Non canonical Wnt ligands and cytokine-driven myelopoiesis

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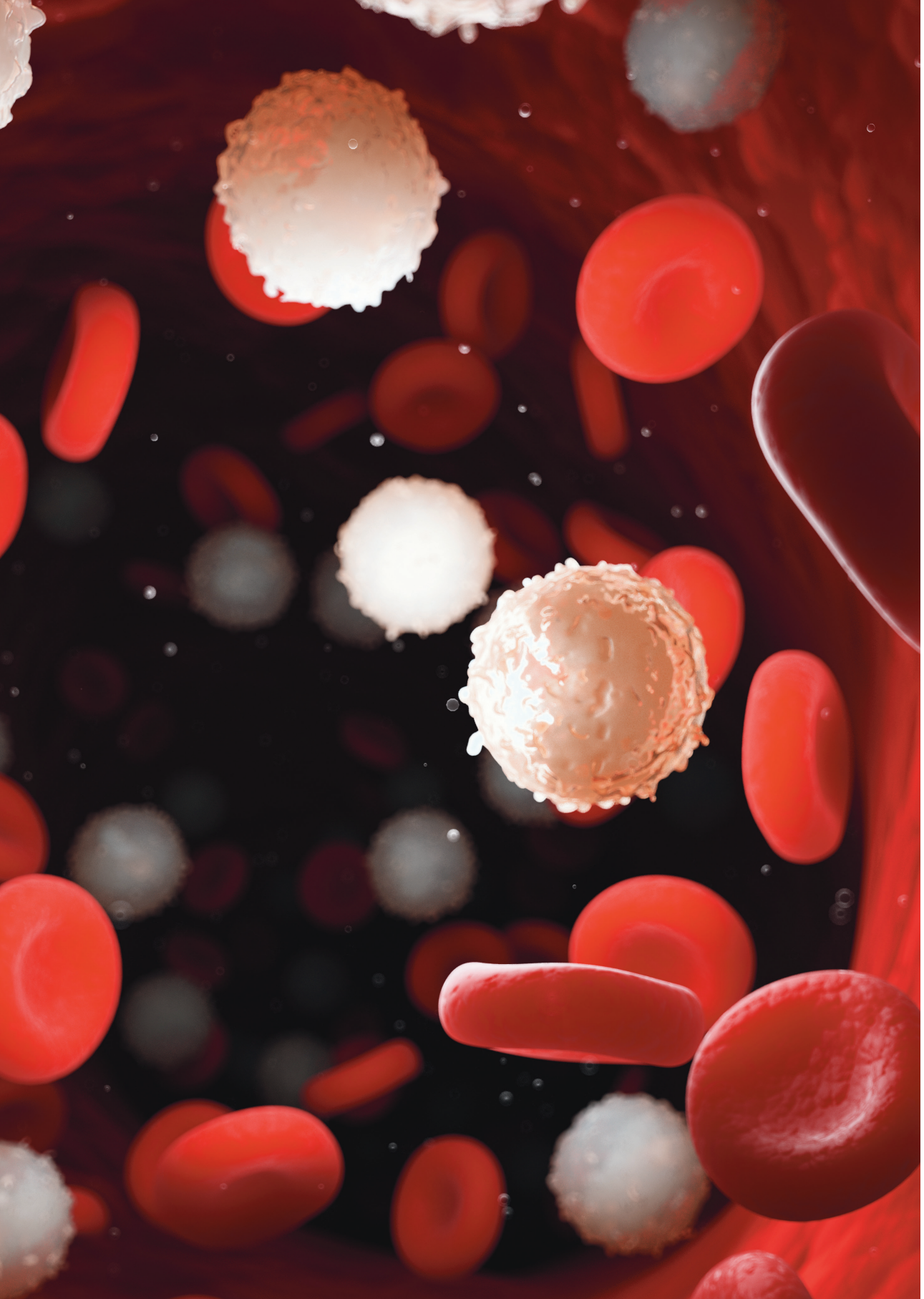

Leukemia stem cell immunophenotyping - Tools for diagnostic, prognosis and therapeutics

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3. LEUKEMIA STEM CELL IMMUNOPHENOTYPING – TOOLS FOR DIAGNOSTIC, PROGNOSIS AND THERAPEUTICS

3.1. ABSTRACT

The existence of cancer stem cells is debatable in numerous solid tumors, yet in leukemia, there is compelling evidence of this cell population. Leukemic stem cells (LSCs) are altered cells in which accumulating genetic and/or epigenetic alterations occur, resulting in the transition between the normal, preleukemic and leukemic status. These cells do not follow the normal differentiation program; they are arrested in a primitive state but with high proliferation potential, generating undifferentiated blast accumulation and a lack of a mature cell population. The identification of LSCs might guide stem cell biology research and provide key points of distinction between these cells and their normal counterparts. The identification and characterization of the main features of LSCs can be useful as tools for diagnosis and treatment. In this context, the aim of the present review was to connect immunophenotype data in the main types of leukemia to further guide technical improvements.

Keywords: leukemia, leukemic stem cell, surface markers, cytometry, immunophenotype.

3.2. INTRODUCTION – LEUKEMIC STEM CELLS (LSCS) AS A TOOL TO UNDERSTAND LEUKEMIAS

Leukemia comprises a diverse set of malignant diseases that share the common feature of sustained leukocytosis on the bone marrow or peripheral blood[1,2]. This cellular accumulation affects people of all ages and both sexes[2] and can target hematopoietic cells broadly, from primitive to mature and from the myeloid to lymphoid lineage, including mixed cells in some cases. The affected cell population has an intimate relationship with diagnosis and prognosis. The main diagnostic tool used thus far is based on morphology and cytogenetic techniques; however, leukemia subtype identification and its link to risk stratification and prognosis are limited.

A fundamental component of a poor prognosis and decreased overall survival is relapse occurrence[3], that is, the reappearance of disease after treatment. The main cause of relapse is the persistence of a bulk of malignant cells that are resistant to treatment[4]. This reservoir for relapse is believed to be formed by quiescent cells with primitive characteristics – leukemic stem cells (LSCs).

The existence of cancer stem cells is not widely accepted in cancer research[5]; however, in leukemia, there is substantial evidence of their existence. These cells were first described in the 1960-70s[6] in clonal experiments in which colony formation was observed in a rare cancer population. This ability is understood as a matter of primitiveness because of self-renewal potential. Nonetheless, the concept was not validated until the 1990s[7,8] and was then introduced into cancer and leukemia research, mainly considering acute myeloid leukemia (AML), a disease in which the LSC theory was initiated[5].

As the normal counterpart of LSCs, hematopoietic stem cells (HSCs) are characterized by their self-renewal potential and broad differentiation capability, as well as quiescence and primitive morphological features[9-12], although unregulated proliferation and differentiation might be present in addition to aberrant cell production[13].

These cells are often referred to as leukemia-initiating cells (LICs)[14,15], as they reliably reproduce the donor's disease in xenograft models[3,10,16]; however, engraftment potential assays are needed to justify such denomination[16,17], and these cells are widely used in experimental cancer research but less frequently in diagnosis and in the clinic.

Experiments with transplantation and xenograft models have been extensively used and are the basis for population and surface marker definitions. These methods are able to establish whether a population is capable of initiating leukemia and indicate the hematopoietic lineage-biased leukemic formation and primitiveness of LICs[16], which are related to diagnosis and treatment outcomes.

Lineage-biased cell formation and the primitiveness of LSCs have led to a discussion on the origin of LSCs, and there are different theories about it. It is well accepted that tumorigenesis occurs by the accumulation of numerous genetic alterations, which alone are harmless[18-20], but account for the preleukemic status[2,21]. Genetic alterations (e.g., chromosomal translocations[19,20]) and DNA damage (by complications of a previously diagnosed malignancy[2]) are common primary alterations. Genes related to epigenetic control and intracellular signaling have been described as targets of secondary leukemic alterations, although the molecular basis of these mutations is not completely understood[18].

Considering that numerous alterations target the genetic cellular content, it was proposed that cycling cells would be more likely to accumulate damage, as their replication machinery is continuously active[19]. This hypothesis was reinforced by the idea that these cycling cells reside in the vascular niche, where contact with noxious stimuli and substances are more likely compared with the endosteal niche. In this hypothesis, ST-HSCs (short-term HSCs) or progenitors would reacquire HSC properties, such as self-renewal and long-term engraftment potential, in addition to differentiation arrest (or even dedifferentiation) and proliferation gain[3,22,23]. On the other hand, there is evidence that transformation could occur in HSCs as well, since these cells are maintained throughout life, with a low turnover rate[9,12]. In this hypothesis, stemness would be maintained, but a proliferative pool would be generated[23,24] and responsible for disease propagation[18,22].

Both transformation hypotheses are presumably accurate, as leukemia is a heterogeneous malignancy[20]. In fact, there is evidence that AML is commonly caused by transformation in primitive cells[9,13,18,24,25], whereas in chronic myeloid leukemia (CML) and myelodysplastic syndrome (MDS), multipotent progenitors are affected[9]. However, the existence of LSCs in acute lymphoid leukemia (ALL) is debatable[9,25]. In any case, it is important to highlight that even when considering individual leukemia types, there is a wide range of heterogeneity, which is related to transformed cells[9,18,24], as well as molecular alterations.

Primitively altered cells have similarities with their normal counterparts, and some properties favor their survival[9,26]. As discussed earlier, endosteal niche occupation is proposed to be one resistance mechanism[27] that functions through the mechanical protection of chemotherapeutic drugs[28,29]. Their quiescent status is a key point as well, since it protects against drugs that target actively cycling cells, making them resistant to most available tyrosine kinase inhibitors[24,30]. Additionally, apoptosis entrance protection[11] and aging avoidance[12] have been described, among other mechanisms, to explain cell persistence and relapse occurrence[3-5,11,15,26,28].

Based on this evidence, the need for a technical development that characterizes and isolates the LSC population (highlighting its discrepancies from the HSC population) is compelling[5,14,16,21,31] to promote advancements in diagnosis and therapies[24,32]. In this context, multiparametric flow cytometry is convenient[1], because this technique is reliable, fast and provides isolated alive cells that can be used for subsequent experiments. The aim of the present review was to describe potential markers to recognize LSCs and support forthcoming research in the field.

3.3. THE OUTSTANDING HEMATOPOIETIC MALIGNANCY OF ACUTE MYELOID LEUKEMIA (AML)

AML is the most prevalent leukemia in adult humans[17,23], and it is an aggressive, complex and heterogeneous disease originating from genetic and epigenetic alterations in which differentiation ability is lost by primitive cells, compromising mature cell production and accumulating myeloid primitive cells on the bone marrow and peripheral blood[3,5,10,15-18,20,22,28,32-35].

Typical genetic alterations associated with AML are chromosomal translocations and abnormal fusion protein formation, such as BCL-ABL, AML1-ETO, RUNX1/RUNX1T1, PML/RARα and DEK/NUP214[34,36]. These abnormalities, in addition to blast morphology and differentiation stage arrest, are used for diagnosis, subtype definition and risk stratification[15,20,37]. AML is the most well-known and well described type of leukemia, yet new therapies are needed to cure it and to improve its survival rate[5,15-17,23,26,35,37]. High-dose chemotherapy is effective in less than 50% of patients[38], and this is associated with the survival of a chemotherapy-resistant pool and relapse occurrence[5,17,21,22,26,32,33,35,38,39].

As discussed earlier, this resistant pool is composed, in part, of LSCs, which were first described in AML. In fact, all cancer stem cell hypotheses were established on this condition[17], which is now seen as a clonal disease[32,33,40,41], possibly organized hierarchically, as normal hematopoiesis[22,33,42]. Nevertheless, in AML, additional issues have been described, such as multiple leukemic-initiating populations, leukemic advantage and dynamic immunophenotypic characteristics, which might guide our understanding of leukemogenesis[18,24].

Multiple leukemic-initiating populations refer to the possibility that inside the heterogenic pool of altered cells, more than one type – with different immunophenotypes – can initiate disease in a xenograft model[10,21,22]. This might be explained by multiple preleukemic clones with divergent alterations or origins in the leukemic process. It is hypothesized that when HSCs are transformed, accumulated blasts exhibit less atypical membrane proteins, resembling HSCs and

having more primitive characteristics[24]. When progenitors are transformed (and in this case, the granulocyte-monocyte progenitor is mainly affected), leukemic cells exhibit morphology and membrane protein expression associated with more mature cells[22,26,40]. Importantly, regardless of morphology and membrane protein expression, the proportion of LSCs varies, which also reflects the prognosis[3,41].

Leukemic advantage is related to chemotherapy resistance and relapse, with LSCs outcompeting HSCs for the niche[9] or disturbing HSC maintenance[33], and dynamic immunophenotypic characteristics are associated with changes in the surface protein expression on LSCs due to therapeutic treatment[13,21]. In fact, it is well documented that during relapse, LSCs commonly change their surface protein expression, reinforcing dynamic behavior[13,43]. Thus, considering the singularities of LSCs in AML, it is possible to discuss potential flow cytometry panels and immune therapy targets.

3.3.1. HETEROGENEITY IN THE PROGNOSIS AND IMMUNOPHENOTYPE OF LSCs IN AML

The most accepted HSC marker in humans is CD34, which is also the most accepted marker of AML-LSCs. When considering leukemic cell lines, this marker is also suitable for LSC separation. As CD34 positivity is not specific for LSCs (gathering progenitors in variable differentiation stages as well)[20], CD38 is also widely used[3,5,9,10,14,16-18,20,21,24,29,38,40,41], as it increases population selectivity; however, $CD34^+CD38^-$ is not enough to have a homogeneous population and, more importantly, is not enough to separate LSCs from HSCs[9,14,35]. The enrichment strategies used and surface markers investigated are diverse; however, it is clear that some strategies are used more frequently than others, such as $CD34^+$, $CD34^+CD38^-$, $CD34^+CD38^-Lin^-$, $CD34^+CD38^-CD123^+$, $CD34^+CD38^-CD133^+$, and $CD34^+CD38^-CD123^+CD90^+$, but the results might vary according to the gate selection strategy used[21]. Additional surface markers are shown in **Figure 1**, and a complete list of these markers is presented in **Supplementary Table 1**.

Lineage-specific and mature cell markers can be used to distinguish LSCs from HSCs, but they may not be efficient, as there are overlaps, and the atypical presence of mature cell markers in LSCs varies considerably due to their differentiation disarray[23]. One way to increase the use of lineage (Lin)-specific and mature cell markers is by using a cocktail (Lin cocktail) to further enrich the sample for LSCs. By mixing surface markers, we can use the atypical presence of mature markers as a convenient way to improve LSC and HSC separation, as they are absent on HSCs[21]. Moreover, in patients with the $CD34^+CD38^-Lin^-$ LSC population, the lack of CD90 expression (also known as Thy-1) may enrich for the primitive population[3,17,22,31], although its positivity has been observed by other researchers[31].

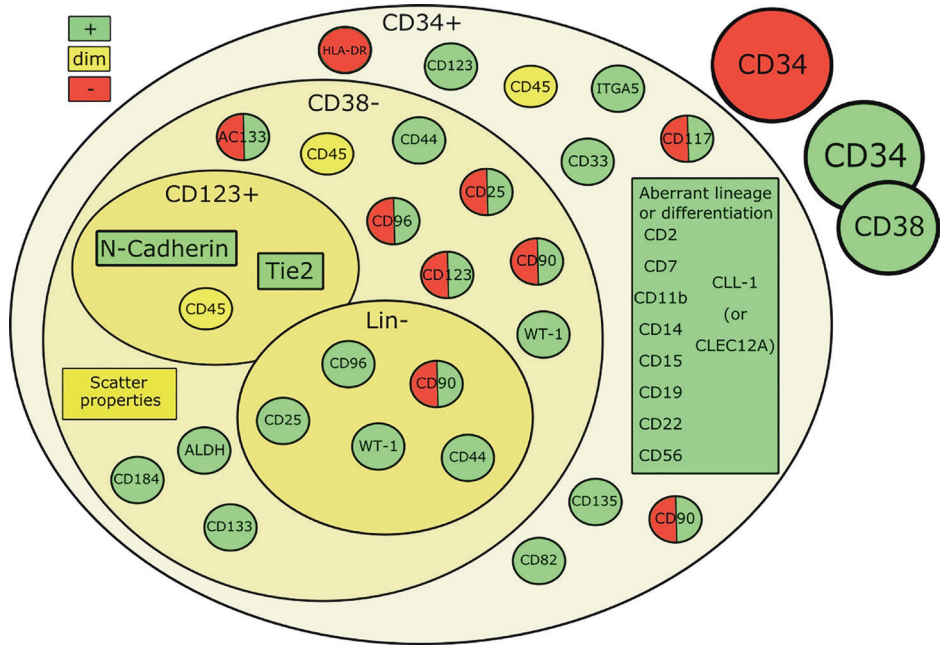

Figure 1. Schematic representation of strategies used to enrich the AML-LSC population. Red circles refer to the absence of marker expression, and green circles refer to their presence. Yellow circles indicate weak expression or a variable form of analysis (in the case of scatter properties). Three main groups can be observed, $CD34^+$, $CD34^-$ and $CD34^+CD38^+$, although the most investigated one is $CD34^+$, with the following subdivisions: $CD34^+CD38^-$, $CD34^+CD38^-CD123^+$ and $CD34^+CD38^-Lin^-$. ALDH refers to aldehyde dehydrogenase activity, and WT-1 refers to Wilms tumor 1.

When considering CD45RA, the human homolog of B220 in mice[24], it is important to highlight that its expression and signal on flow cytometry are not strong ($CD45^{dim}$) on LSCs[3], which may hamper its utilization for LSC gating and gate selection, influencing the sample purity in cell sorting. The expression of CD45 concomitantly with that of CD90 may be used as a strategy, since in more than 90% of AML patients, LSCs are contained in the $Lin^-CD34^+CD38^-CD45^+CD90^-$ population[22].

As shown in **Figure 1**, other lineage-specific and mature cell markers have also been described[40], such as CD2, CD7, CD11b, CD13, CD14, CD15, CD19, CD22, CD33, CD56, CD123 and lectin-like C-like molecule-1 (CLL-1)[3,5,10,14,16,21,29,35]. It is worth discussing the expression of CD123 and CLL-1. CD123 is not only a myeloid lineage marker but also the IL-3 receptor, playing roles in HSC and LSC intracellular pathway activation[3,17,20,41]. CD123 was the first described LSC-specific antigen[11,35]. The panel $CD34^+CD38^-CD123^+$ is widely used for LSC characterization[11,16,20,27,28,31,40,44], and its presence is associated with a poor prognosis[17,35,44], since this population appears to have chemotherapy escaping skills[37,38] and is associated with unfavorable cytogenetics[35].

CLL-1, also known as C-type lectin domain family 12 member A (CLEC12A) 21 or CD123, is highly expressed on AML-LSCs[21,29,40] and absent on HSCs and normal progenitors; it is also suitable for LSC characterization and is important in leukemic cell lines such as KASUMI, KG1 and TF1[16]. Other markers associated with lineage-specific and mature cells that are aberrantly expressed in LSCs appear in less than 50% of AML patients with CD34^{+} LSCs[21], except for CD33[21].

In addition to CD123, CD25 is another cytokine-related receptor that can also be present in AML-LSCs[5,14,17,29,35,41]. In this context, it likewise exerts intracellular signal triggering activities and is associated with a poor prognosis[17,35] because of its link to unfavorable cytogenetics. Both CD123 and CD25, aside from CD96, appear to be equally and highly predictive of a poor prognosis[32,35]. CD96, also known as TIM-3 (T-cell Ig mucin 3) or Tactile[5,17,21,31,35], in contrast to CD123 and CD25, does not participate in cytokine signaling and is related to homing properties[17,31,41], similar to CD44, which was described as possible surface marker of LSCs[5,15-17,21,29,35,41,42].

Flow cytometry also permits the use of other parameters in addition to surface proteins, and they are also reliable for distinguishing LSCs from HSCs. Higher values of forward scatter (FSC) and side scatter (SSC) in LSCs have been observed in comparison with HSCs[14], which indicate that malignant primitive cells are larger and have more cytoplasmic complexity than their normal counterparts for unknown reasons[14]. In some reports, SSC was enough to segregate these populations[14,26,40,41], although LSCs were observed in the SSC-low population[26,40]. Aberrant aldehyde dehydrogenase (ALDH) activity is also widely associated with the LSC population[14,26,37], although it is not a surface marker.

Noteworthy, some surface markers fit into more than one functional category, and some are closely associated with chromosomal alterations, such as the aberrant expression of CD2, the chromosomal inversion inv(16), the HLA-DR and translocation t(15;17) and the N-cadherin and translocation t(8;21)[27,35]. In fact, N-cadherin expression also seems to be able to discriminate the subtype of AML 27 and is related to a poor prognosis.

Novel markers are continuously being described, and proteomic studies are crucial for a complete view of surface proteins in LSCs. In this context, CD82, CD97 and CD99 were also included as possible targets on LSCs, in addition to PTH2R, ESAM, MET and ITGA6[32], although the prevalence of these new markers is unknown.

Remarkably, there is abundant evidence that the CD34^{-} population might also exhibit stem cell properties[16,20,22,29,38,41,44]. In fact, upon comparing CD34^{+} and CD34^{-} populations in AML, only 9 protein-coding genes were differentially expressed[23], reinforcing the similarity between the populations, although this view is not unanimous[11]. It is unknown whether the absence of CD34 is due to its loss[9] or initially

altered cells. Considering the second option, lineage-specific markers might not be aberrant, as a differentiated cell (such as a committed progenitor) could be targeted by transformation. In this case, there is a gain of primitive functions and the maintenance of lineage-specific markers. In fact, there is evidence that LSCs are transcriptionally more related to the differentiated normal population as progenitors (in particular, granulocyte-monocyte progenitors) than HSCs independent of CD34 expression[22,23].

3.3.2. AML-LSCs IN RELAPSE: THE SAME CELLS TELLING DIFFERENT STORIES

An important event related to changes in surface markers, mainly considering CD34 and CD38, is relapse[13,21]. As discussed earlier, this event is related to a poor prognosis and LSC existence, since this population is responsible for relapse[3,5,11,15,17,21,32,35].

Numerous AML patients achieve remission after standard chemotherapy, yet relapse is equally common[5,21,32,37]. In these situations, a high proportion of patients show immunophenotypic changes to a more primitive stage[13,21,37]. The hypothesis behind this phenomenon is that the remaining cells are more quiescent and more resistant and leukemogenic.

It is unknown whether surface marker changes are due to treatment[13,43], the maintenance of different populations[13], or the involvement of distinct preleukemic clones[21], but it is critical to establish suitable markers to distinguish LSCs in relapse, as it is intimately associated with the prognosis, survival and treatment response[15,16,21]. In this context, CD123^{+} CD44 and CD90^{-17} have been described.

Although the presence of CD34 is commonly associated with primitiveness, glucocorticoids and chemotherapies can inhibit its expression[43], and the disappearance of this marker signal (due to its downregulation) is not uncommon during relapse[21,43]. On the other hand, other markers appear to be maintained, even when protein expression changes significantly[13] (mostly CD123 and CLL-1[21, 44]).

Interestingly, a particular class of surface markers appears to have importance in relapse. Embryonic markers usually change during relapse and are associated with a poor prognosis in solid tumors[3]. OCT4, NANOG, SOX2, SSEA1, and SSEA3 have been investigated and might be involved in pluripotency gain, in addition to being present in leukemia cell lines, such as KG-1, KASUMI and ME-1[3].

Noteworthy, the heterogeneity between patients is high; therefore, AML subtypes and disease evolution must be considered to better establish the immunophenotypic panel used in each situation. Zeijlemaker and colleagues[21] beautifully developed an assay for 8-color flow cytometry reliable for diagnosis and relapse, combining markers with high variance between patients (such as lineage- and differentiation-specific markers – CD7, CD11b, CD22, CD56, CD96 and CLL-1), reducing the need for more colors, and analyzing the most described markers, such as CD34, CD38, CD123 and CD45.

3.4. CML – SPECIFICITY IN MYELOID MALIGNANCES

CML, as well as AML, also affects the myeloid lineage and primitive cells, producing altered mature cells in a clonal manner, although lymphoblasts may also be produced[12,18,45-47]. This subtype of leukemia is widely associated with the appearance of the Philadelphia chromosome, with the t(9;22)(q34;q11) chromosomal translocation[12,18,29,39,45-47] and formation of the BCR/ABL1 fusion protein, which produces a 210 kDa (or 190 kDa) constitutively active tyrosine kinase protein[18,29,45,47]. This functional alteration leads to elevated proliferative, adhesive and antiapoptotic potential[12,45]. Tyrosine kinase inhibitors, as would be expected, are effective for CML[46], although in some cases, the translocation is not reciprocal, producing unbalanced altered chromosomes with particular outcomes[36].

In its chronic phase, this malignancy barely causes symptomatology[48], but when most patients are diagnosed and treated, with favorable chances of remission, chemotherapy alone has insufficient effects[29,47], and the complete eradication of cells positive for the Philadelphia chromosome is not usually achieved[46,47].

Again, disease is maintained by a reduced pool of LSCs that is also responsible for relapse. The chromosomal translocation target cell can be stem cells or multipotent progenitors[29,30], but in both cases, the LSCs are quiescent, self-renewing and chemotherapy resistant[29,30].

3.5. LSCS IN CML: SIMPLE OR UNKNOWN?

CD34 is a key primitive marker; thus, its expression is remarkable for CML-LSC immunophenotyping[45], although leukemia-initiating activity has been described in the negative population as well[45]. In fact, transcriptional similarity was described between the human $CD34^{-}Lin^{-}$ LSC population and the normal HSC population ($CD34^{+}CD38^{-}Lin^{-}$) than between the $CD34^{+}Lin^{-}$ population and the HSC population[45]. Most described markers and gating strategies are represented in **Figure 2**.

An analysis of the $CD34^{+}$ population has shown an (almost) complete predominance of BCR/ABL1-positive cells[36,47], which appear to be reduced in the more differentiated population[36]. A lack of or reduced CD38 expression[29], as well as the Lin cocktail[29], is also important for LSC enrichment[29,46,47], but several parameters change in comparison with AML-LSCs. Indeed, CLL-1 and CD96, important markers for AML-LSCs, are absent in CML-LSCs[29]. In fact, the negativity of these markers can be used to further enrich the LSC population, being used in a channel other than CD25, CD33, CD52, CD117 and IL-1RAP[29]. This panel has been described as effective for BCR/ABL1-positive cell isolation, although Landberg and colleagues[46] showed that only IL-1RAP is sufficient

to isolate this population ($CD34^{+}CD38^{-}IL\text{-}1RAP^{+}$), although further enrichment is possible with the use of CD26[46]. CD26 was also used alone and was able to show a high percentage of BCR/ABL1-positive cells[29], which seems to vary between patients or disease phases[29]. In the context of disease phase, CD19 and CD20 may also improve diagnosis in specific situations[29].

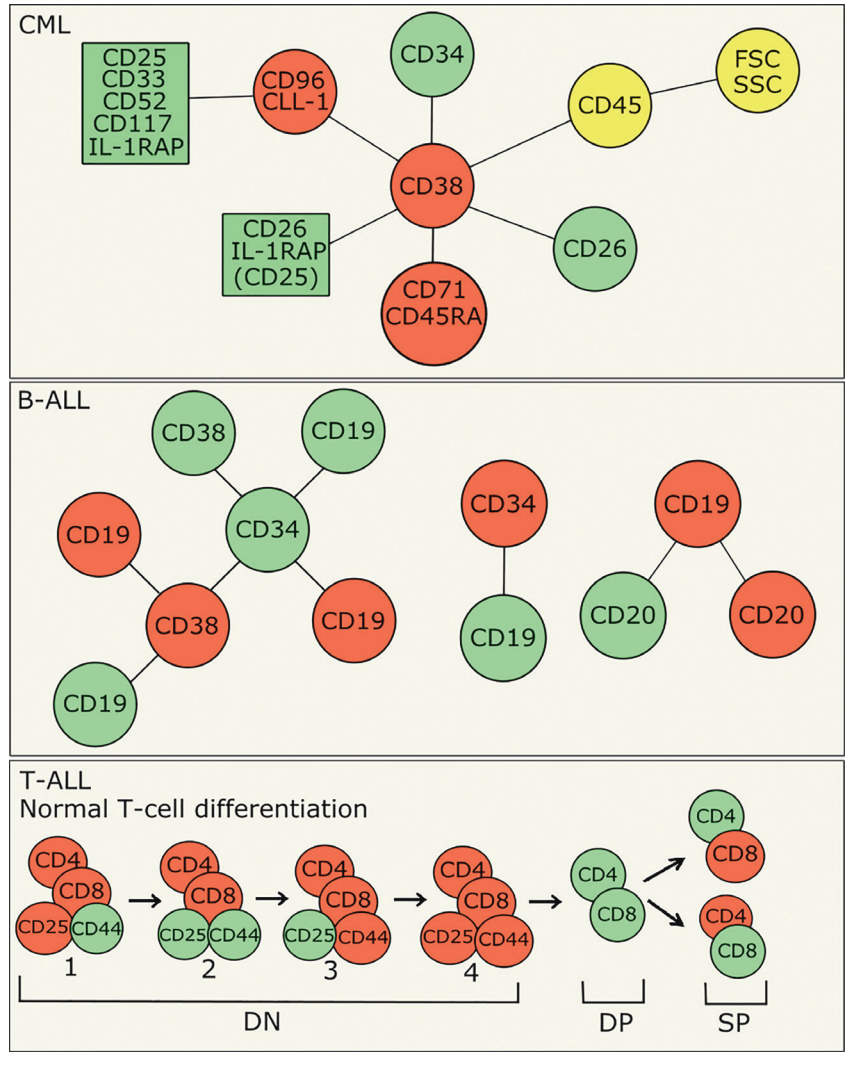

Figure 2. Schematic representation of possible gating strategies for LSC flow cytometry in CML and ALL (B and T subtypes). Red forms refer to the absence of marker expression, and green forms refer to its presence. Yellow forms refer to weak expression or a variable form of analysis (in the case of scatter properties). In the T-ALL subdivision, DN refers to double negative for the CD4 and CD8 population, DP refers to double positive, and SP refers to single positive for the same markers.

CD25 has also been described as useful; however, it seems to discriminate only partially BCR/ABL1-positive cells[46]. Other markers, such as CD45RA and CD71, are widely absent in BCR/ABL1-positive and $CD34^+CD38^-$ populations, which might also serve as negative controls for population cell sorting[47].

The presence of CD13, CD44, CXCR4, CD33, CD1117, CD123, CD133 and HLA-DR has also been described in CML-LSCs; however, it does not seem to drastically improve enrichment[12,29,46]. Neurotransmitter receptors are also increased in CML-$CD34^+$ cells[12], but their roles are unknown.

Light scattering properties were previously described[49], in combination with CD45, and present reduced values for both parameters. The combination of strategies may improve diagnosis, and in the case of CML, it seems significantly simpler than AML, although method validation is needed.

3.6. THE OTHER FACE OF LEUKEMIAS – THE LYMPHOID-BIAS

Lymphoid leukemias, as the nomenclature defines, affects mostly cells from the lymphoid branch of hematopoiesis, which give rise to B and T lymphocytes, and dendritic cells, among others. In addition to myeloid leukemias, lymphoid leukemias are divided into acute and chronic, although other divisions are also used to further specify committed lineage-biased alterations. Similarities and differences between lymphoid and myeloid leukemias have been well described and will be discussed when addressing LSC immunophenotyping.

3.6.1. ALL – DIVERGENT STEPS IN DIFFERENTIATION

ALL is heterogeneous when considering its genetic alterations and prognosis[9], and it is the prevailing type of leukemia in pediatric subjects[25]. In addition to CML, ALL is also widely associated with Philadelphia chromosome formation due to the t(9;22)(q34;q11) chromosomal translocation, which can occur in HSCs or progenitors[9,18,19], resulting in the formation of a 190 or 201 kDa fusion protein. In fact, the size of the fusion protein may indicate a preleukemic origin[9]. Other fusion proteins are also associated with ALL development, such as TEL-AML1 (or ETV6/RUNX1), resulting in t(12;21)(p13;q22)[9] and CALM/AF10[24], and it may affect HSCs or progenitors, which echo the differentiation stage of blast accumulation, although it is debatable whether only HSCs can be targeted by preleukemic events and whether the differentiation stage reflects blast capabilities[18].

Although ALL presents good responses to current treatments and a high percentage of long-term survival, in relapse patients, the rates drop drastically[25] and appear to be due to the presence of LSCs. As previously discussed, the existence of an LSC population in ALL[9,25] is debatable, although a rare population capable of in

vitro survival and proliferation[25] has been demonstrated. However, there is a lack of appropriate in vitro and in vivo models in which this disease can be investigated[25].

$CD34^+CD38^-$, again, appear to reliably enrich for LSCs, although the expression of these markers is heterogeneous between patients[18]. The selections of markers for ALL are represented in **Figure 2** and summarize populations with described leukemia-initiation activity[25]. CD19 presence is also a crucial marker for ALL, since it is a regulator of lymphocytic signaling[9], although its expression changes according to altered genetic profiles[9,25,43]. In fact, $CD19^+$ clones have shown to possess leukemia-initiating capability independent of CD34 expression[25].

In BCL-ABL-ALL subtypes, the $CD34^+CD38^-CD19^-$ population is usually normal, but it might be a target for transformation, mainly considering the 210 kDa fusion protein type[9]. It would generate an aberrant population of $CD34^+CD38^-CD19^+$ cells that harbor a few types of translocations[9], in addition to CD26 expression[29].

In B-biased ALL (B-ALL), in which the B-lymphocyte differentiation lineage is compromised, the Philadelphia chromosome is the most common alteration[18]; therefore, CD19, CD26 and the size of the BCR-ABL fusion protein are essential characteristics for LSC investigation and risk stratification.

In contrast, for T-cell biased ALL (T-ALL), most of the altered genes are related to regulatory pathways and transcription factor expression[19]. In more than 50% of patients, the Notch1 gene is translocated[19], but SCL/TAL1, LMO1 and LMO2 are also widely present[19]. In fact, the retroviral transduction of constitutively active Notch1 and LMO2 was able to initiate T-ALL[19] without other genetic alterations, highlighting the importance of these protein alterations in T-ALL leukemogenesis.

The normal T-cell differentiation workflow is represented in **Figure 2** and is the basis for the T-ALL LSC search. Clinically, there are commitment double negative (DN) and double positive (DP) populations, although only the DN population recreates T-ALL in the xenograft model[19,50], indicating LSC potential that is absent in the DP population. As shown in **Figure 2**, in T-cell differentiation, cells pass through surface marker alterations, starting from a more primitive population, which is DN for CD4 and CD8 ($CD4^-CD8^-$), and gain and lose the expression of CD25 and CD44. The next stage involves the gain of CD4 and CD8, defining the DP population; therefore, it is expected that the DP population is less capable of leukemia initiation, since this population is a result of the differentiation process. Interestingly, higher potential has been described in the DN3 and DN4 populations[19]. One explanation for this might finding be the presence of CD25 (in DN3), a target of Notch[19]. As observed for the $CD34^+CD38^-$ population, the DN3 immunophenotype is not enough to distinguish the leukemic counterpart from its normal counterpart. Aberrant CD25 expression on DN4 was described as one point of distinction[19].

Oddly, single positive populations ($CD4^+CD8^-$ or $CD4^-CD8^+$) are also capable of initiating leukemia after transplantation[50], mainly considering $CD8^+$ [50], but there is still a lack of differential markers that can identify LSCs. Lymphocytic leukemias are less known than myeloid leukemias considering stem cell biology, but a consensus seems to be drawing close. The links between genetic alterations and surface markers are more straightforward, easing therapeutic target development.

The information gap between these 3 main types of leukemia is enormous and sometimes contradictory, which reinforces the heterogeneity of leukemias, even considering each type individually. In this context, it is crucial to adjust technical tools to reduce errors and misinterpretation. LSC identification is one key point to diagnosis and therapeutic advances in addition to trace cellular alterations linked to leukemogenesis. Thus, we gathered scientific data about this population with the hope of aiding in technical improvements.

3.7. CONCLUSION

Leukemias are heterogenous diseases in which the cause, incidence and prognosis change significantly. Thus, it is imperative to avoid simplistic views and extrapolations and address each subtype of leukemia by its peculiarities, such as the blood lineage involved, targeted cells and genetic or epigenetic transformation. Transformations involved in leukemia development commonly affect primitive cells (stem cells or progenitors), and processes such as proliferation and potential maintenance are disturbed, giving rise to LSCs. It is believed that alterations reflect the cell surface, disarraying membrane molecules, which was the topic of this review. Relapse is the main cause of the poor cure rate and prognosis; therefore, it is essential that the cells responsible for relapse – LSCs – are investigated. For this purpose, the identification and isolation of a pure population isolated alive is important, despite the challenge in its identification. In this context, the use of surface markers and flow cytometry is suitable to identify and isolate cells with LSC or HSC phenotypes.

In the case of AML, the most known leukemia subtype, CD34, CD38, CD123, CD90 and the Lin cocktail are widely used, although overlap with the HSC immunophenotype may occur. Numerous other markers and panels have been proposed, and in this context, Zeijlemaker and colleagues[21] proposed a comprehensive test in addition to drawing attention to peculiarities in variations in AML and their reflection on marker choice. For CML, the panels proposed vary less than those for AML but follow similar patterns, widely using CD34 and CD38 in combination with mature markers, such as CD25, CD26 and CLL-1, among others. For the lymphoid branch of leukemias, CD19 is

broadly used on B-ALLs, whereas T-ALLs depend more on the differentiation step in which genetic transformation occurs.

In summary, the isolation of a live LSC population is needed for the investigation of its drivers, as well as its intracellular alterations and signaling activation. In addition, the population immunophenotyping establishment can be used for development of new therapies based on antibodies, aside from increasing specificity of diagnosis and prognosis. Thus, compelling investigations on LSC panels are essential for leukemia research and therapy development.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Described altered surface markers by population enrichment. WT-1 refers to Wilm's tumor 1, FSC to forward scatter, SSC to side scatter

Level of enrichment	Markers	Signal	Observation	Ref
CD34^{+}	CD33	+		10,29
	CD45	dim		3
	CD71	-		10
	CD82	+		32
	CD90	+ / -		10,20
	CD97	+		32
	CD98	+		32
	CD117	+ / -		10
	CD123	+		10
	CD135 (FLT3)	+		32
	Lineage markers	+	CD2, CD7, CD11b, CD13, CD14, CD15, CD19, CD22, CD56, CD33, CLL-1 (CLEC12A)	1,3,9,14,21,22,35,44
	DR4	+		39
	ESAM	+		32
	FCGR1A	+		32
	GPR114	+		32
	HLA-DR	-		3,10
	ITGA5	+		32
	ITGA6	+		32
	MET	+		32
	PTH2R	+		32
	TMEM5	+		32
	TNFRSF10B	+		32
CD34^{+}CD38$^{-/low}$	AA4.1	+	Mice	24
	AC133	+	Further enrichment: CD44^{+} or CD24^{-}	3,12,42
	AC133	-		29
	ALDH	High		14,33,35
	CD13	+ / -		21
	CD19	+		9,21
	CD24	+	Mice	24
	CD25 (IL-2RA)	+ / -		5,14,17,21,29,35,41
	CD26 (DPPIV)	-		29
	CD32	+		5,14
	CD33	+		10
	CD43	+	Mice	24
	CD44	+		3,5,14-17,21,29,32,35,41,42
	CD45	dim	Further enrichment: embryonic markers	3
	CD45RA	+		22

Supplementary Table 1. Continued

Level of enrichment	Markers	Signal	Observation	Ref
	CD47	+		5,14,16,29,32,34,35
	CD48	dim		26
	CD52	+		29
	CD71	+		10,20
	CD82	+		32
	CD90	+	Further enrichment: SSEA1^{dim}	3,20,24,29,41
	CD90	-	Further enrichment: CD96^{+}	17,29,31
	CD93	+		16
	CD96	+ / -		3,5,14,16,17,21,29,31,32,35,41
	CD98	+		16
	CD114	+ / -		29
	CD117	+ / -		29
	CD123	+ / -		3,5,11,14,16,17,20,21,24,28,31,32, 35,38,40,41,44
	CD184 (CXCR4)	+		29
	GMP-like phenotype	+	Further enrichment: AC133^{+}	22,42
	HLA-DR	-		3,20,35,37
	IL-1RAP	+ / -		29
	Lineage markers	+	CD2, CD7, CD11b, CD14, CD15, CD19, CD22, CD56, CD13, CD33, CLL-1 (CLEC12A)	1,3,9,14,21,22,35,44
	Sca-1	-	Mice	24
	WT-1	+		17,21,41
	Scatter properties		Further enrichment: high FSC/SSC, low side population	14,21,40
CD34^{+}CD38$^{-/low}$ Lin^{-}	CD25	+		17
	CD44	+		17
	CD90	+		31
	CD90	-	Further enrichment: CD45RA^{+}	3,17,22,31
	CD96	+		17
	CD123	+		17
	WT-1	+		17
CD34^{+}CD38$^{-/low}$ CD123^{+}	CD45	Dim	Further enrichment: JAM-C	16
	N-Cadherin	+		27
	Tie2	+		5
CD34^{-}				11,21,22,29,32,35,41,44
CD34^{+}CD38^{+}	AC133	+		42
	CD123	+ / -		11,38

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