Molecular Pathways

The Molecular Basis of Lmo2-Induced T-cell Acute Lymphoblastic Leukemia

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Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is commonly caused by the overexpression of oncogenic transcription factors in developing T cells. In a mouse model of one such oncogene, LMO2, the cellular effect is to induce self-renewal of committed T cells in the thymus, which persist long-term whilst acquiring additional mutations and eventually giving rise to leukemia. These precancerous stem cells (pre-CSCs) are intrinsically resistant to radiotherapy, implying that they may be refractory to conventional cancer therapies. However, they depend on an aberrantly expressed stem-cell-like self-renewal program for their maintenance, in addition to a specialised thymic microenvironmental niche. Here we discuss potential approaches for targeting pre-CSCs in T-ALL by using therapies directed at oncogenic transcription factors themselves, downstream self-renewal pathways and the supportive cell niche.

Background

T-cell acute lymphoblastic leukemia (T-ALL) is a common pediatric leukemia representing approximately 20% of all ALL (1). Around half of T-ALL cases harbour recurrent chromosomal translocations leading to aberrant T-cell expression of a variety of oncogenic transcription factors. Most commonly, these involve the homeobox genes TLX1 or TLX3, or components of basic helix-loop helix (bHLH) complexes, including bHLH factors themselves (SCL/TAL1, LYL1) and their binding partners, the LIM-domain only factors LMO1 or LMO2 (2). Expression profiling of T-ALL reveals distinct gene signatures according to the underlying chromosomal abnormality, suggesting that the mechanism of leukemogenesis is oncogene-specific (3). Despite this genetic heterogeneity, current therapies for both B- and T-cell ALL are essentially identical using multi-drug combinations, prolonged low dose maintenance chemotherapy and allogeneic bone marrow transplant for high-risk or relapsed disease. Unfortunately, with this generic approach, 20% of children and 50% of adults succumb to relapsed disease (1). A better understanding of how these chromosomal translocations cause leukemia will provide rational approaches for identifying new therapeutic strategies.

Aberrant expression of the LMO2 gene in T-cells accounts for about 9% of T-ALL (4). In addition, activation of LMO2 by retroviral insertion was the cause of T-ALL in 4 patients receiving gene therapy for X-linked Severe Combined Immunodeficiency (X-SCID) (5-8). In normal hematopoiesis, expression of LMO2 is restricted to adult hematopoietic stem cells (HSCs) and the erythroid lineage, and is tightly regulated by a network of transcription factors including LMO2...
itself, SCL, GATA1 and ETS factors (9). LMO2 is essential for the formation of yolk sac erythropoiesis, leading to embryonic death at E10.5 (10). In contrast, LMO2 expression is extinguished early in T cell development and is not required for normal development of this lineage (11). In erythroid cells, Lmo2 functions as a bridging molecule assembling a multi-protein transcriptional complex that includes hetero-dimers of E proteins and Scl or Ly11, as well as Gata1 and Ldb1 (12). This complex binds to DNA through the bHLH factors and GATA1, recognising a bipartite DNA sequence comprising an E box and a GATA site. This core bHLH-GATA complex can act as a repressor or activator of transcription depending upon the recruitment of other partner proteins, which occurs in a cell stage-specific manner.

In a mouse model of Lmo2-induced T-ALL, a different multi-protein complex was identified in the immature CD4^+ CD8^- double negative (DN) T-cells of the thymus, comprising Lmo2, Scl, E47 and Lbd1, which binds to a bipartite E-box motif (13). Differing multi-protein complexes in normal and T-ALL cells provides a window for specificity of therapeutics that could disrupt the leukemic Lmo2 complex but spare the normal complex that may be essential for maintenance of HSCs and/or erythropoiesis.

Animal models of Lmo2-induced T-ALL provide an invaluable tool for understanding the mechanism of leukemogenesis and testing new therapies. Transgenic mice expressing Lmo2 in T-cells develop T-ALL, albeit after a long latency period (14, 15). A similar mouse model of T-ALL involves the overexpression of Scl with or without Lmo1 (16-18). Due to the similarity between Lmo1 and Lmo2, and the fact that Scl binds to Lmo2 and can accelerate Lmo2-driven leukemogenesis (19, 20), we will consider these models together, although the possibility remains that distinct molecular mechanisms underlie the two models. In both transgenic models, there is a delayed onset of T-ALL due to the requirement for additional genetic mutations, which include activating mutations of Notch signalling and loss of the Cdkn2a locus, both of which are also observed in LMO2-associated human T-ALL (7, 8, 21-23).

To understand the mechanism of leukemogenesis by SCL/LMO proteins, a number of groups have examined the thymi of pre-leukemic transgenic mice. These studies reveal a pre-leukemic phenotype characterised by a small thymus with increased apoptosis and an expanded proportion of immature DN T-cells (20, 24). Gene expression studies of these pre-leukemic thymocytes reveals a loss of T-cell differentiation genes, most notably the pre-T cell receptor alpha subunit (pTα, Figure 1), which is required for progression of DN T-cells to the CD4^+ CD8^- double-positive (DP) stage (17). The phenotype of E-protein deficient mice is similar to that of Scl/Lmo transgenic mice and
reduced E protein expression in Scl transgenic mice accelerates T-cell leukemogenesis\(^{(25, 26)}\). Together, these studies have concluded that the SCL and LMO oncogenes induce leukemia by perturbed T-cell differentiation through loss of E protein function.

Given the multigenic nature of LMO2-induced T-ALL, we reasoned that impaired differentiation would not alone be sufficient for DN thymocytes to acquire the requisite additional genetic mutations. Indeed, blocked T cell development due to impaired TCR recombination or expression is insufficient to induce T-ALL in mouse models\(^{(27)}\). Accordingly, using a combination of \textit{in vivo} cell marking and transplantation of thymocytes from young Lmo2-transgenic mice, we have recently demonstrated that the primary cellular effect of Lmo2 is to induce self-renewal committed T cells in the thymus at the DN3 stage of T cell development (CD4\(^{-}\)CD8\(^{-}\)CD44\(^{-}\)CD25\(^{-}\))\(^{(28)}\). This population of cells has many features of cancer stem cells (CSCs) including the ability to serially transplant, the ability to generate mature T-cells and the expression of a stem cell signature. However, as these self-renewing thymocytes were evident soon after birth, up to one year before overt T-ALL, we refer to them as precancerous stem cells (pre-CSCs).

Evidence for CSCs in T-ALL is provided by the ability to propagate human T-ALL in NOD-SCID mice\(^{(29)}\). Similar to AML and B-ALL, CSC activity is enriched in the rare CD34\(^{+}\)CD4\(^{-}\)CD7\(^{-}\) sub-fraction of T-ALL\(^{(30-32)}\). The CSC hypothesis posits that the stem cell-like properties of CSCs make them more resistant to therapies including chemo-radiation. Whilst there is limited experimental evidence for this we have shown that self-renewing Lmo2-transgenic thymocytes can survive high dose irradiation\(^{(28)}\). This intrinsic resistance suggests that pre-CSCs may survive conventional cancer therapies and mediate disease relapse. In support of this, array CGH studies of matched diagnosis and relapse samples show that a significant number of relapses arise from cells that are ancestral to the predominant clone at diagnosis\(^{(33)}\).

To elucidate the molecular basis of LMO2-induced self-renewal, we performed gene expression analysis using DN thymocytes from young, preleukemic Lmo2 transgenic mice. Surprisingly, this revealed fewer than 200 gene expression changes, many fewer than similar analyses of overt leukemic cells, likely reflecting the lack of further genetic mutations in Lmo2-induced pre-CSCs. In addition to the previously reported loss of T-cell differentiation genes, we identified 81 upregulated genes, of which 48 were more highly expressed in normal HSCs than in immature thymocytes, implying that Lmo2 may induce self-renewal via upregulation of an HSC-associated gene expression program. Gene Set Enrichment Analysis (GSEA) of this stem cell signature with overt human T-cell ALL showed that this was significantly associated with the LMO2/LYL1-expressing
subset of T-ALL. This suggests that LMO2-induced self-renewal pathways persist into overt T-ALL in humans and implies that studying pre-CSCs provides a rationale for understanding how to overcome therapeutic resistance. We will discuss potential avenues for targeting pre-CSCs in T-ALL including targeting the transcription factors themselves, downstream self-renewal pathways and microenvironmental survival signals (Figure 1).

**Clinical-Translational Advances**

*Targeting the Lmo2-containing transcription complex*

The Lmo2 complex provides a rational target for therapy based upon the observation of oncogenic addiction that has been best demonstrated experimentally by inducible models of c-myc driven lymphoma (34, 35). Oncogenic addiction infers that secondary mutations are unlikely to replace the function of the initiating oncogenic event. Accordingly, it has been shown that Lmo2 function is essential for the maintenance of Lmo2-induced T-ALL in mice (36).

When considering therapeutic targeting of transcription factors, eliminating their expression poses a major technical hurdle, whilst targeting critical protein-protein interactions is a more viable strategy. In the case of Lmo2, it is a bridging molecule, hence there are at least two protein-protein interactions that can serve as targets. Firstly, LMO2 does not bind to DNA directly, but rather interacts with the DNA-binding bHLH factors LYL1 or SCL. This interaction should be essential for the oncogenic function of Lmo2. Secondly, LMO2 interacts with the Lim-domain-binding protein LDB1. The phenotype of Ldb1 knockout mice suggests that it is an essential cofactor for Lmo2 (37).

With regard to targeting LMO2, two successful approaches have been recently developed by the Rabbitts laboratory. Firstly, intracellular single chain antibodies, or intrabodies, with LMO2 binding specificity have been shown to effectively block leukemic T-cell growth (38). As intracellular delivery of such large molecules remains problematic, a peptide library screen was undertaken and an 8 amino acid peptide aptamer was shown to disrupt the Zn binding CysXXCys motif of the LIM finger 4 domain of LMO2 (36). Like the intrabody-mediated approach, this peptide aptamer could block both normal and leukemic functions of Lmo2. These studies provide proof of principle that targeting the LMO2 complex may be an effective therapy for LMO2-induced T-ALL.
**Targeting critical downstream mediators of self-renewal**

An alternate approach to targeting the LMO2 complex directly would be to target critical downstream components of the LMO2-induced self-renewal program. Our gene expression analysis revealed a small number of HSC-associated candidate genes that may be responsible for self-renewal (28). The most compelling of these is the hematopoietic expressed homeobox gene *Hhex*, which is overexpressed 10-fold in Lmo2-transgenic thymocytes. The central homeodomain of Hhex is most similar to the T cell oncogene TLX1, with an identical threonine at position 47 that determines DNA-binding specificity (39). Enforced expression of Hhex was able to induce self-renewal of T-cells similarly to Lmo2 and this gene has previously been shown to induce T-ALL in mice, implying that it could be a critical downstream component of the Lmo2-induced gene expression program (28, 40). The N-terminal domain of HHEX localises it to the nucleolar PML body through interactions with the PML protein (41). Whilst there are no current therapeutics that target homeodomain transcription factors, a speculative possibility is that arsenic trioxide, which eradicates CSCs in promyelocytic leukemia by targeting the PML body for degradation, might likewise reduce HHEX expression (42, 43).

The NF-κB signalling pathway is commonly deregulated in a variety of cancers. It is activated early in Scl-induced leukemogenesis, possibly through transcriptional activation of IKK or repression of p50 (44, 45). Moreover GSEA of the Lmo2-induced pre-CSC gene signature revealed enrichment for targets of the NF-κB pathway (our unpublished data). Irrespective of the mechanism, the NF-κB pathway is activated in the majority of T-ALL patient samples and has been shown to be a major mediator of Notch-driven T-ALL (46, 47). Whilst NF-κB signaling was shown to be dispensable for maintenance of Scl-induced T-ALL cell lines, its role in the maintenance of self-renewing pre-CSCCs remains to be investigated (44).

**Targeting the pre-CSC niche**

Whilst Lmo2-induced pre-CSCCs show inherent capacity for self-renewal, this can only occur within the thymus, implying that survival signals are provided by the thymic epithelium to provide an supportive environment in which self-renewal can occur. Amongst the likely candidates for such niche signals are two stromal-derived signals that are key components of normal thymic T cell development.
Lmo2-transgenic thymocytes self-renew specifically the DN3 stage of T cell development (28). Amongst the signals required for normal DN3 cell survival is Stem Cell Factor (SCF), which is presented in membrane-bound form by the thymic stroma (48). The Stem Cell Factor receptor Kit is overexpressed in Lmo2-induced pre-CSCs, as are two targets of Kit-mediated signaling, the transcription factors Stat3 and Stat5 (28). Together, these data suggest that SCF-induced Stat signaling may be a critical survival signal for these cells. Indeed STAT5 has been previously associated with T-ALL through activation of the kinases JAK1 and JAK2 by activating mutations and chromosomal translocations, respectively (49, 50). If Stat proteins are required for Lmo2-induced pre-CSC survival, they represent attractive targets for therapy, either directly, through the use of Stat-inhibitory drugs, or via inhibition of upstream Kit signalling using the kinase inhibitor Imatinib.

Another critical survival factor at the DN3 stage of T cell development is Notch signaling (51, 52). The Notch ligand DL4 expressed on the thymic stromal epithelial cells is required for survival and proliferation of DN3 cells through regulation of multiple pathways including c-myc, the IL-7 receptor, and the PI3K and NFkB pathways (47, 53-56). Activation of the Notch signalling pathway by mutations in either the γ-secretase or PEST domains are found in more than 50% of all T-ALL including Lmo2-induced T-ALL (23). In mouse models, activation of Notch co-operates with Scl/Lmo1 in inducing T-ALL (57). Notch signaling can also be increased by loss of function mutations of FBW7, a component of the E3-ubiquitin-ligase complex required for degradation of active intracellular Notch (58). Constitutive Notch signalling allows T-cell development to occur independent of the thymic cell niche (59). In Lmo2 transgenic mice, Notch signalling is not increased in young thymocytes, supporting the premise that Notch mutations appear to be secondary events (60, 61). Taken together, these results suggest that Notch mutations allow pre-CSCs to escape the cell niche requirement of DN3 thymocytes. However, it remains to be determined whether, like normal DN3 cells, Lmo2-induced pre-CSCs require cell niche-derived Notch signals for survival.

Chemical γ-secretase inhibitors (GSIs) can effectively inhibit Notch signalling and show efficacy in T-ALL cell lines and in xenograft models (62). However the use of GSIs in vivo is limited by gut toxicity from inhibition of both NOTCH1 and NOTCH2 (63, 64). Such toxicity can be largely ameliorated by concomitant treatment with the anti-T-ALL therapeutic dexamethasone, suggesting that combined use of these two agents may be a more effective therapy (62). Moreover, recently small molecule inhibitors and antibodies targeting specific Notch receptors have been developed that are likely to have less toxic side-effects (64, 65).
Finally, in addition to inducing self-renewal, SCL/LMO proteins inhibit T cell differentiation through transcriptional repression of target genes required for progression through the beta-selection checkpoint. Foremost amongst these is the pre-T cell receptor alpha chain (pTα), which is a direct transcriptional target of these oncogenes (17). Restoration of these molecules could foreseeably force differentiation of pre-CSCs and inhibit the self-renewal process.

Although relevant mouse models have existed for almost 20 years, much remains to be learned about how transcription factor oncogenes such as LMO2 cause leukemia. A similar time lag between the discovery of the BCR-ABL chromosomal translocation and effective tyrosine kinase inhibitors provides the fortitude to continue this pursuit. Through this understanding we can hope to develop CSC-targeted therapies that will improve the treatment of resistant or relapsed disease.
References


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Figure Legend

**Fig. 1. Molecular pathways in Lmo2-induced self-renewing thymocytes.** LMO2-LDB1 forms a bridge between heterodimers of E47 and LYL1. This leads to repression of T-cell differentiation genes including pTα, perhaps through loss of E47 homodimers. The LMO2 complex also activates expression of the homeodomain protein HHEX that resides in the nucleolar PML body, and the NFκB signalling pathway. These changes induce a self-renewal program that is restricted to DN3 thymocytes, which receive survival/proliferation signals such as Notch ligands and SCF from the thymic stroma. Self-renewal of these pre-CSCs allows acquisition of other genetic mutations such as activating mutations of Notch or PI3K pathways, which may allow the pre-CSCs to expand and escape from their thymic niche.
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