human antigens. This is an important difference from transgenic mouse models where certain novel therapeutics (such as antibodies against human PD-L1) cannot be evaluated without the development of antibodies that cross-react with murine antigens. Additionally, xenograft models have the advantage of creating a humanized environment, allowing for the evaluation of drug effects on CLL cells as well as on the tissue microenvironment (either directly or indirectly). Further, the use of human cells allows for a better evaluation of off-target effects as signaling pathways between mice and humans may differ⁸³. Secondly, the gene and activated protein pattern observed in the lymph node and bone marrow of patients can be recapitulated in some xenograft models⁸⁰. This is important as many targeted agents (especially kinase inhibitors) seem to exhibit a majority of their effect through disruption of signaling between tumor cells and the microenvironment. Lastly and potentially most importantly, the injection of mixed cell populations in the primary xenograft models allows for evaluation of cell-cell interactions in a partially humanized microenvironment. This aspect is becoming increasingly important with novel targeted agents as many of these have mechanisms of action that may involve accessory cells. There is extensive *in vitro* evidence that accessory cells of the lymphoid, myeloid and mesenchymal cell lineages play a role in maintaining CLL cells^{24, 28, 36, 84, 85}. In the primary xenograft models these interactions can not only be evaluated in the context of the microenvironmental architecture, but also dissected through the selective addition, removal or modification of accessory cells. Further, Oldreive and colleagues recently demonstrated that the biological properties of CLL CD4+, CD8+ and regulatory T-cells are maintained in a xenograft model of primary CLL⁸⁶. This makes primary xenograft models a suitable pre-clinical model for evaluating novel therapeutics that directly target accessory cells, such as nivolumab (anti-PD-1 antibody), which has shown impressive results in Hodgkin lymphoma and mainly acts by restoring the ability of T-cells to eliminate tumor cells⁸⁷.

Primary CLL xenograft models as a tool for pre-clinical modeling of therapeutics for the treatment of CLL has been validated by Herman et al. utilizing ibrutinib⁸⁰. Interesting, not only did ibrutinib reduce tumor burden in the xenograft mouse model, but an initial efflux of cells from the microenvironment into the peripheral blood was seen in the treated mice similar to the observation of ibrutinib-induced lymphocytosis in clinical trials^{5, 15, 88}. Additionally, reductions in proliferation, signal transduction (specifically BCR and NF- κ B signaling) and increased rates of cell death were observed, mirroring what was seen in patients^{15, 80}. This suggests that primary xenograft models have the potential to be valuable tools for the pre-clinical modeling of CLL in the era of targeted therapies.

The utility of xenografted mice is somewhat limited by the short time frame from xenograftment to model limiting end points. Although these models have been demonstrated to be dependent on T-cells, within 12 weeks of engraftment mice expired, at which time an infiltration of human T-cells into the liver was noted, suggestive of a graft-vs-host response⁷⁹. This somewhat limits the application of xenograft mice as pre-clinical tools for drug development as the demise of mice is not due to tumor progression and not a reflection of drug activity. New variations on these models utilizing autologous activated T-cells may eliminate some of the confounding factors such as the need for allogeneic human cells, potentially eliminating graft vs. host disease⁸⁹. It remains to be seen whether evaluation of survival will become a meaningful endpoint for primary xenograft models of CLL.

Primary CLL xenograft models appear to mimic the state of disease at the time of sample acquisition (the disease state of the donor patient) and do not appear to evolve over time; perhaps due to the short follow up time available with these models. This limits their application as tools to study therapeutics that could prevent progression of disease. Advancements have been made to overcome this limitation by Kikushige et al. by xenografting CLL-HSCs into NSG mice allowing for the evaluation of the emergence and progression of the tumorigenic B-cell lineage. This model demonstrated that neither CD19+ CLL cells nor ProB-cells could produce expansion of human cells; however CLL-HSCs could give rise to secondary-HSCs, progenitor HSCs, myeloid cells and B-cells in the bone marrow of xenografted mice⁹⁰. Many of these mice developed a MBL like disease, with B-cells frequently expressing CD5 as well as *IGHV* gene rearrangements that were unrelated to the one in the original CLL clone⁹⁰. Notwithstanding, more work is still needed to develop a xenograft model that can fully mimic the development and progression of human CLL.

Xenograftment of either primary CLL cells or cells lines has been reviewed in Bertilaccio, et al. and Chen et al.^{74, 91}. Supplementary to xenograft models utilizing primary CLL cells, Bertilaccio et al. developed a xenograft model using a well characterized human CLL like cell line (MEC1)⁹². In this model 10×10^6 MEC1 cells were subcutaneously injected into the left flank of *Rag2*^{-/-} γ_c ^{-/-} mice (an immunocompromised mouse strain similar to the NSG mouse in that it lacks NK cells). Solid discoid subcutaneous masses formed at the site of injection, but the presence of MEC1 cells was also found in the spleen, kidney, liver and most notably in the axillary tumor-draining lymph node and bone marrow⁹². Similar to the primary xenograft models, the injected cells formed focal aggregates that displayed a high proliferation rate; although in this model they formed predominately in the kidney⁹². The

key limitation of this model is that it utilizes an EBV transformed cell line; which can drive proliferation independent of mechanisms active in human CLL. The MEC1 xenograft model has been validated as a tool for drug development, mimicking the response to fludarabine observed in the TCL-1 transgenic mouse⁹². Thus, this model may also represent a simple and reproducible tool for pre-clinical testing of CLL therapeutics.

“De novo” mouse and canine models

In contrast to all other mouse models of CLL, the New Zealand Black (NZB) mouse naturally develops a CLL-like disease without the induced expression of oncogenes or deletion of regulatory regions. Similar to human CLL, NZB mice present with an age-associated expansion of CD5+ B-cells that appears to be preceded by an MBL like state^{93, 94}. Although similar in many regards to CLL, the biggest difference is that MBL in the NZB mice always progresses to CLL while in humans only 1% of patients with MBL will progress to CLL, requiring treatment each year. The expanded B-1 population is typically IgM+B220^{dim}CD5^{dim} and is correlated with increased serum levels of IL-10, a cytokine also up-regulated in human CLL cells⁹³. In addition to mimicking disease progression and general phenotype, the disease in NZB mice is often associated with chromosomal abnormalities, ZAP-70 expression and genetic alterations of the mir-15a/16-1 locus, resulting in a decrease in mature miR-15a and miR16; reflecting the 13q14 deletion⁹⁵. Another parallel between human CLL and the NZB mouse model is some degree of autoimmunity. NZB mice spontaneously develop autoimmune hemolytic anemia, which also occurs in a subset of patients with CLL⁹³. Although the NZB model has not been widely utilized as a tool for pre-clinical drug development it could be particularly useful to study strategies to prevent progression of MBL to CLL.

Cancers in companion animals, such as dogs, arise spontaneously and may present with anatomical and physiological similarities to their human counterparts⁹⁶. As there is currently no defined standard of care established for canine cancers, early upfront testing of novel therapies is warranted. In addition to being an outbred model with more genetic similarity to humans (as compared to mice), canine models also offer the advantage of allowing for serial biopsies and blood collections which is not always obtainable in mice, allowing for study designs similar to their human counterpart⁹⁷. For these reasons, treating canine B-cell NHL is commonly being used as a pre-clinical model for novel therapeutics. As an example, the first published report evaluating ibrutinib *in vivo* was in canine B-cell NHL, demonstrating impressive results in both previously treated and treatment naïve dogs⁹⁸.

CONCLUSIONS

Of the many drugs that enter pre-clinical testing for the treatment CLL only a small percentage successfully transition into clinical use. Although this may reflect a competitive market, it also may reflect flaws of classical *in vitro* models to adequately predict the most effective drugs. This is especially important in the age of novel therapeutics that do not directly induce cell death, but work through disruption of signaling pathways or tumor-microenvironmental signaling. There are many diverse *in vitro* and *in vivo* models available

for studying CLL; the key is to choose the model that is most applicable for the therapeutic agent under investigation.

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Figure 1. Recapitulation of the CLL tissue microenvironment *in vitro*

In vivo CLL cells receive signals from multiple cell types leading to cellular activation, proliferation and survival. This can be recreated *in vitro* by the addition of accessory cells such as stromal cells, NLCs or other PBMC subsets (represented in green) that alter CLL cell signaling through either direct cell-cell contact or through the release of cytokines or chemokines such as IL-4, IL-8 or CXCL12. Additionally, CLL cell activation and signal transduction that occurs in the microenvironment can be replicated by the addition of soluble factors (represented in red) such as anti-IgM, which ligates the B-cell Receptor (BCR), CpG-ODN, which activates toll-like receptor 9 (TLR9) in endosomes, or cytokines such as sCD40L or IL-4. Binding of these soluble factors to their respective receptors leads to activation of key CLL signaling pathways, including BCR, PI3K, NF- κ B and JAK/STAT, leading to a multitude of downstream events including the up-regulation of pro-survival proteins (such as MCL-1, BCL-2, XIAP and survivin).

Figure 2. Recreation of the human lymph node microenvironment in the spleen of NSG mice (A) PBMCs were collected from the lymph node (hLB) or the peripheral blood (hPB) of patients with CLL. PBMCs from the hPB were then xenografted into NSG mice. After 3 weeks human PBMCs were recollected from the mouse peripheral blood (mPB) or the mouse spleen (mSP). (B) CLL cells collected from the mouse or the human microenvironment display similar expression of BCR and NF-κB gene signatures (the average of validated BCR and NF-κB target genes) (B) surface activation markers CD38, CD69 and CXCR4 (C) and proliferation (D) compared to peripheral blood (PB). All readouts were significantly increased in both microenvironments compared to peripheral blood ($P < 0.05$), but there was no significant difference between the up-regulation induced by the mouse spleen and the human lymph node; statistics done by student t-test. All data is shown as the mean $\pm$ SEM normalized to matched peripheral blood. Adapted from Herman et al.⁸⁰

Table 1

Benefits and potential drawbacks of co-culture and conditioned media model systems as tools for drug development.

Microenvironmental stimuli	Model type	Benefits	Drawbacks	References
Stromal cell	Co-culture	Prolonged CLL cell survival; increased PI3K and NF- κ B signaling; increased expression of anti-apoptotic proteins; expression of antigens recognizable by stereotypic IgM receptors, activating BCR signaling	Requires cell-cell contact	25–27, 31 and 32
NLC	Co-culture	Prolonged CLL cell survival; activation of signaling pathways (p44/42 MAPK, BCR and NF- κ B); increased expression of anti-apoptotic proteins; activates CXCR4; increases production of CCL3 and CCL4.	Requires cell-cell contact	35, 37 and 40
CXCL12	Soluble factor	Prolonged CLL cell survival; activation of signaling pathways (p44/42 MAPK and PI3K); plays a role in the switch to a pro-angiogenic phenotype; induces cell migration	Requires expression of CXCR4 – which is internalized upon binding CXCL12	35 and 39
anti-IgM	Soluble factor	Increased BCR and NF- κ B signaling, increased expression of anti-apoptotic proteins; up-regulation of gene signatures (including metabolism and cell-cycle), increased proliferation and cell survival; increases production of CCL3 and CCL4.	Response varies by <i>IGHV</i> gene mutational status and method of IgM crosslinking	41–43 and 45
CpG-ODN	Soluble factor	Induces proliferation and cell survival; increases the production of pro-survival cytokines (IL-6 and TNF α); increases the expression of surface activation molecules (CD40, CD80, CD86); induces interactions with T-cells	Response varies by <i>IGHV</i> gene mutational status	47, 48, 50 and 51
CD40L	Soluble factor	Induces pro-survival proteins and signaling pathways (NF- κ B); increases proliferation and drug resistance; increases adhesion to stromal cells	Takes a combination of cytokines to elicit a strong effect	52, 53, 55, 57 and 58
IL-4	Soluble factor	Induces pro-survival proteins and signaling pathways (JAK/STAT); increases proliferation and drug resistance; increases adhesion to stromal cells	Takes a combination of cytokines to elicit a strong effect	52, 54, 55, 57 and 58

Table 2

Advantages and disadvantages of different transgenic mice for the pre-clinical modeling of targeted therapies.

Model	Model type	Description	Advantages	Disadvantages	Reference
TCL-1	Transgenic	TCL1 gene under the control of Ig VH promoter/IgH-Eμ enhancer	Aggressive CLL (disease apparent in mice 1–1.5 yrs. old); express BCR, PI3K and anti-apoptotic proteins; BCR responsive	Time to disease onset; overexpression of TCL-1; wildtype p53	60
SCID (TCL-1)	Transgenic	Adoptive transfer of TCL-1 leukemic cells into SCID mice	Aggressive CLL; express BCR, PI3K and anti-apoptotic proteins; BCR responsive; validated as a tool for drug development	Time to disease onset; overexpression of TCL-1; wildtype p53	64
SCID (TCL1-192)	Transgenic	Adoptive transfer of TCL1-192 clonal cells into SCID mice	Very aggressive CLL; express BCR, PI3K and anti-apoptotic proteins; heightened BCR responsiveness; validated as a tool for drug development	Time to disease onset; overexpression of TCL-1; wildtype p53	67
BCL-2;Traf2DN	Transgenic	Double transgenic of the BCL-2 overexpressing mouse and the Traf2 dominant negative mouse	Refractory like CLL (disease apparent in mice 6 months old); display resistance to chemotherapy; constitutive activation of NF-κB	No increase in leukemic cell proliferation	61
DLEU2 MDR	Transgenic	Deletion of the conditional MDR allele (containing both Dleu-2 and miR151/16-1)	Mimics indolent CLL with del13q14 (disease apparent in mice 1–1.5 yrs. old); displays unmutated <i>IGHV</i> genes with stereotypic antigen binding regions; leukemic cells are actively proliferating	Can develop CLL or NHL; no clear dysregulation of anti-apoptotic proteins; low penetrance; long latency	62
miR15a/16-1 cluster-deleted	Transgenic	Deletion of only the miR151/16-1 cluster	Mimics indolent CLL with del13q14 (disease apparent in mice 1–1.5 yrs. old); displays unmutated <i>IGHV</i> genes with stereotypic antigen binding regions; leukemic cells are actively proliferating	Can develop CLL or NHL; no clear dysregulation of anti-apoptotic proteins; no survival disadvantage compared to littermates; low penetrance; long latency	62
IRF-/-Vh11	Transgenic	Vh11 knock-in allele bred into the IRF4 knockout mouse	Develop MBL followed by CLL (all mice have CLL like disease by 10 months); develop both indolent and aggressive disease; increased expression of MCL-1; heightened BCR responsiveness; link to NOTCH signaling	No dysregulation in BCL-2	63

Table 3
Xenograft and de novo mouse models for the evaluation of targeted agents for CLL.

Strain	Model type	Protocol	Description/Advantages	Disadvantages	Reference(s)
NOD/SCID	Primary xenograft	i.v. injection of 100e6 CLL PBMCs	Stable engraftment of spleen, bone marrow and peritoneal cavity through week 8; proliferation of CLL cells in the spleen; reproduces clinical heterogeneity of CLL; humanized microenvironment	No survival data	77
NSG	Primary xenograft	i.v. injection of 100e6 CLL PBMCs with either 10 ⁵ hCD34+ cells or 20e6 normal allogeneic APCs	T-cell dependent expansion of CLL cells; engraftment of spleen, bone marrow and peritoneal cavity; proliferation of CLL cells in the spleen; reproduces CD38 expression seen in patients; humanized microenvironment	No survival data; graft-vs-host response	78
NSG	Primary xenograft	i.v. injection of 100e6 CLL PBMCs	Engraftment of spleen and bone marrow; Spleen microenvironment mimics the proliferation, cell activation and signal transduction observed in the human lymph node; humanized microenvironment; validated as a tool for drug development	No survival data; graft-vs-host response; minimal bone marrow engraftment	79
Rag2 ^{-/-} γc ^{-/-}	Cell line xenograft	subcutaneous injection of 10e6 MEC1 cells	Solid subcutaneous masses form at injection site; presence of MEC1 cells in spleen, kidney, liver, axillary tumor-draining lymph node and bone marrow; validated as a tool for drug development	EBV transformed cell line; no humanized microenvironment	91
NZB	de novo	mouse strain develops CLL spontaneously	Naturally occurring CLL that progresses from an MBL state; displays similar clinical features as CLL	Long latency; no human cells or microenvironment	92 and 94