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Targeting Immunophenotypic Markers on Leukemic Stem Cells: How Lessons from Current Approaches and Advances in the Leukemia Stem Cell (LSC) Model Can Inform Better Strategies for Treating Acute Myeloid Leukemia (AML)

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Therapies targeting cell-surface antigens in acute myeloid leukemia (AML) have been tested over the past 20 years with limited improvement in overall survival. Recent advances in the understanding of AML pathogenesis support therapeutic targeting of leukemia stem cells as the most promising avenue toward a cure. In this review, we provide an overview of the evolving leukemia stem cell (LSC) model, including evidence of the cell of origin, cellular and molecular disease architecture, and source of relapse in AML. In addition, we explore limitations of current targeted strategies utilized in AML and describe the various immunophenotypic antigens that have been proposed as LSC-directed therapeutic targets. We draw lessons from current approaches as well as from the (pre)-LSC model to suggest criteria that immunophenotypic targets should meet for more specific and effective elimination of disease-initiating clones, highlighting in detail a few targets that we suggest fit these criteria most completely.

Leukemias are hematologic malignancies originating in the bone marrow (BM) that lead to abnormal expansion of white blood cells in an acute or chronic manner. Acute myeloid leukemia (AML) represents around one-third of leukemias and about 10,000 deaths occur yearly in the United States as a result of this disease (Noone et al. 2018). AML is characterized by blocked differentiation and clonal overproliferation of myeloid hematopoietic precursor cells, leading to features of BM failure, and if not treated, results in death within months. Although chemotherapeutic treatments (most commonly cytarabine [ara-C] and anthracyclines) can eliminate detectable leukemic cells in the BM and blood and successfully lead the majority of

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patients into remission, most patients still fatally relapse (Ferrara and Schiffer 2013; Döhner et al. 2015), necessitating the discovery of novel approaches for treating AML.

Findings throughout the past 25+ years provide evidence that AML and the closely related "pre-leukemic" myelodysplastic syndromes (MDS) arise from primitive hematopoietic cells that harbor mutations and epigenetic aberrations, initially leading to formation and/or expansion of pre-leukemic cells and ultimately to transformed leukemic stem cells (LSCs) capable of regenerating and maintaining the disease. Experimentally, LSCs are most commonly functionally defined by their ability to initiate and maintain AML in mice. Sequencing studies have provided evidence that these disease-initiating cells are not eliminated by chemotherapy and eventually prompt MDS and AML relapse. Therefore, to achieve permanent cures of AML and MDS and prevent relapse, elimination of LSC is essential. The eradication of pre-LSC (hematopoietic stem cells [HSCs] primed for transformation while retaining the ability to contribute to multilineage differentiation) is also likely necessary. In this review, we will summarize the evolving LSC model and analyze current and prospective strategies for treating AML by targeting LSC populations via aberrantly expressed surface antigens.

THE LEUKEMIA STEM CELL MODEL

Significant evidence has been gathered supporting the existence of LSCs capable of initiating, maintaining, and regenerating AML (for review, see Passegue et al. 2003; Pandolfi et al. 2013; Corces et al. 2017; Thomas and Majeti 2017). To validate this model, several areas have been examined, including (1) the cell of origin for AML, (2) functional heterogeneity in the organization of AML, and (3) contribution of LSCs to relapse.

The Cell of Origin for AML

Initial Evidence from Cytogenetics Studies

Cytogenetic examination of human AML cells provided the first evidence of a stem cell origin in this cancer. The coupling of karyotyping with colony-forming potential assays in primary human AML samples identified cytogenetically abnormal cells that were capable of long-term culture and/or multilineage differentiation, suggesting the presence of these aberrations in stem cells (Hogge et al. 1987; Tamura et al. 1993). In addition, cytogenetic analysis together with immunophenotyping uncovered identical aberrations in bilineage leukemias, supporting the origin of these leukemias as an immature cell with both myeloid and lymphoid potential (Sun et al. 1991; Carbonell et al. 1996; Hyakuna et al. 1998). Finally, cytogenetics coupled with fluorescence-activated cell sorting (FACS) detected chromosomal rearrangements in immunophenotypically defined HSCs and hematopoietic progenitor cells ($CD34^+CD38^-$ and $CD34^+CD38^+$ cells, respectively) in primary MDS and AML samples at diagnosis (Haase et al. 1995, 1997; Feuring-Buske et al. 1999; Nilsson et al. 2000, 2002; Barreyro et al. 2012; Will et al. 2012; Elias et al. 2014; Woll et al. 2014; Shastri et al. 2017) and in the relapse setting (Engel et al. 1999; Diez-Martin et al. 2000).

Early Evidence from Mouse Modeling of Pre-Leukemia

Modeling of frequent molecular changes in MDS/AML in murine models provided important early conceptual insight into pre-leukemic stages at the hematopoietic stem/progenitor level and their functional relevance for leukemia development and maintenance. These studies include reduction in expression of the transcription factor PU.1 (Steidl et al. 2006; Will et al. 2015), overexpression of the MLL-AF9 and CBFβ–SMMHC fusion oncogenes (Kuo et al. 2006; Somervaille and Cleary 2006; Pulikkan and Castilla 2018), and introduction of CEBPα mutations (Kirstetter et al. 2008; Bereshchenko et al. 2009), all resulting in distinct pre-leukemic phases characterized by myeloid-biased HSC, blocked differentiation, and accumulation of immature myeloid or myelomonocytic cells in the BM and blood. AML development occurred in these mice with a latency of 2–6 months, which indicated for the first time that a distinct

pre-leukemic phase with definable cell-intrinsic properties exists, and that stem and immature progenitor cells play a key role in this process. In addition, it was found that some pre-leukemic molecular alterations, such as down-regulation of the transcription factors PU.1 and JunB, remain functionally critical for fully transformed LSC at later stages, which indicated that targeting pre-leukemic/founding aberrations may represent a promising therapeutic avenue (Steidl et al. 2006).

Evidence from Gene Expression Studies

Further support for the HSC origin of AML in humans was initially collected through studies of gene expression in purified cell populations. Gene expression analysis of phenotypic short-term HSCs ($Lin^-CD34^+CD38^-CD90^{low}$) from AML patients identified dysregulated expression of genes in this population such as the JunB and PU.1 transcription factors (Steidl et al. 2006), and the epigenetic regulator SATB1 (Steidl et al. 2007). More broadly, microarray transcriptional profiling studies of immunophenotypically defined HSCs from MDS and AML versus normal BM showed that MDS/AML HSCs exhibit gene expression profiles resembling normal HSCs (Nilsson et al. 2007; Gentles et al. 2010; Eppert et al. 2011), but that multiple specific genes and pathways are dysregulated at the stem cell level such as those involved in apoptosis, adherens junctions, interleukin (IL), Jak-STAT, and interferon (IFN) signaling (Nilsson et al. 2007; Majeti et al. 2009a; Barreyro et al. 2012; Will et al. 2012), suggesting involvement of HSCs in human AML pathogenesis.

Evidence from Sequencing Studies Using Single-Nucleotide Variants as Markers of Subclonality

A logical argument for the stem cell origin of leukemia is that the number of mutations and therefore time required for cellular transformation must occur in a long-lived cell with high proliferative potential, as mutations would not have sufficient time to accumulate in progenitor and more differentiated cells because of their transient nature. Single-colony and single-cell sequencing technologies have allowed for direct evidence of this phenomenon. A cell-surface immunophenotype has been identified ($Lin^-CD34^+CD38^-CD99^-TIM\text{-}3^-$) that distinguishes functionally normal residual HSCs from AML patient samples (as defined by transplantation experiments) (Jan et al. 2011). FACS and targeted sequencing of these normal HSCs from AML patient samples showed that genetic lesions serially accumulate in HSCs, as multiple clones existed in the HSC pool containing the partial spectrum of all mutations seen in AML cells from that patient (Jan et al. 2012). Likewise, whole-exome sequencing on paired diagnosis/remission/relapse AML samples identified that $CD34^+$ progenitors as well as mature cells harbor some of the mutations from the original leukemia at the time of remission (Corces-Zimmerman et al. 2014). The persistence of these mutations in remission indicates that they originate from a stem cell that survived chemotherapy.

An HSC origin is further supported by the detection of the same *DNMT3A* mutations in both the dominant leukemic clone and in normal mature blood cells such as T cells (Shlush et al. 2014). The presence of identical mutations in AML and T cells strongly suggests that the cell of origin in AML is long-lived and has multilineage differentiation potential. In addition, in at least two documented occasions, transplantations performed with donor BM retrospectively determined to harbor a *DNMT3A* mutation resulted in AML in the recipient with a *DNMT3A*-mutant leukemic clone (Yasuda et al. 2014; Hahn et al. 2015). These anecdotal findings suggest an HSC origin as these cells are responsible for long-term repopulation of the immune system.

Single-cell sequencing of $CD34^+CD38^-IL1RAP^+/CD123^+/CD45RA^+$ BM cells from longitudinal MDS and secondary AML samples from individual patients revealed that MDS and AML arise from different but ancestrally related clones, further supporting a stem cell origin for these diseases (Chen et al. 2019). Importantly, the stem cell origin theory has been tested prospectively as well. It was recently shown that a succession of stages ultimately leading to a serially transplantable leukemia could be modeled

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by creating induced pluripotent stem cells from primary MDS and AML samples with increasing numbers of driver mutations (Kotini et al. 2017).

It has been estimated that HSCs acquire between 1 and 2 exonic mutations per decade, and while most of these do not manifest in clonal expansion or disease, several studies have found that at least 5%–10% of individuals over age 65 have disproportionate expansion of single hematopoietic clones, with the percentage further increasing with age. Detection of mutations associated with myeloid malignancies at a variant allele frequency (VAF) of 2% or greater in the peripheral blood currently defines this phenomenon, termed clonal hematopoiesis (CH) (Genovese et al. 2014; Jaiswal et al. 2014; Xie et al. 2014; Shlush 2018). CH mutations found in BM HSCs and hematopoietic progenitors further hints that CH represents a premalignant event preceding myeloid malignancies (Fig. 1A; Arends et al. 2018).

The nature of the transition from CH to myeloid malignancies is the subject of intense

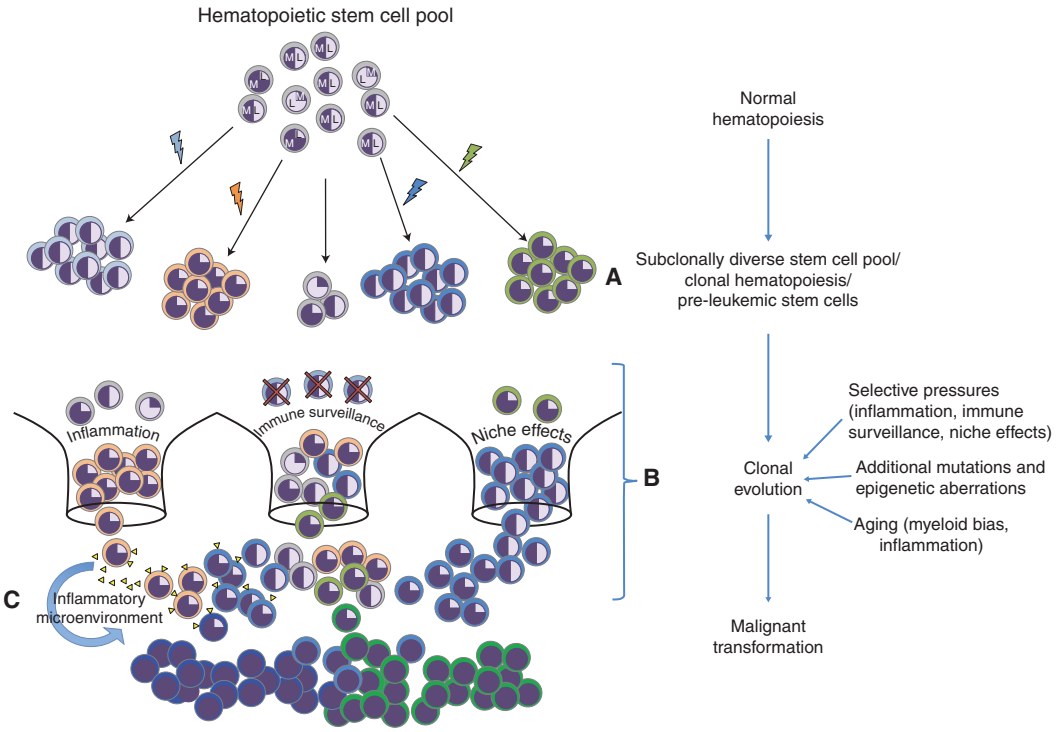

Figure 1. Selective pressure model of acute myeloid leukemia (AML). A proposed model showing transformation of the hematopoietic stem cell (HSC) pool from normal to malignant. "Normal" HSCs are shown in gray. Myeloid (M) and lymphoid (L) biased stem cells are present, as well as unbiased HSCs (represented as equal proportion M and L). Different aberrations such as mutations (colored lightning bolts) are associated with certain HSC subclones that can be detected, for example, by sequencing (clonal hematopoiesis) (*A*). These aberrations can occur in a lineage-biased stem cell or may confer a lineage bias. Selective pressures such as inflammation, immune surveillance, and niche effects may give certain HSC clones an advantage over others (i.e., inflammation gives the clone with an orange mutation a selective advantage over normal HSCs). Clonal evolution is also potentially further driven by additional mutations and aging-associated phenomena such as chronic inflammation and myeloid bias (*B*). For example, an inflammatory microenvironment created by pre-leukemic clones can promote selective expansion of mutated clones over normal HSCs (*C*). Together, these factors can eventually lead to malignant transformation and achievement of clonal dominance of particular stem cell subclones, and consecutive uncontrolled proliferation of leukemic bulk clones (shown as royal blue and bright green cells).

investigation (for review, see Bowman et al. 2018). One study estimated that any detectable CH is associated with an 11-fold greater risk of hematologic cancer and this risk increases to almost 50-fold with a greater clone size (VAF of 10% or greater) (Jaiswal et al. 2014; Steensma et al. 2015; Corces et al. 2017). However, the overall risk for hematologic malignancy in individuals with CH has been estimated to be only 4% (Jaiswal et al. 2014), indicating that further clonal evolution is required for cancer development. Two independent longitudinal analyses detected AML-relevant mutations in whole blood samples several years before AML diagnosis and found that higher mutational burden correlated with increased rates of AML progression (Abelson et al. 2018; Desai et al. 2018). While these studies were not done in BM or in stem-cell-enriched populations, the presence of these mutations years before diagnosis indicates their occurrence in long-lived cells and represents indirect evidence for the accumulation of mutations in HSCs or expansion of HSCs with existing mutations to a detectable limit. It would also be informative, albeit logistically challenging, to perform single-HSC sequencing on these samples to identify co-occurring mutations and their association with development and time-to-development of AML.

Disruption of Hematopoietic Stem Cell Function and Integrity as an Early Step in the Pathogenesis of Myeloid Malignancies

In the BM niche, HSCs are generally maintained in a dormant state with infrequent cell divisions to limit the opportunity for mutations to arise from the byproducts of ATP production (reactive oxygen species [ROS]) or errors during genome replication (Ito et al. 2006; Pietras et al. 2011). Studies have estimated that human HSCs enter the cell cycle on average every 40 weeks (Catlin et al. 2011), implying that there is tight regulation on maintaining HSCs in a noncycling "quiescent" state the vast majority of the time. Accordingly, it has been shown that DNA damage accumulates in HSCs with age, and cell-cycle-associated replication stress contributes to functional impairment in aging HSCs, including reduced proliferative and regenerative capacity (Rossi et al. 2008; Flach et al. 2014).

Although HSCs are usually kept dormant, they must be poised to undergo cell division when an injury or infection cannot be controlled locally. In this case, increased proinflammatory cytokines and Toll-like receptor ligands directly and/or indirectly stimulate hematopoietic stem and progenitor cells (HSPCs) to proliferate and differentiate to regain homeostasis and meet the blood and immune system demands (King and Goodell 2011; Takizawa et al. 2012; Pietras 2017). Different combinations of cytokines and ligands stimulate particular subsets of HSCs to differentiate along specific lineages. For instance, in response to viral infections, IFN-γ is released, promotes differentiation of myeloid-biased HSCs (Matatall et al. 2014) and leads to expression of IL-6 by the BM niche. IL-6 then acts specifically on the multipotent progenitor (MPP) population to regulate hematopoietic output, driving myeloid output at the expense of lymphoid production (Reynaud et al. 2011; Mirantes et al. 2014). Direct cytokine-mediated activation of HSPCs has been demonstrated to be an efficient alternative to cytokine release by mature cells and subsequent paracrine stimulation of HSPCs for rapid myeloid recovery (Zhao et al. 2014).

Although proinflammatory signals can recover homeostasis in acute settings, chronic exposure has been shown to lead to HSC exhaustion and BM failure. For instance, IL-1 exposure in mice resulted in an HSC pool with limited ability to differentiate into nonmyeloid lineages and reduced self-renewal potential as assessed by competitive transplantation (Pietras et al. 2016). Chronic IFN-γ compromised long-term HSC reconstitution capacity after transplantation (Baldridge et al. 2010) and chronic tumor necrosis factor α (TNF-α) has also been shown to inhibit HSC growth and survival, reducing reconstitution ability of human $CD34^{+}CD38^{-}$ cells (Baldridge et al. 2011). These drastic effects of chronic inflammation are likely lessened in the endogenous, nontransplant setting where chronic inflammatory signaling and the resultant cytokine microenvironment have been pos-

tulated to instead cause continual disruption of HSC quiescence, frequent cell cycling, and an increased probability of accumulating DNA replication-associated mutations (Cohen et al. 1991; Pietras 2017). This idea is supported by studies showing that injection of mice with the double-stranded RNA mimic pI:pC, which mimics viral infection, induces HSCs to exit from quiescence in an IFN-α-receptor-dependent manner and accumulate DNA damage (Pietras et al. 2014; Walter et al. 2015). Similar effects were seen upon chronic LPS exposure (Esplin et al. 2011; Takizawa et al. 2017). In line with this, large epidemiological studies demonstrated that a prior history of infectious disease led to a 1.3-fold increased risk of AML and MDS and a prior history of autoimmune disease was associated with 1.7- and 2.1-fold increased risks for AML and MDS, respectively (Anderson et al. 2009; Kristinsson et al. 2011).

For a malignancy to develop, a mutated HSC conceivably must still retain its ability to sustain itself. Aside from triggering cell cycling and, consequently, mutations in HSCs, chronic inflammation can also act as a selective force and promote expansion of HSCs that are resistant to the detrimental effects of a proinflammatory environment (Fig. 1B). This phenomenon was elegantly shown in a mouse model of pre-leukemic myeloproliferation with variable expressivity. Specifically, upon genetic knockout of *Tet2*, the amount of myeloproliferation seen was positively associated with the presence of bacterial dissemination (potentially because of $Tet2^{-/-}$-related intestinal defects), and the presence of bacteria or of TLR agonists were sufficient to cause this myeloproliferation through increased IL-6 secretion. Further, $Tet2^{-/-}$ HSPCs were specifically sensitive to IL-6, in part because of up-regulation of the IL-6 receptor compared to $Tet2^{+/+}$ HSCs, providing an example of mutated HSCs acquiring properties that give them a competitive advantage in a proinflammatory environment (Meisel et al. 2018). Likewise, in the human setting, HSCs from patients with myeloproliferative neoplasms harboring activating *JAK2* mutations were found to be resistant to suppressive growth signaling from chronic TNF-α stimulation and were clonally selected in this environment (Fleischman et al. 2011; Takizawa et al. 2012; Craver et al. 2018).

Contrastingly, pre-leukemic or CH mutations may foster an inflammatory environment. For instance, although only described in mature hematopoietic cells, Tet2 was shown to repress expression of inflammatory cytokines including IL-1β and IL-6. Loss of function mutations in *Tet2* therefore derepressed these signals and contributed to an inflammatory environment, which in the leukemia setting could promote disease progression (Zhang et al. 2015; Fuster et al. 2017; Jaiswal et al. 2017). CH is also associated with increased risk of diseases with inflammatory components, including coronary heart disease and various cancers (Coombs et al. 2017; Jaiswal et al. 2017); thus, a positive feedback loop between an inflammatory microenvironment and the development of and selection for aberrant HSCs may be a causative factor in AML development (Fig. 1C).

The question remains why certain mutations, such as those providing a proliferative advantage, would necessarily result in myeloid malignancies. One theory for this is the changing capacity and characteristics of HSCs as they age. It is well described in human and mouse hematopoiesis that deficiencies in the adaptive immune system occur with aging. This is accompanied by myeloid skewing, specifically demonstrated by increased myeloid repopulation potential in HSC transplantation experiments from young versus old mice and young versus old human BM specimens (Rossi et al. 2005; Rundberg Nilsson et al. 2016; Yamamoto et al. 2018). Accordingly, the majority of pediatric leukemias are lymphoid, whereas adult leukemias are most frequently myeloid, even when initiated by the same oncogene, for instance t(9;22) (MLL-AF9), suggesting an alteration in the potential of HSCs with age (Meyer et al. 2013). This increased propensity toward myeloid differentiation provides insight into the phenomenon of age-related myeloid malignancies (Fig. 1B), yet the cell-intrinsic mechanisms for this occurrence must still be investigated further. Perhaps an age-related inflammatory environment could provide a link to explain this propensity for myeloid disease.

Functional Heterogeneity in the Organization of AML

The first description of the heterogeneous composition of AML was provided by transplantation of subpopulations of cells from leukemic BM specimens into immunodeficient mice. Whereas $CD34^+CD38^-$ cells were capable of initiating disease in recipient animals, $CD34^-$ cells were not, providing the first indication that all leukemia cells are not created equal, and that a hierarchy exists within a cancer cell population (Lapidot et al. 1994; Bonnet and Dick 1997). These studies were validated in vitro by long-term proliferation and colony output assays of immunophenotypic subpopulations from AML samples (Sutherland et al. 1996). Higher-resolution experiments were done by using lentiviral transduction to mark single cells from AML specimens and performing serial transplantations. These experiments led to the identification of a more complex hierarchy, with subpopulations of cells that were capable of short-term or long-term repopulation, and/or whose repopulation only reached a detectable limit after secondary or tertiary transplantation (indicating initial quiescence). Tracking clones via a lentiviral integration site identified that quiescent long-term leukemia-initiating cells (LICs) gave rise to long-term LICs, which gave rise to short-term LICs, eventually generating bulk tumor cells that lose the ability to self-renew, but are also unable to differentiate, leading to their uncontrolled accumulation (Hope et al. 2004). Of note, expanded studies with a higher number of primary AML specimens have identified that the leukemia-initiating population is not limited to the $CD34^+CD38^-$ immunophenotype and in fact the $CD34^+CD38^+$ and $CD34^-$ populations are also in some cases capable of leukemia initiation (Martelli et al. 2010; Taussig et al. 2010; Goardon et al. 2011; Sarry et al. 2011; Quek et al. 2016). Although these studies have provided pivotal insight into the heterogeneity that exists in AML, the use of xenotransplantation into mice to determine human tumor heterogeneity may select for different functional properties or may underestimate (or overestimate) the actual LSC frequency. These methodological caveats should be kept in mind in our interpretations with regard to the precise architecture of human AML.

Contribution of LSCs to Relapse

LICs have been shown to exhibit stem cell characteristics that contribute to chemotherapy resistance—including quiescence and drug efflux potential (Terpstra et al. 1996; Costello et al. 2000). Indeed, human AML xenograft studies in mice found that certain cell-cycle-quiescent leukemia cells localize in the BM endosteal region, remain after chemotherapy, and can transplant AML to secondary recipients (Ishikawa et al. 2007; Saito et al. 2010). Similarly, sequencing studies have demonstrated that HSCs harboring leukemia-associated mutations persist after standard chemotherapy in AML patients (Miyamoto et al. 2000; Ding et al. 2012; Welch et al. 2012; Corces-Zimmerman et al. 2014). However, in light of evidence that nonleukemic clones expand following induction chemotherapy (Wong et al. 2016), distinguishing CH with a high VAF from incomplete elimination of leukemic clones will be crucial for elucidating the nature of relapse. Considering that it is currently not possible to detect whether chemotherapy has eliminated every leukemic cell because of sensitivity limitations in minimal residual disease (MRD) detection (Hourigan et al. 2017; Hollein et al. 2018), whether relapse is being driven by residual LSCs (dominant or minor subclones), by chemotherapy-induced mutations that lead to drug resistance, or by chemotherapy-induced clonal evolution of pre-LSCs, is not entirely clear (Jan and Majeti 2013), but current evidence supports the former (Corces et al. 2017; Shlush et al. 2017). Specifically, sequencing of paired diagnosis and relapse samples has demonstrated several cases of minor subclones at diagnosis that present as the dominant subclone at relapse (Parkin et al. 2013; Corces-Zimmerman et al. 2014; Shlush et al. 2017). Because of detection limits of sequencing, even in studies where new subclones appeared at relapse (Ding et al. 2012; Corces-Zimmerman et al. 2014), it cannot be ruled out that these clones

were present in low frequency at diagnosis and expanded after chemotherapy.

Until more sensitive techniques are available, it may not become entirely clear what the nature of relapse is. Despite these limitations, the suspected stem cell origin of AML relapse suggests that eradication of both malignant and premalignant HSPC, but not necessarily HSCs in a CH stage, holds promise for achieving lasting cures. Thus, an ideal therapeutic target should be present not only on LSC-enriched populations at diagnosis, but also in the relapse setting, and with expression during complete remission being predictive of relapse.

PRESENT AND FUTURE STRATEGIES FOR TARGETING LSCs IN AML

Associated Limitations of Current Targeted Approaches in AML

In AML, heterogeneity in the cellular compartment of origin and the varied mutational spectrum across patients have challenged the identification of commonly dysregulated pathways that are relevant in the initiation and maintenance of the disease (Sarry et al. 2011; Papaemmanuil et al. 2016). Sequencing studies have determined that AML is genetically complex, with patients typically having multiple driver mutations, and despite there being overlap in these mutations, no single genetic aberration is found in more than 35% of patients (Papaemmanuil et al. 2016). Thus, a treatment designed to eliminate cells carrying a particular mutation will likely only be relevant in a minority of patients. Further, individual patients have high intratumor heterogeneity; any detected mutation is usually only present in a subset of tumor cells and focusing a therapeutic strategy on a particular mutation may leave many subclones (especially smaller ones) unaccounted for (Ding et al. 2012; Welch et al. 2012; Parkin et al. 2013; Chen et al. 2019). For this reason, several groups have sought to identify common characteristics of leukemia cells across AMLs with different genetic profiles—most notably cell-surface protein markers—that might be exploited for therapeutic targeting in most patients (Jordan et al. 2000b; Majeti et al. 2009b). Three of the most commonly sought-after targets in AML cells are CD33, FLT3, and CD123, but clinically, toxicities caused by lack of specificity of expression and resistance mechanisms are presenting considerable challenges.

CD33

Currently, the only approved immunotherapy in AML is gemtuzumab ozogamicin, an anti-CD33 antibody conjugated to a cytotoxic derivative of calicheamicin (Sievers et al. 1999, 2001), but this treatment has been on and off the market because of hematologic and nonhematologic toxicities (Baron and Wang 2018). In 2017, it was reapproved for use at lower doses in combination with chemotherapy and as a single agent in patients not eligible for intensive chemotherapy or with relapsed/refractory AML (Amadori et al. 2010; Castaigne et al. 2012). Whereas CD33 is often expressed on LSC-enriched populations (Griffin et al. 1984; Hauswirth et al. 2007; Sadovnik et al. 2017), hematopoietic toxicity of anti-CD33 treatments relate to its expression on normal hematopoietic cells, including HSCs (Taussig et al. 2005; Kikushige et al. 2010; Sadovnik et al. 2017; Haubner et al. 2019). Several creative approaches are being tested to circumvent this toxicity. For instance, a bispecific antibody targeting CD47 and CD33 was developed and reported to be effective in an AML xenograft model (Boyd-Kirkup et al. 2017). Another approach under development is the use of CRISPR-Cas9 technology to delete CD33 in HSCs and subsequent use of anti-CD33 targeting approaches such as CAR T cells to eliminate leukemic cells (Kim et al. 2018).

FLT3

FLT3, or CD135, is a highly sought-after therapeutic target in AML because of the substantial fraction of patients harboring activating mutations in this gene (up to 35%) (Gilliland and Griffin 2002; Patel et al. 2012; Papaemmanuil et al. 2016). In-frame length polymorphisms (resulting from internal tandem duplications) in the juxtamembrane domain of *FLT3* (*FLT3*-ITD)

were discovered in AML patients in 1996, and led to a constitutively active kinase by destabilizing the inhibitory functions of this domain (Chan 2011). *FLT3* mutations are demonstrated to be driving mutations in leukemogenesis (Lee et al. 2005; Schessl et al. 2005; Kim et al. 2008; Mallardo et al. 2013), but detection of minor leukemic subclones with *FLT3*-ITD mutations indicate it is preferentially a late event (Corces-Zimmerman et al. 2014; Shouval et al. 2014). Targeted sequencing of single-LSC-derived colonies has resolved the likely order of acquisition of mutations in several patients, concluding that FLT3 mutations occur late, as they never occur as the sole aberration but only co-occur with other leukemia-relevant mutations (Jan et al. 2012). Meanwhile, sequencing of residual normal HSC-derived colonies showed that *FLT3* mutations never occur in these cells (Jan et al. 2012; Corces-Zimmerman et al. 2014), and *FLT3* mutations are not found in CH (Abelson et al. 2018; Desai et al. 2018), suggesting they are not early, or pre-leukemic events. In addition, *FLT3* mutations are often gained or lost between diagnosis and relapse, further evidence that they are not a part of the founding AML clone (Krönke et al. 2013; Corces-Zimmerman et al. 2014; Shlush et al. 2014; Shouval et al. 2014).

Over 60 clinical trials have been conducted to test FLT3 inhibitors alone or in combination therapy in AML. Currently, only midostaurin has FDA approval for treatment of AML patients with *FLT3*-ITD mutations in combination with standard induction therapy, as it modestly improved event-free and overall survival in clinical trials (Stone et al. 2017). Although most *FLT3*-ITD subclones are sensitive to tyrosine kinase inhibitor (TKI) treatment, particularly circulating leukemic cells, BM leukemic cells are not effectively eliminated and MRD often contains *FLT3*-ITD$^+$ cells. Studies of samples from patients who relapsed while under treatment with the TKI quizartinib have uncovered resistance mechanisms. These include suboptimal pharmacokinetics and pharmacodynamics of TKIs, acquisition of additional mutations in *FLT3* (commonly in the kinase domain) leading to decreased inhibitor efficacy (Alvarado et al. 2014; Ghiaur and Levis 2017; Smith et al. 2017), and activation of compensatory signaling pathways, including stromal-derived fibroblast growth factor and CXCL12, which lead to activation of mitogen-activating protein kinases (MAPKs) and Bcl2 family survival pathways (Yang et al. 2014; Traer et al. 2016; Ghiaur and Levis 2017; Smith et al. 2017). In addition, high FLT3 ligand levels in the BM that occur during chemotherapy have been proposed to stimulate FLT3-ITD$^+$ cells through their remaining FLT3 wild-type (WT) copy, facilitating cell survival amid quizartinib treatment, which preferentially targets the mutated receptor (Sato et al. 2011; Yang et al. 2014; Chen et al. 2016; Taylor et al. 2017).

Elimination of MRD is not apparent under the use of FLT3 inhibitors because of one or many of these mechanisms, and the findings that FLT3 mutations are not early events in leukemogenesis may be the driving reason behind their limited sustained efficacy. Whereas proposed immunotherapeutic approaches (Reiter et al. 2018) may prevent some mechanisms of resistance by eliminating any FLT3-expressing leukemic cells, expression on normal HSCs (Sadovnik et al. 2017) will likely prevent progression of this tactic to wider use in the treatment and cure of AML.

CD123

Several studies have reported high expression of CD123 (IL-3Rα) on CD34$^+$CD38$^-$ populations from AML samples (Jordan et al. 2000a; Munoz et al. 2001; Graf et al. 2004; Yalcintepe et al. 2006; Sadovnik et al. 2017). The expression of CD123 on normal HSPC is somewhat conflicting. Most analyses of CD123 protein expression by flow cytometry in normal human hematopoietic cells have reported that CD123 is expressed on a low–mid proportion of normal BM CD34$^+$ cells, attributed to expression on myeloid progenitors (common myeloid and granulocyte-macrophage progenitors [CMPs and GMPs]), but with no expression on CD34$^+$CD38$^-$ cells (Sato et al. 1993; Jordan et al. 2000a; Munoz et al. 2001; Graf et al. 2004). However, other studies have detected expression in HSCs (Taussig et al. 2005; van Rhenen et al. 2007a; Sadovnik

et al. 2017; Haubner et al. 2019) and when side population (SP) stem cells from normal BM were analyzed, CD123 was detected in up to 82% of SP cells with a median of 27% (Moshaver et al. 2008). CD123 was also highly expressed in $CD34^+CD38^-$ cells in regenerating BM, which may be particularly relevant for patients undergoing chemotherapy (van Rhenen et al. 2007a).

CD123 targeting by monoclonal antibodies has been modestly effective in preclinical models, at least in part via inhibition of IL-3 signaling and Fc-mediated recruitment and cell killing by innate immune effector cells (Jin et al. 2009). Several CD123-directed agents have been tested in phase I/II clinical trials, with results awaited (NCT02113982, NCT02152956, NCT02715011 (suspended), NCT02730312, NCT02848248 (terminated). The CD123 monoclonal antibody talacotuzumab reached a phase III clinical trial to be tested in combination with decitabine in patients with AML not eligible for intensive chemotherapy, but was discontinued (NCT02472145), and another phase II clinical trial with talacotuzumab as a monotherapy (NCT02992860) was terminated because of risk to patients. Toxicity to normal HSPC may limit whether CD123-directed therapies will progress to clinical approval (Thomas and Majeti 2017), particularly given the importance of IL-3 for hematopoietic cell viability and proliferation (Barreda et al. 2004).

Considerations for Prospective LSC Targets

Advances in the LSC model imply conditions that therapeutic targets in AML should meet. For instance, studying the cell of origin and architecture of AML indicate that a $Lin^-CD34^+CD38^-CD90^-$ immunophenotype is most characteristic of LICs, thus proteins expressed on this population in AML samples have therapeutic promise. Further, studies of the subclonal composition of relapse suggest that the timing in which a target is expressed in the transformation process should be of high importance. Toxicities associated with current targeted therapies reinforce the importance of minimal expression on normal tissues and hint at the benefits and drawbacks associated with different classes of biopharmaceutical agents. These and other considerations are detailed below.

Immunophenotype

Several proteins expressed on immunophenotypically defined LSCs have been discovered by transcriptional profiling and subsequent validation of protein overexpression in HSC-enriched populations ($CD34^+CD38^-$ cells) from AML versus normal BM samples. Tables 1 and 2 list these targets, expression on LSC-enriched populations and in normal tissues, described functions in AML, and exploration of targeting strategies. The immunophenotypic populations profiled in many studies are somewhat heterogeneous, containing only a fraction of true LSCs; thus, there have been efforts to improve immunophenotypic enrichment of LSCs. Studies coupling immunophenotyping of AML samples and xenotransplantation have identified that LSC populations in over 90% of cases bear a $Lin^-CD34^+CD38^-CD90^-$ immunophenotype (Goardon et al. 2011; Thomas and Majeti 2017). Ideally, a therapeutic target for AML should be highly expressed in this compartment to increase accuracy and reduce targeting irrelevant populations that may contain many normal clones. TIM-3, IL1RAP, and CD96 have been specifically shown to be up-regulated in this population in AML specimens (Jan et al. 2011; Barreyro et al. 2012). Aside from intratumor heterogeneity, contamination with some normal HSPC has not been fully avoided by most profiling approaches. Most studies have hence analyzed mixed populations of leukemic and normal stem cells, leaving unexplored whether expression is fully specific to leukemic cells or to some extent reflects a systemic feature of (otherwise normal) HSPC in a leukemic microenvironment. Transplantation studies combining index FACS sorting and single-clone sequencing can potentially improve this by identifying which surface markers are in fact marking stem cells that are part of the leukemic clone(s) (Jan et al. 2011), keeping in mind that xenograft models leave room for "false negatives" such as LSCs or relevant pre-leukemic clones that do not engraft into immunodeficient mice for various reasons.

Expression on Normal HSC and Other Tissues

As demonstrated by toxicities associated with CD33- and CD123-directed therapies, the expression (albeit often lower) of LSC antigens on normal HSC complicates the therapeutic efficacy of many otherwise potent targets. Therefore, studies have sought to identify markers of LSCs that truly distinguish them from normal HSCs to minimize off-tumor, on-target toxicity. Some LSC antigens found to be absent from normal BM HSC include CD32, CLL-1, and IL1RAP (see Tables 1 and 2 for relevant references), so developing therapies against these proteins may result in reduced toxicity. Although some studies have detected its expression on normal HSC, TIM-3 expression has been demonstrated to distinguish residual, normal HSCs from LSCs in AML patient specimens using xenotransplantation (Jan et al. 2011), providing evidence of a strong therapeutic window for this target as well. Minimal expression of therapeutic targets on normal HSC and hematopoietic progenitors is particularly important for immunotherapeutic approaches as cells with any expression of the target may be eliminated. Along these lines, an ideal therapeutic target should be minimally expressed in normal tissues, particularly those that are vital.

Timing in AML Pathogenesis

A relationship between the order of mutations in AML and whether these mutations predict relapse has begun to be established. As *DNMT3A*, *TET2*, and *ASXL1* mutations (which often occur early and are associated with CH) detected in MRD were found not to be predictive of relapse while mutations in *NPM1* were predictive (Jongen-Lavrencic et al. 2018), mutations acquired after the initial CH stage in leukemogenesis may be better markers of MRD than CH markers. Likewise, immunophenotypic markers expressed after the CH stage may be more efficient and less toxic therapeutic targets than early markers whose expression may occur on non-diseased, clonal hematopoietic cells. One indication that aberrant expression of a target commonly occurs in this timeframe is if expression predicts relapse when detected at the complete remission/MRD stage; this has been shown to be the case for CLL-1 (van Rhenen et al. 2007b). It has been proposed that FLT3 mutations may be useful in MRD diagnosis because of their presence only on frank, leukemic clones (Corces et al. 2017); however, the limited efficacy of FLT3 targeting in AML suggests that very late hits in the leukemic progression may not be ideal targets either. Therefore, a target that is not commonly expressed on cells only harboring CH-associated mutations but that is expressed in later pre-leukemic stages may be most efficient at eliminating malignant and relevant premalignant stem cells while sparing normal hematopoietic clones. Whereas the order of genetic hits in AML has been elucidated by various model systems and methods, future studies should monitor the timing of aberrant cell-surface protein expression on LSCs throughout the process of leukemic transformation. For instance, the expression of potential LSC targets could be measured by flow cytometry in serial samples collected from patients progressing from CH to AML, and in cells harboring progressive numbers of leukemia-relevant driver mutations.

Target Functionality

Aside from differential expression on leukemic versus normal HSCs, functionality is also prudent for an LSC target. Whereas, in principle, certain targeted therapies such as immunotherapeutic approaches do not depend on a target having a cancer cell-intrinsic function to be effective, without functionality, down-regulation or mutation of the target, could readily lead to resistance to any type of therapeutic agent (Fig. 2A). Functionality also provides an opportunity for a larger therapeutic window in the case that a therapeutic target is expressed in normal HSPC or other tissues; if a leukemic cell is more dependent on the target for cell survival than a normal cell, a targeting strategy that inhibits the target's function could be preferentially cytotoxic to the leukemic cells while sparing normal cells (Fig. 2B). CD44, CD47, TIM-3, CLL-1, and IL1RAP have been found to play a functional role in AML cells, while the roles of many other LSC targets have yet to be explored (Tables 1 and 2).

Table 1. Antigens expressed on LSC-enriched populations with limited specificity or functionality

Gene	Evidence as a leukemic stem marker	Described function in AML?	Investigation of targeting strategies in AML	Expression in normal tissues[a]
CD9	Protein expressed on AML $CD34^+CD38^-$ cells in 75%–90% of patients, expressed on normal BM $CD34^+CD38^-$ cells in 50%–75% of healthy donors (Sadovnik et al. 2017) Protein up-regulation on $CD34^+CD19^-CD13^+/CD33^+$ AML versus normal BM cells in 29% of patients, expression persists at relapse (Coustan-Smith et al. 2018) Protein expression correlated with failure of induction therapy (Wu et al. 2016)	Not described	N/A	Ubiquitous
IL2RA (*CD25*)	Protein up-regulation on $CD34^+CD38^-$ AML versus normal BM or CB cells in ~25% of samples; no expression on normal $CD34^+CD38^-CD133^+$ BM cells (Saito et al. 2010; de Boer et al. 2018) Protein expressed on AML $CD34^+CD38^-$ cells in 50%–75% of patients, expressed on normal BM $CD34^+CD38^-$ cells in 15%–50% of healthy donors (Herrmann et al. 2014; Sadovnik et al. 2017) Protein expression correlates with treatment failure (Terwijn et al. 2009; Cerny et al. 2013; Allan et al. 2018)	Not described	Phase I clinical trial: NCT02588092 (Flynn et al. 2016; Madhumathi et al. 2017)	Hematopoietic (some HSCs, activated T cells, activated B cells, basophils) (Vincenti et al. 1998; Brisslert et al. 2006; Saito et al. 2010)
FCGR2A (*CD32*)	Protein up-regulation on $CD34^+CD38^-$ AML versus normal BM or CB cells in ~34% of samples; expression on <2% of normal $CD34^+CD38^-CD133^+$ BM cells (Saito et al. 2010) Protein expression distinguishes leukemic versus normal $CD34^+CD38^-$ cells (Ho et al. 2016) Protein up-regulation on $CD34^+CD19^-CD13^+/CD33^+$ AML versus normal BM cells in 31% of patients; expression persists at relapse (Coustan-Smith et al. 2018)	Not described	N/A	Hematopoietic (macrophages, B cells)
CD44	Protein expression on $CD34^+CD38^-$ AML cells in all patients analyzed (Florian et al. 2006; Sadovnik et al. 2017) Protein up-regulation on $CD34^+CD19^-CD13^+/CD33^+$ AML versus normal BM cells in 35% of patients; expression persists at relapse (Coustan-Smith et al. 2018) Some protein expression on normal BM $CD34^+CD38^-$ and $CD34^+CD38^+$ cells (Reuss-Borst et al. 1992; Liesveld et al. 1994; Jin et al. 2006; Liu and Jiang 2006; Sadovnik et al. 2017)	Homing, engraftment, proliferation, and cell survival signaling (Jin et al. 2006; Hertweck et al. 2011)	Phase I clinical trial: NCT01641250 (Song et al. 2004; Jin et al. 2006; Quéré et al. 2011)	Ubiquitous (Liu and Jiang 2006)

Continued

Table 1. *Continued*

Gene	Evidence as a leukemic stem marker	Described function in AML?	Investigation of targeting strategies in AML	Expression in normal tissues[a]
CD47	Significant protein up-regulation on AML lymphoid$^-$ CD34$^+$CD38$^-$CD90$^-$ cells versus normal BM HSC (lymphoid$^-$CD34$^+$CD38$^-$CD90$^+$) and MPP (lymphoid$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^-$); some protein expression on normal BM HSC and MPP (Majeti et al. 2009b) Protein expressed on AML CD34$^+$CD38$^-$ cells in >90% of patients, expressed on normal BM CD34$^+$CD38$^-$ cells in >90% of healthy donors (Sadovnik et al. 2017) Expressed on 100% of normal BM CD34$^+$ cells (de Boer et al. 2018) No significant protein up-regulation on CD34$^+$CD19$^-$ CD13$^+$/CD33$^+$ AML versus normal BM cells (Coustan-Smith et al. 2018)	CD47-SIRPα interaction inhibits phagocytosis by macrophages (Jaiswal et al. 2009)	Phase I clinical trials: NCT02641002 (terminated), NCT02663518, NCT02678338, NCT03248479 (Majeti et al. 2009b; for review, see Murata et al. 2018)	Ubiquitous (Majeti et al. 2009b)
CD52 (Campath-1)	Protein up-regulation on CD45$^+$CD34$^+$CD38$^-$ MDS and AML cells versus AML complete remission samples (Blatt et al. 2014) Protein expressed on AML CD34$^+$CD38$^-$ cells in 50%–75% of patients, expressed on normal BM CD34$^+$CD38$^-$ cells in 50%–75% of healthy donors (Sadovnik et al. 2017) Protein up-regulation on CD34$^+$CD19$^-$CD13$^+$/CD33$^+$ AML versus normal BM cells in 35% of patients; expression persists at relapse (Coustan-Smith et al. 2018) Some protein expression on normal BM CD34$^+$CD38$^-$ cells (Olweus et al. 1994)	Not described	Saito et al. 2011; Blatt et al. 2014	Not described
CD93	Protein up-regulation on CD34$^+$CD38$^-$ AML versus normal BM or CB in ∼7% of samples (Saito et al. 2010) Specific protein expression on CD34$^+$CD38$^-$ MLL-rearranged AML cells versus non-MLL-rearranged AML cells; absence of protein expression on CD34$^+$CD38$^-$ CB cells; increased leukemia initiating frequency in CD34$^+$CD38$^-$CD93$^+$ versus CD34$^+$CD38$^-$CD93$^-$ AML (MLL-AF9) cells (Iwasaki et al. 2015)	Not described	Iwasaki et al. 2015	Hematopoietic (platelets, neutrophils, monocytes, macrophages, some B-cell precursors, some dendritic and NK cells, HSCs); microglia; endothelial cells (Fonseca et al. 2001;

Continued

Table 1. *Continued*

Gene	Evidence as a leukemic stem marker	Described function in AML?	Investigation of targeting strategies in AML	Expression in normal tissues[a]
	Protein expressed on AML $CD34^+CD38^-$ cells in 75%–90% of patients, expressed on normal BM $CD34^+CD38^-$ cells in 50%–75% of healthy donors (Sadovnik et al. 2017) Protein expression on 99% of CB $Lin^-CD34^+CD38^-$ cells (Danet et al. 2002) Protein up-regulation on $CD34^+CD19^-CD13^+/CD33^+$ AML versus normal BM cells in 15% of patients, expression persists at relapse (Coustan-Smith et al. 2018)			Danet et al. 2002; McGreal et al. 2002)
CD96 (tactile)	Significant protein up-regulation on $CD34^+CD38^-$ AML versus normal BM cells (Gramatzki et al. 1998; Kikushige et al. 2010; Du et al. 2015) Significant protein up-regulation on $CD34^+CD38^-$ AML cells (specifically $CD90^-$) compared to normal $Lin^-CD34^+CD38^-$ BM cells in 2/3 of AML samples examined; >85% of $CD34^+CD38^-CD96^+$ AML cells did not express lineage markers; increased leukemia initiating frequency in $CD34^+CD38^-CD96^+$ versus $CD34^+CD38^-CD96^-$ AML cells (Hosen et al. 2007) Expressed on 5%–20% of normal BM $CD34^+CD38^-$ cells (Hosen et al. 2007) Protein expressed on AML $CD34^+CD38^-$ cells in 15%–50% of patients, expressed on normal BM $CD34^+CD38^-$ cells in <15% of healthy donors (Sadovnik et al. 2017) Protein up-regulation on $CD34^+CD19^-CD13^+/CD33^+$ AML versus normal BM cells in 49% of patients; expression persists at relapse (Coustan-Smith et al. 2018) Complete remission rate lower in patients with higher percentage of $CD34^+CD38^-CD96^+$ cells (Du et al. 2015); high expression associated with poorer overall survival (Jiang et al. 2017)	Potential inhibitory role in immune function (for review, see Georgiev et al. 2018)	Mohseni Nodehi et al. 2012; Kellner et al. 2013	Hematopoietic (T cells, NK cells, some HSCs); epithelial (mucosal, vascular) (Perna et al. 2017)

Continued

Table 1. *Continued*

Gene	Evidence as a leukemic stem marker	Described function in AML?	Investigation of targeting strategies in AML	Expression in normal tissues[a]
CD99	Protein expression distinguishes functionally normal from leukemia-initiating $Lin^-CD34^+CD38^-$ cells in AML samples (Jan et al. 2012) $CD99^-CD34^+CD38^-$ AML cells lack leukemia-relevant mutations and have multilineage potential (Chung et al. 2017) Expressed on 100% of normal BM $CD34^+$ cells (de Boer et al. 2018) Protein overexpression on $CD34^+CD19^-CD13^+/CD33^+$ AML versus normal BM cells in 26% of patients (Coustan-Smith et al. 2018)	Not described	Chung et al. 2017	Ubiquitous
ADGRG1 (*GPR56*)	Protein highly expressed on AML cells that positively engrafted in immunodeficient mice; protein expressed on healthy $CD34^+$ cells (Pabst et al. 2016) Ectopic expression of Gpr56 significantly accelerated HOXA9-induced leukemogenesis in mice (Daria et al. 2016)	Not described	Daria et al. 2016; Saha et al. 2018	Hematopoietic (cytotoxic T cells, NK cells); epithelial (lung, GI tract, epidermal cells, reproductive and endocrine tissues); hepatocytes; kidney cells

AML, Acute myeloid leukemia; BM, bone marrow; CB, cord blood; GI, gastrointestinal; HSC, hematopoietic stem cell; MPP, multipotent progenitor; NK, natural killer.

[a]Expression data was gathered from Human Protein Atlas (Uhlen et al. 2015), Proteomics Database (Kim et al. 2014), and Human Proteome Map (Wilhelm et al. 2014), as well as the noted references.

Table 2. Functional LSC targets with no expression on normal/functional HSC

Gene	Evidence as a leukemic stem marker	Described function in AML?	Investigation of targeting strategies	Expression in normal tissues[a]
HAVCR2 (*CD366, TIM-3*)	Significant protein up-regulation on $CD34^+CD38^-$ AML versus normal BM cells; no expression on normal HSC (Kikushige et al. 2010; Haubner et al. 2019) Protein up-regulation on $CD34^+CD19^-CD13^+$/$CD33^+$ AML versus normal BM cells in 41% of patients; expression persists at relapse (Coustan-Smith et al. 2018) Significantly higher expression on $lymphoid^-CD34^+CD38^-CD90^-$ AML versus $lymphoid^-CD34^+CD38^-CD90^+$ normal BM cells; $lymphoid^-CD34^+TIM\text{-}3^+$ AML cells gave rise to leukemia in immunodeficient mice, whereas $lymphoid^-CD34^+TIM\text{-}3^-$ did not; expression on ~30% of normal BM $lymphoid^-CD34^+CD38^-$ cells (Jan et al. 2011) TIM-3 expression marks functional LSCs and distinguishes leukemic versus normal $CD34^+CD38^-$ cells (Ho et al. 2016)	Galactin-9-mediated signal transduction leading to MCL-1 expression and cell survival (Kikushige et al. 2015)	Kikushige et al. 2010	Hematopoietic (monocytes, some NK cells, GMPs) (Haubner et al. 2019)
CLEC12A (*CD371, CLL-1*)	Protein expression on $CD34^+CD38^-$ AML cells; absence of protein expression on normal BM, regenerating BM, and mobilized peripheral blood $CD34^+CD38^-$ cells (Bakker et al. 2004; van Rhenen et al. 2007a,b; Kikushige et al. 2010; Sadovnik et al. 2017; Bill et al. 2018; de Boer et al. 2018; Haubner et al. 2019) $FSC^{low}SSC^{low}CD38^{low}$ side population cells from AML patients were 2%–100% positive for CLL-1 and cytogenetically abnormal, $FSC^{low}SSC^{low}CD38^{low}$ side population cells from normal BM were 0%–4% positive for CLL-1 (Moshaver et al. 2008)	Potential negative regulation of immune system (Han et al. 2004; Marshall et al. 2004; Chen et al. 2006; Gagné et al. 2013; Neumann et al. 2014)	Phase I clinical trial: NCT03038230 (Zhao et al. 2010; Lu et al. 2014; Laborda et al. 2017; Leong et al. 2017; Tashiro et al. 2017; Jiang et al. 2018; Wang et al. 2018; Zheng et al. 2019; for review, see Morsink et al. 2019)	Hematopoietic (myeloid) (Bakker et al. 2004; Perna et al. 2017; Haubner et al. 2019)

Continued

Table 2. *Continued*

Gene	Evidence as a leukemic stem marker	Described function in AML?	Investigation of targeting strategies	Expression in normal tissues[a]
	$CD34^+CD38^-CLL\text{-}1^+$ cells gave rise to leukemia in immunodeficient mice, protein expression persists in remission and relapse (van Rhenen et al. 2007b)			
IL1RAP	Significant protein overexpression on AML versus normal $Lin^-CD34^+CD38^-$ BM cells; IL1RAP protein is absent from normal $Lin^-CD34^+CD38^-$ cells; cytogenetically abnormal cells were contained in the $IL1RAP^+$ fraction of AML specimens (Barreyro et al. 2012) Increased IL1RAP expression on AML $CD34^+CD38^-$ cells versus normal BM $CD34^+CD38^-$ cells (Askmyr et al. 2013) IL1RAP protein expression distinguishes leukemic versus normal $CD34^+CD38^-$ cells (Ho et al. 2016) Protein expressed on $CD34^+CD38^-$ AML cells in 50%–75% of all AML samples and absent from normal $CD34^+CD38^-$ cells (Jaras et al. 2010; Sadovnik et al. 2017; de Boer et al. 2018) Protein expression persists in relapse (Ho et al. 2016; de Boer et al. 2018)	Facilitates IL-1 and receptor tyrosine kinase signaling (Ågerstam et al. 2015; Mitchell et al. 2018)	Askmyr et al. 2013; Ågerstam et al. 2015; Mitchell et al. 2018	Hematopoietic (monocytes/macrophages, lowly expressed on myeloid progenitors, lymphocytes, NK cells, and mast cells) (Jaras et al. 2010); epithelial (lowly expressed in GI tract, reproductive, and endocrine tissues) (Ågerstam et al. 2015); lowly expressed on skin fibroblasts and keratinocytes

AML, Acute myeloid leukemia; BM, bone marrow; HSC, hematopoietic stem cell; CB, cord blood; GI, gastrointestinal; MPP, multipotent progenitor; NK, natural killer; GMP, granulocyte-macrophage progenitor; IL, interleukin.

[a]Expression data was gathered from Human Protein Atlas (Uhlen et al. 2015), Proteomics Database (Kim et al. 2014), and Human Proteome Map (Wilhelm et al. 2014), as well as the noted references.

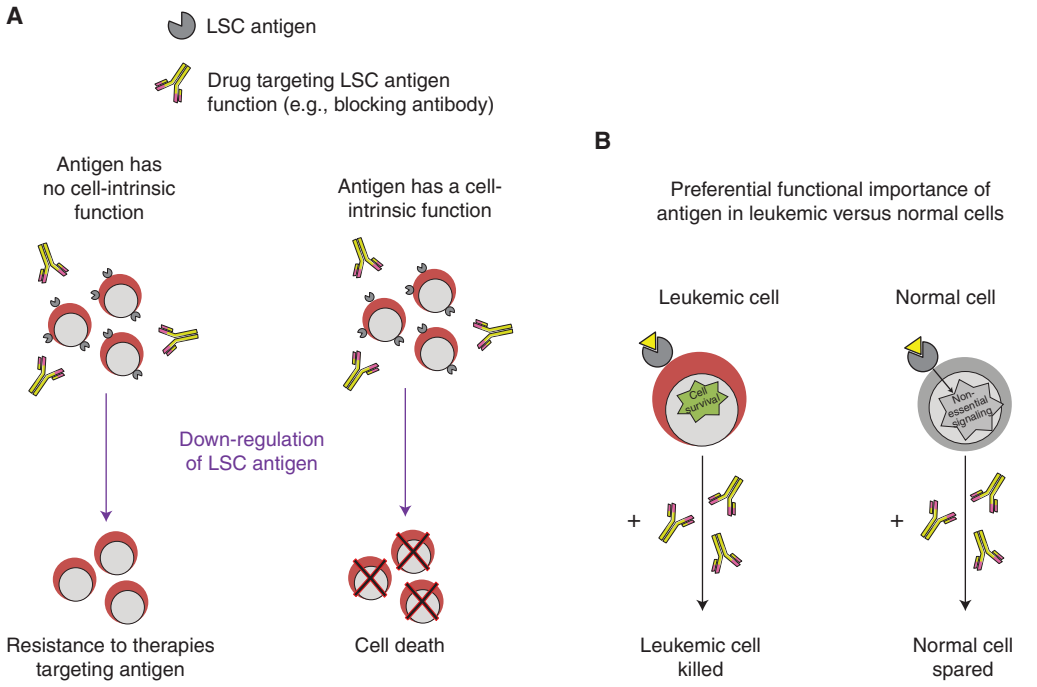

Figure 2. Importance of target functionality for therapeutic targeting. (*A*) If a therapeutic target does not have a cell-intrinsic function in leukemic cells, leukemic cells may be able to escape elimination by the drug through down-regulating the target. (*B*) If a target provides essential signaling in leukemic cells but is dispensable for normal cells, even normal cells expressing the target could be spared by a drug that inhibits the target's function (but would likely be eliminated with an immunotherapeutic approach or antibody–drug conjugate).

Targeting Strategy

Target function and relative expression in leukemic and normal cells are important factors for determining which therapeutic modality will create a proper balance between elimination of leukemic cells and sparing of normal cells. A direct targeting approach (such as a blocking antibody or small molecule inhibitor) may reduce on-target off-tumor toxicity in the presence of lower levels of antigen on normal HSPC than on LSCs or preferential dependence of leukemic versus normal cells on the antigen for cell survival. For instance, if a normal cell expresses the target but is not reliant on it for its function, this cell could be spared by a direct targeting approach but would be killed by an immunotherapeutic approach or antibody–drug conjugate (Fig. 2B). In addition, while immunotherapeutic approaches (such as CAR T cells, bispecific T-cell engager antibodies, and antibodies capable of Fc-mediated recruitment) in other hematologic malignancies have shown effectiveness in eliminating cells with even minimal expression of the relevant target (Cheson and Leonard 2008; June and Sadelain 2018), patients with AML often have aberrant immune responses, thus, antileukemic immune activation may not be effective (Le Dieu et al. 2009). It may therefore be important to explore AML therapies with mechanisms of action dominated by direct targeting and functional inhibition for this reason as well.

Of the LSC therapeutic targets that have been proposed, three targets that best fit the criteria described above are TIM-3, CLL-1, and IL1RAP.

TIM-3

TIM-3 protein was found to be significantly upregulated on lymphoid^{-}CD34^{+}CD38^{-}CD90^{-}

AML versus lymphoid$^-$CD34$^+$CD38$^-$CD90$^+$ normal BM cells (Jan et al. 2011), and more broadly in CD34$^+$CD38$^-$ (Kikushige et al. 2010; Ho et al. 2016) and myeloid CD34$^+$ cells (Coustan-Smith et al. 2018) in AML versus normal BM samples. TIM-3 has been shown to identify CD34$^+$ AML cells with leukemia initiating activity upon xenotransplantation (Kikushige et al. 2010; Jan et al. 2011; Ho et al. 2016).

There is conflicting data on the expression of TIM-3 in normal HSCs. One study found expression on ~30% of normal BM lymphoid$^-$ CD34$^+$CD38$^-$ cells, but further showed that within this population, TIM-3$^-$ cells contributed to significantly increased multilineage engraftment in immunocompromised mice than TIM-3$^+$ cells, leading to the conclusion that functional HSCs are primarily TIM-3$^-$ (Jan et al. 2011). However, other studies found no TIM-3 expression on normal BM CD34$^+$CD38$^-$ cells (Kikushige et al. 2010; Haubner et al. 2019). TIM-3 expression is restricted to the hematopoietic system, within which TIM-3 is only expressed on monocytes and a fraction of natural killer cells and GMPs (Kikushige et al. 2010; Haubner et al. 2019).

A TIM-3-directed monoclonal antibody reduced engraftment of primary AML cells in immunodeficient mice and minimized AML engraftment upon secondary transplantation, indicating LSCs were targeted. Normal CD34$^+$ cord blood cells were not affected in similar xenograft/antibody experiments. The authors attributed the effects on AML cells to antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Kikushige et al. 2010).

TIM-3 has been described to be a receptor for a molecule secreted by AML cells, galactin-9 (Gal-9). Kikushige and colleagues demonstrated that TIM-3 activation by Gal-9 led to activation of Akt, ERK, and NF-κB, and to nuclear translocation of β-catenin in primary AML cells. They also showed that Gal-9 is functionally important for LSCs as antibody-mediated neutralization of Gal-9 reduced engraftment of CD34$^+$ AML cells in xenograft models (including the CD34$^+$ CD38$^-$ population) but did not affect engraftment of normal human cord blood cells (Kikushige et al. 2015). Finally, because expression of TIM-3 persists at relapse in most patients, these cells are likely part of a therapy-refractory clone (Ho et al. 2016; Coustan-Smith et al. 2018).

CLL-1

Expression of CLL-1 has been reported on CD34$^+$CD38$^-$ as well as FSC^{low}SSC^{low}CD38^{low} SP AML cells by several groups; meanwhile, expression is absent on these populations in normal BM, on regenerating BM CD34$^+$CD38$^-$ cells, and on mobilized peripheral blood CD34$^+$CD38$^-$ cells (Bakker et al. 2004; van Rhenen et al. 2007a,b; Moshaver et al. 2008; Kikushige et al. 2010; Sadovnik et al. 2017; Bill et al. 2018; de Boer et al. 2018; Haubner et al. 2019). Further, CLL-1$^+$ AML cells were found to be cytogenetically abnormal (Moshaver et al. 2008) and CD34$^+$CD38$^-$CLL-1$^+$ AML cells gave rise to leukemia in immunodeficient mice (van Rhenen et al. 2007b).

CLL-1 expression has only been detected in the hematopoietic system (Bakker et al. 2004), where it is expressed by some normal myeloid cells, including monocytes, macrophages, dendritic cells, granulocytes, and myeloid precursors (primarily GMPs, but also on a smaller percentage on CMPs and megakaryocyte–erythrocyte progenitors) (Bakker et al. 2004; Perna et al. 2017; Bill et al. 2018; de Boer et al. 2018; Haubner et al. 2019). No significant increase in CLL-1 expression was found in immunophenotypic myeloid progenitors in AML versus normal samples (Coustan-Smith et al. 2018; de Boer et al. 2018) and expression of CLL-1 is lower in LSCs compared to bulk AML cells and normal mature myeloid cells (de Boer et al. 2018). These may be important caveats for therapeutic targeting of this protein.

Several CLL-1-directed therapies have been developed that lead to cytotoxicity in AML cell lines and primary AML cells, as well as in AML cell line xenografts, while sparing normal HSCs (for a review, see Morsink et al. 2019). These include monoclonal antibodies (Zhao et al. 2010), antibody–drug conjugates (Jiang et al. 2018; Zheng et al. 2019), CLL-1/CD3 bispecific antibodies (Lu et al. 2014; Leong et al. 2017), and

anti-CLL-1 CAR T cells (Laborda et al. 2017; Tashiro et al. 2017; Wang et al. 2018). A CLL-1/CD3 bispecific antibody (MCLA-117) is being tested in a phase I clinical trial in patients with AML (NCT03038230), as is a compound CLL-1/CD33 CAR T-cell therapy (Liu et al. 2018).

Given the high expression of CLL-1 in monocytes and granulocytes, CLL-1-directed therapies may have toxicity issues. This is evidenced by studies showing that only bispecific antibodies with low (vs. high) affinity for T cells were tolerated in preclinical trials in monkeys (Leong et al. 2017), and CLL-1 antibody–drug conjugates and CAR T cells were cytotoxic to mature normal myeloid cells (Tashiro et al. 2017; Zheng et al. 2019). CAR T approaches against this target must incorporate a suicide system for safety reasons (Tashiro et al. 2017). It will be important to test the relative functional relevance of CLL-1 in these normal cell types versus AML cells to determine whether a strategy that blocks the function of CLL-1 instead of recruiting the immune system might limit toxicity. In this light, studies on CLL-1 (Clec12a) knockout mice provide some encouragement. Although the number of *Clecl12a*$^{-/-}$ mice born is lower than Mendelian ratios, once born, *Clecl12a*$^{-/-}$ mice have no major abnormalities, a normal life span, and no signs of disease. These mice show normal cellularity in hematopoietic organs and no changes in frequency of mature hematopoietic cells compared to WT mice (Neumann et al. 2014). These studies suggest a broadly nonessential role of CLL-1.

Although a specific role for CLL-1 has not been studied in leukemia or cancer in general, studies in normal hematopoietic cells indicate it plays a role in negative regulation of the immune system. For instance, CLL-1 was found to physically associate with the signaling phosphatases SHP-1 and SHP-2, inhibiting activation of immune cells through a cytoplasmic inhibitory motif (Han et al. 2004; Marshall et al. 2004). CLL-1 was shown to be involved in Toll-like receptor and T-cell activation pathways (such as CD40L) through regulating cytokine production (Chen et al. 2006). It was also found that CLL-1 dampens immune responses triggered by damage-associated molecular patterns (Gagné et al. 2013; Neumann et al. 2014). Together, these studies suggest that CLL-1 normally inhibits some inflammatory processes, and suggest the role of CLL-1 in leukemia could be to modulate immune responses as an immune-evasion mechanism.

Lastly, CLL-1 expression persists in remission and relapse in most patients, implying CLL-1 is part of a chemotherapy-resistant clone (van Rhenen et al. 2007b; Coustan-Smith et al. 2018; Haubner et al. 2019). Specifically, CLL-1 expression on CD34^{+}CD38^{-} cells at complete remission was associated with relapse in several patients and more strongly correlated with relapse than did standard MRD detection by leukemia-associated (immune) phenotype expression. Furthermore, lasting remissions occurred in patients with low or absent CLL-1 expression in the stem cell population at complete remission (van Rhenen et al. 2007b).

IL1RAP

IL1RAP gene expression was specifically shown to be significantly up-regulated in AML Lin^{-}CD34^{+}CD38^{-}CD90^{-} cells (a population highly enriched for LSCs; Goardon et al. 2011) compared to this population in age-matched healthy BM samples (Barreyro et al. 2012). Multiple additional studies have identified IL1RAP as a target for LSC-directed therapy in AML based on its surface expression in HSC-enriched populations from AML specimens (Askmyr et al. 2013; Bonardi et al. 2013; Ho et al. 2016; Sadovnik et al. 2017).

IL1RAP protein expression is absent from normal HSCs (Jaras et al. 2010; Barreyro et al. 2012; Ho et al. 2016; Sadovnik et al. 2017; de Boer et al. 2018), but is present in mature cells of the immune system, primarily monocytes and macrophages, and to a lesser extent lymphocytes, natural killer cells, and mast cells. It is lowly expressed on some myeloid progenitors as well (Jaras et al. 2010; Barreyro et al. 2012; de Boer et al. 2018). It is known that the canonical ligand for the IL1RAP/IL-1 receptor complex, IL-1β, acts on epithelial cells, fibroblasts, keratinocytes, endothelial cells, muscle cells, and neurons (Striz 2017); thus, IL1RAP may be

expressed in these tissues, at least in the inflammatory setting. Protein array data confirms low IL1RAP expression in some of these tissues (Kim et al. 2014; Wilhelm et al. 2014; Ågerstam et al. 2015; Uhlen et al. 2015). Toxicity in these cell types would be important to monitor if IL1RAP-directed therapies move forward. However, given that total body *Il1rap*$^{-/-}$ mice do not have overt abnormalities, and *Il1rap*$^{-/-}$ BM is capable of complete and long-term (>20 wk) reconstitution of lymphoid and myeloid hematopoietic cells (Mitchell et al. 2018), on-target, off-tumor toxicity may be limited.

Several groups have targeted IL1RAP in AML using different strategies. Some have focused on the utility of IL1RAP as a surface label and target for immune effector cells in AML. For instance, an IL1RAP-directed monoclonal antibody significantly reduced leukemic burden in BM and spleens of mice transplanted with *MLL-AF9* and activated *NRAS* cotransduced cord blood cells as well as mice transplanted with primary AML cells, with effects attributed to natural killer cell ADCC activity (Ågerstam et al. 2015). These studies have led to testing of an IL1RAP-directed antibody in a phase I clinical trial (CANFOUR trial) where dose escalation will first be tested in patients with solid malignant tumors (NCT03267316). Jiang and colleagues showed in a xenograft of the AML cell line EOL-1 that several different IL1RAP-directed monoclonal antibodies completely eliminated AML engraftment in BM and blood. These antibodies induced CDC of primary AML blasts when incubated with cells and complement in vitro (Jiang et al. 2016). Additionally, an IL1RAP/CD3 bispecific antibody is in development, and both in vivo and in vitro effects on the MV4;11 AML cell line were presented at the 2017 American Society of Hematology meeting (Meng et al. 2017).

The functional importance of IL1RAP in leukemic cells has also been tested. shRNA-mediated knockdown of IL1RAP in primary AML cells led to reduced clonogenic growth and in AML cell lines led to reduced engraftment in immunodeficient mice. In addition, IL1RAP-directed antibodies increased apoptosis of primary AML cells in the absence of immune effector cells but spared normal hematopoietic cells. These IL1RAP-directed antibodies nearly eliminated leukemic cells in the BM in AML cell line xenografts. Finally, *Il1rap* loss impaired LSC function/frequency in MLL-AF9-driven AML. These studies imply that IL1RAP is functionally important for growth and survival of AML cells (Mitchell et al. 2018).

Specific functions of IL1RAP in AML cells have been recently explored. IL1RAP antibodies inhibited IL-1-induced AML cell growth (Ågerstam et al. 2015) and signaling (Mitchell et al. 2018) in vitro. In MLL-AF9 AML, shRNA-mediated knockdown of IL1RAP led to reduced degradation of the WT MLL protein, which normally competes with the MLL-AF9 fusion; thus, IL1RAP targeting reduced MLL-AF9 occupancy and aberrant transcription (Liang et al. 2017). Whereas the canonical role of IL1RAP is to facilitate signaling through the IL-1 pathway, several lines of evidence suggest that IL1RAP has alternative or at least additional mechanisms in AML. *FLT3 WT* and *FLT3* mutant AML cells were found to be differentially sensitive to IL1RAP antibody-mediated inhibition, which led to studies demonstrating that IL1RAP modulates FLT3 ligand-mediated signaling and that growth and these two proteins physically interact. This relationship was also demonstrated for the receptor tyrosine kinase c-KIT (Mitchell et al. 2018). Thus, AML cells may use up-regulation of IL1RAP as a way to amplify multiple signaling pathways involved in cell survival and proliferation.

High *IL1RAP* expression in AML correlates with poor prognosis (Barreyro et al. 2012; de Boer et al. 2018), and IL1RAP expression persists at relapse (de Boer et al. 2018). IL1RAP expression may be associated with the acquisition of certain mutations, as it was found to mark genetically distinct AML subclones such as those with *NRAS* mutations (de Boer et al. 2018). In monosomy 7 AML patient samples, the clone size of IL1RAP^{+} AML HSPCs included and exceeded that of cells bearing the chromosomal aberration, suggesting IL1RAP overexpression preceded loss of chromosome 7, potentially marking a pre-leukemic cell population. The timing of IL1RAP overexpression in

leukemic transformation should be further explored in future studies.

CONCLUDING REMARKS

For AML patients over the age of 65, which represent the majority (Noone et al. 2018), the 5-year overall survival is currently only estimated to be ~14%, and has improved marginally in the last 45 years (Mokdad et al. 2017; Lancet 2018). In recent years, therapeutic advances leading to improved patient outcomes in AML are a result of the optimization of existing drugs rather than the use of novel therapeutics (Fernandez et al. 2009; Lancet et al. 2017; Wei and Tiong 2017). Clearly, improved and more specific treatments are necessary in this disease, yet a universal antigen that fits all criteria for an ideal LSC target has been challenging to find.

Because of their lack of expression on normal HSC and minimal expression/essentiality on other normal tissues, their functional importance in AML cells, and persistent expression at relapse, we would propose TIM-3, CLL-1, and IL1RAP as the most promising current therapeutic targets to safely eliminate LSCs. Recent findings appear to favor the development of direct targeting strategies against these targets to reduce toxicities and minimize potential resistance mechanisms (Fig. 2). In addition, while CD25 only marks LSCs in a fraction of patients, it appears to be a strong predictor of relapse and may represent another potent drug target in a smaller population of patients.

Although targeting a common immunophenotypic antigen at the stem/progenitor cell level is a potential means to overcoming the daunting genetic complexity, inter- and intrapatient heterogeneity of AML, therapies may still need to be tailored to distinct molecular subtypes of AML, and, accordingly, more studies will be required to relate mutational profiles to cell-surface expression of putative LSC antigens in larger cohorts of patients. Targeting several antigens to eliminate leukemic cells that persist at remission may be necessary (Haubner et al. 2019). Overall, advances in our understanding of AML pathogenesis provide insight into the improvement of LSC-targeted therapies in AML: continued development of the LSC model, including further understanding of the progression from CH to pre-leukemia and AML, hold promise in informing better targets and therapeutic agents for the treatment and cure of AML.

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