Mechanisms of extramedullary relapse in acute lymphoblastic leukemia Reconciling biological concepts and clinical issues

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32

Abstract

Long-term survival rates in childhood acute lymphoblastic leukemia (ALL) are currently above 85% due to huge improvements in treatment. However, 15-20% of children still experience relapses. Relapses can either occur in the bone marrow or at extramedullary sites, such as gonads or the central nervous system (CNS), formerly referred to as ALL-blast sanctuaries. The reason why ALL cells migrate to and stay in these sites is still unclear. In this review, we have attempted to assemble the evidence concerning the microenvironmental factors that could explain why ALL cells reside in such sites. We present criteria that make extramedullary leukemia niches and solid tumor metastatic niches comparable. Indeed, considering extramedullary leukemias as metastases could be a useful approach for proposing more effective treatments. In this context, we conclude with several examples of potential niche-based therapies which could be successfully added to current treatments of ALL.

Keywords: acute lymphoblastic leukemia; niche; cellular microenvironment; children; relapse; extramedullary; sanctuary; bone marrow; central nervous system; testis

I. Introduction

Acute lymphoblastic leukemia (ALL) is a hematological malignancy defined by the clonal expansion and accumulation of abnormal immature lymphoid precursor cells in the bone-marrow (BM) compartment. This abnormal cell population replaces normal hematopoietic cells and can spread from the BM to the entire body through systemic circulation. Relatively rare relative to other hematological malignancies in adults, ALL is the most frequent malignancy found in children and accounts for approximately 80% of childhood leukemia, with a maximal incidence rate between one and six years [1]. Over the last decades, the use of multi-agent chemotherapy protocols, better patient stratification into risk-adapted groups for treatment, and improved treatments have drastically reduced the mortality of this young population. Indeed, survival rates have risen from approximately 10% in the 1960s to above 90% for children in high-income countries in the 2000s.

However, approximately 15 to 20% of children and approximately 50% of adult patients still relapse [2–6]. This situation is defined as the resurgence of blast cells after the achievement of complete remission. Relapses are generally classified and stratified according to three prognostic factors: time of relapse, site of relapse, and immunophenotype B or T [7].The time of relapse, relative to the beginning and end of treatment, is the most important prognostic factor: the earlier the relapse occurs, the worse the prognosis. The definition of relapse is slightly different depending on the classification used (Berlin-Frankfurt-Münster [BFM] or Children Oncology Group [COG]) [3,7]. The most important factor after timing is probably the anatomical sites of relapse, which are commonly divided into bone marrow (BM) alone (worst prognosis), extramedullary alone (best prognosis), and combined relapses (intermediate prognosis). Although immunophenotype T was initially associated with poor survival and is still used in tumor classification, its prognostic value appears to be less important due to better stratification [8]. Furthermore, the assessment of early minimal

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72   residual disease (MRD) is also used to predict the prognosis of patients presenting a relapse,

73   as it is for newly-diagnosed ALL [9–12]

74   Given the considerable therapeutic progress that has been made, one of the main goals

75   is to attenuate treatment-related sequelae by reducing their toxicity, while continuing to

76   improve current survival rates. Decreasing the incidence of relapse and identifying optimal

77   treatments and therapeutic strategies when relapse occurs, are critical goals as well. Indeed,

78   deciphering the underlying mechanisms that lead to relapse and understanding how and why

79   relapses occur is a prerequisite to defining new treatment approaches.

80   In this review, we will begin with a brief overview of the principal mechanisms

81   involved in ALL relapses. Second, we will describe where ALL cells reside within some sites

82   of relapse and compare medullary, extramedullary, and metastatic niches, using a few

83   examples. We will show how their microenvironments can be considered to be quite similar.

84   Third, we will discuss the interplay between ALL cells and the microenvironments they

85   colonize. Fourth, we will discuss the potential predisposition of some ALL cells to reside in

86   specific niches. Finally, we will conclude with therapeutic perspectives related to

87   microenvironment targeting.

88   **II. Cellular origin of relapse**

89   Multiple biological factors may contribute to the emergence of ALL relapses [13].

90   Cumulative evidence suggests a clonal origin of ALL [14]. It has previously been shown that

91   clonal evolution is involved in relapse, with the possible emergence of subclones derived

92   either from the major clone found at initial diagnosis, minor subclones, or a common ancestral

93   pre-leukemic clone (Figure 1) [15–20]. Several studies have described chemoresistance-

94   related mutations in the BM samples of relapsed patients, such as those that affect the

95   glucocorticoid response pathway (mutations of the glucocorticoid receptor gene *NR3C1* or

6

96 other genes, interfering with its function, such as *BTG1*) or nucleotide metabolism (for

97 example, *NT5C2* or *PRSP1* genes [13,21–25]. Clonal heterogeneity is a common feature in

98 ALL. Clonal evolution is likely to be a dynamic process in which selection plays a central

99 role, as in the Darwinian model [14,26]. Both intrinsic and extrinsic factors, such as

100 microenvironmental conditions or chemotherapeutic pressure, may favor a clone from which

101 the relapse can develop.

102   Relapses may originate from a chemoresistant clone, either already present as a minor

103 clone at diagnosis or resulting from the acquisition of secondary mutations during treatment.

104 Aside from chemoresistance mechanisms, relapse can also be explained by the presence of

105 dormant cells within the bulk population. These rare, non-dividing, quiescent cells are less

106 sensitive to cytotoxic agents. They can therefore resist treatment and give rise to a new

107 population after reentering the cell cycle, leading to relapse. Aside from the quiescent state

108 itself, cells may also exert their resistance by residing in niches where the microenvironment

109 confers protection from chemotherapy. Indeed, drug concentrations may be lower in niches, as

110 suggested for brain and testicular tissues [27,28]. Local conditions, the cell matrix, cell-cell

111 interactions, and soluble factors may also maintain quiescence. The origin of such dormant

112 cells is still an open question, but recent studies have provided some clues to their existence at

113 diagnosis [29,30]. Why and how they reenter the cell cycle and proliferate to induce relapse

114 still needs to be addressed for understanding the evolution of these dormant cells. How such

115 cells reach and invade distant favorable microenvironments from their native marrow niche is

116 also yet to be elucidated.

117   Metastasis is the spread of cancer cells from the tissue where they first originate to

118 another distant one. ALL relapses occur most often in the BM, whereas extramedullary

119 relapses are less commonly reported. Nevertheless, the occurrence of relapse is generally

120 thought to be a leukemic event, depending mainly on internal properties of the blast,

121 regardless of the type of relapse. However, the occurrence of relapse may not depend only on

122 leukemic cell features, but also on the microenvironment. In the next section, we will describe

123 the most frequently-reported sites of relapse and the concept of niches, proposing that

124 extramedullary leukemia relapse is similar to that of solid tumor metastasis, anticipating a

125 major role of the microenvironment of extramedullary sites.

126 **III. Sites of relapse**

127 Relapse can occur in the BM, the native niche from where the disease spreads at the

128 onset. It can also emerge from extramedullary sites, such as the central nervous system (CNS)

129 or gonads. Both used to be called "sanctuary sites", because of the relative protection these

130 sites appear to offer to blast cells from systemic chemotherapy. At these sites, the tissue-blood

131 barrier is an obstacle to drug delivery [27,31,32]. Although CNS and testicular relapses may

132 be more common in children, they also concern adults. These sites may represent the first

133 extramedullary sites colonized by leukemic cells but can also be colonized secondary to other

134 extramedullary sites that may have evaded clinical detection. Prophylactic cranial irradiation

135 was added to systemic chemotherapy treatment in the 1970s to attempt to prevent CNS

136 relapses [33]. Intrathecal chemotherapy and the use of high-dose methotrexate for neuro-

137 meningeal prophylaxis have progressively replaced irradiation, with good results for

138 diminishing neurological sequelae, showing that the blood-tissue barrier problem can be

139 bypassed [34]. Similarly, treatment intensification, with the introduction of intermediate and

140 high-dose methotrexate, has significantly decreased the incidence of testicular relapses, thus

141 considerably improving the historically worse prognosis for boys [35,36]. However, extra-

142 medullary relapses are still a problem and have been reported in many other organs. Several

143 studies have reported that extramedullary relapses occur in approximately 40% of cases

144 (approximately 20% combined and 20% presumably isolated) [3,8,37]. Here, we summarize

145 the evidence that shows the role of the microenvironment in extramedullary relapses.

146   1. Different microenvironments

147   *a) Bone marrow (Figure 2a)*

148   BM is the most frequent site of ALL relapse and is involved as an isolated or combined

149 site of relapse in 75-90% of cases [3,8,38–41]. BM relapses are most often the unique site

150 involved (approximately 65% of cases) [3,8,34–37]. There is no clear evidence for a

151 privileged BM site in which lymphoblasts primarily reside. Indeed, contrary to BM aspirates,

152 BM biopsy results are generally not given in published studies, and almost all studies

153 presenting BM biopsy data concern patients with advanced disease. In contrast, the

154 engraftment of leukemic cells in BM has been well-studied in murine models [42–45]. In

155 these models, leukemic cells engraft and proliferate in specific and restricted perivascular

156 regions, in which C-X-C motif chemokine ligand 12 (CXCL12) and E-selectin are expressed,

157 and which overlap with perivascular niches of hematopoietic progenitors. CXCL12 appears to

158 be required for the diapedesis of leukemic cells [42]. Leukemic cells migrate and arrest in

159 vessels close to endosteum, where they settle. From these perivascular endosteal niches, they

160 may proliferate and spread to the BM and beyond [44].

161   *b) Gonads (Figure 2b)*

162   Large group studies have reported that isolated testicular relapses could account for

163 3.5-8% of ALL relapses in children (including boys and girls) followed for up to 20 years

164 [3,8,38–41,46]. Most studies mentioning testicular biopsies in the context of ALL did not

165 indicate the precise location of blasts [47–49]. Such information can be retrieved from rare

166 and old clinical studies and animal models [50–56]. Animal models indicate that leukemic

167 cells infiltrate the interstitial testicular tissue mainly at the perivascular and peritubular

168 regions, forming small foci, notably in subcapsular areas. Despite perivascular interstitial

169 invasion, extensive infiltration of the interstitium is rare. On the other hand, the epididymis is

170 more often massively invaded by blast cells [54,55]. Leukemic invasion seems to occur

171 through the hematogenous route, with possible extension to the epididymis *via* the lymphatic

172 route [55]. In these models, although seminiferous tubules were degenerated or destroyed near

173 leukemic infiltrations, leukemic cells were never found in the tubules nor the gonadal duct

174 [54–56]. Unlike these *in vivo* studies, testicular leukemic infiltration was mostly found to be

175 diffuse in a postmortem series of children who died of very advanced leukemia. Leukemic

176 cells were found in the tubules in half the patients with heavily infiltrated testes [57]. This

177 relatively old study included children with uncontrolled leukemia at a time when outcomes

178 were poor and probably does not reflect the route of initial invasion. Thus, it seems that

179 leukemic cells invade the testis by the hematogenous route and first infiltrate the perivascular

180 and peritubular spaces, where they can spread to the epididymis through the local lymphatic

181 system. Without effective treatment, seminiferous tubules may be destroyed and invaded as

182 well. This is concordant with the perivascular and peritubular interstitial infiltration observed

183 in patients with testicular relapse leukemia [50–52].

184   Although less commonly involved than the testis, ovaries may also be involved in

185 ALL [58]. Ovarian relapses are scarcely reported in clinical trials [40,59], but autopsies have

186 provided evidence of ovarian disease in end-stage leukemia [57,60,61]. More recently,

187 ovarian involvement was found in approximately 8% of leukemia cases in an autopsy series,

188 regardless of age. However, the authors did not indicate whether the patients had been treated

189 or not, nor the treatment performed [62]. The effects of chemotherapy could explain the

190 differences observed between and within the reports mentioned above, in which ovarian

191 involvement was found in 3% to up to 50% of cases [57,60,61]. Indeed, Pais *et al.* reported

192 that the number of ovarian relapses dramatically decreased in their cohort from the moment

193 they introduced intermediate-dose methotrexate in the treatment regimen (from almost 10% of

194 all relapses before 1979 to no ovarian relapse noted afterwards, until 1991). As they assumed,

195   isolated ovarian relapse has become an exceptional event, likely representing slightly less than

196   1% of all relapses [40,59]. However, contrary to testicular involvement, which can be

197   clinically suspected with a simple physical examination, there is no overt clinical sign that

198   allows suspicion of ovarian leukemic infiltration. In addition, ultrasound evaluation is not

199   systematically performed during the course of the disease and follow-up, even if it might be

200   [63]. However, it was possible to detect leukemic cells in up to 70% of samples by

201   polymerase chain reaction of cryopreserved ovarian tissue retrieved at diagnosis (or shortly

202   after), even in the absence of enlargement and with a negative histological examination

203   (except in one patient from a study of Soares *et al.*, whose ovarian medulla was invaded) [64–

204   67]. Most published studies concerning ovarian disease consist of case reports, which usually

205   do not report the precise location of leukemic cells in the ovary [58]. In their autopsy series,

206   Reid *et al.* found that leukemic invasion, which was mostly histologically mild, took place

207   predominantly in the medulla in the perivascular spaces [57]. However, leukemic cells have

208   also been detected in the cortex by other authors. Thus, it is difficult to say where the cells

209   preferentially settle [64–67]. Nonetheless, follicles have never been reported to be invaded in

210   the literature.

211   ***c) Central nervous system (Figure 2c)***

212   Isolated CNS relapses represent approximately 7.5-15% of ALL relapse [3,8,38–41].

213   The precise location of leukemic cells in the CNS is rarely indicated in published studies.

214   Autopsy and animal model studies have allowed a better understanding of how the CNS may

215   be invaded by leukemic cells [68,69]. Indeed, Price and Johnson studied 126 brains from

216   children who died of ALL and developed a grading system to evaluate the severity of

217   arachnoid involvement. They detected leukemic cells in the CNS of 55% of patients. Most

218   showed moderate leukemic infiltration and the authors observed that infiltration of the brain

219   parenchyma occurred only in cases of extensive and deep leukemic arachnoid involvement

11

220 with cerebrospinal fluid (CSF) contamination and pia-glial membrane destruction (13.4% of

221 cases). Arachnoid invasion was most often isolated or multifocal, rather than diffuse. They

222 concluded that leukemic cells first invade the superficial arachnoid from the vein wall, then

223 the deep perivascular arachnoid, and finally, the brain parenchyma through disrupted pia-glial

224 membranes [68]. *In vivo* rodent models have provided similar results and suggest that

225 leukemic cells transit through the blood-CSF barrier to reach the leptomeninges [69,70].

226 Although Price and Johnson only studied brain and did not consider the spinal cord or dura

227 matter in their study, diffusion throughout the CNS *via* the CSF has been confirmed by

228 leukemic cell DNA detection, both in the lumbar spinal cord and frontal brain of rats

229 inoculated in the cerebellum [70]. This is consistent with neuroradiological findings, which

230 indicate that meningeal infiltration is the most common manifestation of CNS leukemia, with

231 most often diffuse infiltration [71,72]. However, although much rarer and mostly represented

232 by case reports in the literature, CNS involvement may also consist of epidural masses that

233 can compress the spinal cord [73]. Thus, leukemic cells do not colonize one specific

234 anatomical region within the brain or other CNS sites, but rather appear to preferentially

235 localize to the subarachnoid space, likely in perivascular areas. A very recent study, using a

236 mouse model, has shown that meningeal invasion occurs through the diapedesis of leukemic

237 cells from cranial or spinal bone marrow of adjacent bones. The authors reported that

238 leukemic cells transit through blood vessels, the emissary vessels that bridge the CSF space

239 from the brain or spinal cord to the calvarium or vertebral bodies. They also discovered that

240 this mechanism involves an interaction between a6 integrin and laminin and that it can be

241 blocked by specific PI3Kδ inhibitors [74].

242 **d) Others**

243 Because leukemic cells spread throughout the organism via systemic circulation, other

244 sites of relapse may be considered. Other extramedullary sites have been little studied and

12

245 published data consist mostly of case reports [75]. Although no site can be defined as

246 privileged, since ALL can settle in many tissues, breast involvement may be a privileged site

247 of extramedullary leukemia, mostly described by Isabel Cunningham [75–79].

248   *2. Similarities between sanctuary sites*

249   We chose not to provide an exhaustive comparison between metastatic niches but

250 rather to focus on three representative common actors: the CXCL12/C-X-C chemokine

251 receptor type 4 (CXCR4) axis, hypoxia, and stem cell factor (SCF).

252   ***a) The concept of "niche"***

253   The BM microenvironment has been, and is still, extensively studied [80–84]. First

254 postulated by Schofield in 1978, it is now accepted that hematopoietic stem cells (HSC) reside

255 in specialized compartments, called "niches", within the BM [85]. Such niches allow the

256 maintenance and regulation of HSCs by providing specific local conditions, cytokines,

257 chemokines, and other soluble factors, as well as various types of specialized stroma cells.

258 Different types of HSC niches have been described in the literature and may be named

259 differently, depending on the authors, which can be confusing [80,81,86,87]. Our

260 understanding of the BM microenvironment is constantly evolving and is still a subject of

261 debate. For example, some studies have provided evidence for the existence of an osteoblastic

262 niche [86], but this has been more recently questioned. Indeed, the existence of an authentic

263 osteoblastic HSC niche has been ruled out, even if osteoblastic lineage cells have an indirect

264 role in the regulation of HSCs and appear to be part of a specialized niche for lymphoid

265 progenitors [81,84]. Rather than viewing the BM as a suite of separated niches, it is more

266 likely a unique continuous niche in which some components are shared (and some others not)

267 between more specialized compartments within this niche, as proposed by Hira *et al.* [88].

268   HSCs localize mainly to perivascular zones, as do leukemic cells, with most found close to

269   sinusoids, whereas approximately 10% are found near small arterioles.

270   The microenvironments of the testes and CNS also have niches that maintain and

271   regulate spermatogonia and neural or glial stem cells, respectively[89–93]. It has been shown

272   in several cancer models that cancer cells can migrate from the tissue they originate to

273   predisposed distant niches, which they can then remodel to suit their needs [94,95]. Such

274   metastatic, BM, testicular, and neural niches may share characteristics that attract leukemic

275   cells and offer them a favorable microenvironment for maintenance and growth. We focus on

276   some of these shared components that have been found to play a major role in these

277   microenvironments.

278   *b) CXCL12/CXCR4*

279   CXCL12, also known as stromal derived factor 1 alpha (SDF1α), is one of the most

280   important chemokines in the BM niche [84]. Through binding to its receptor, CXCR4,

281   CXCL12 favors HSC homing to the BM and participates in the maintenance of these cells in

282   the niche. CXCR4 is a seven-transmembrane G-protein-coupled receptor that is expressed on

283   multiple cell types, including, notably, hematopoietic cells [96]. The inhibition of CXCR4 is

284   frequently used clinically to mobilize HSCs into the peripheral blood as a source of stem cells

285   for transplantation [84,97–99]. CXCL12 is highly expressed by specialized cells close to

286   sinusoid vessels within the BM, called CXCL12 abundant reticular cells , and favors HSC

287   retention in the BM [84,97].

288   CXCL12 has been shown to be involved in metastasis in many cancer models, such as

289   breast, prostate, and lung cancer, pancreatic adenocarcinoma, rhabdomyosarcoma, and others

290   [100]. For example, it was shown in an ovarian cancer model that the most advanced stages of

291   the disease and most invasive phenotypes are associated with increased CXCR4 expression at

14

292   the cancer cell surface. More importantly, ascites samples from patients with advanced

293   ovarian cancer showed significantly higher CXCL12 levels [101]. The role of the

294   CXCL12/CXCR4 axis in the dissemination of peritoneal cancer has been clarified more

295   recently in a study using a primarily murine colon cancer model. The authors found that

296   CXCL12-expressing cells, similar to CXCL12 abundant reticular cells and distributed

297   throughout the perivascular zones of adipose tissue, were a privileged site from which cancer

298   cells could disseminate [102]. In another example, Ogawa *et al.* showed that CXCL12

299   expression increased in the regional lymph nodes of mice with intrapulmonary transplanted

300   cancer cells in a Lewis lung carcinoma model, and participated in the formation of a pre-

301   metastatic niche [103].

302   The CXCL12-CXCR4 axis is also involved in the pathogenesis of leukemia, as it favors

303   not only ALL cell homing in the BM, but also their survival and proliferation [42,43,104,105].

304   Some evidence indicates that, in the human testis, CXCL12 is expressed solely by Sertoli

305   cells to promote spermatogonial stem-cell migration through the seminiferous tubules during

306   spermatogenesis [106–109]. Sertoli cells reside in seminiferous tubules, where leukemic cells

307   are generally not found, or only very late during testicular involvement. However, the use of

308   transwell migration assays showed that ALL cells can also migrate towards mouse testis

309   conditioned medium and that such migration was reduced when the cells were treated with

310   plerixafor, a CXCR4 chemical inhibitor [110]. This result suggests that leukemic-cell

311   migration towards the testis might be at least partially mediated (or not exclusively) by the

312   CXCL12/CXCR4 axis. Moreover, McIver *et al.* have shown that although CXCL12

313   expression is restricted to Sertoli cells in the normal testis, it is expressed in the tumor stroma

314   of seminoma samples [107]. Pathological conditions may induce CXCL12 expression in the

315   interstitium of the testis [111]. Thus, it is possible that leukemic cells could be attracted and

316   settle in the testis.

317   The CXCL12/CXCR4 axis is also known to play an important role in neurogenesis and

318   neuron migration during development [112–116] and CXCL12 is constitutively expressed in

319   the adult CNS, where it participates in neuromodulatory functions and acts as a major player

320   in neuro-inflammation by recruiting leukocytes across the blood-brain barrier [112,117–120].

321   McCandless *et al.* showed that normal expression of CXCL12 on the basolateral side (*i.e.*

322   away from vessel lumen) of CNS endothelial cells retains leukocytes in the perivascular

323   space, thus preventing their egress to the CNS parenchyma [119,120]. Leukemic cells may

324   therefore be attracted into perivascular areas by such a CXCL12 expression pattern, where

325   they could stay, possibly in a quiescent state. Indeed, recent studies in a T-ALL model have

326   shown that the CXCL12/CXCR4 axis plays an important role in leukemic-cell migration into

327   the CNS [121,122]. Nevertheless, although treating xenografted mice with plerixafor (a

328   CXCR4 antagonist) reduced engraftment into the BM and liver in a B-ALL model, it failed to

329   prevent blast infiltration of the CNS, suggesting that CNS infiltration may not be mediated by

330   the CXCL12/CXCR4 axis in B-ALL [69]. Other studies indicate that the CXCL12/CXCR4

331   axis is also involved in leukemic-cell infiltration into other extramedullary tissues. Indeed,

332   Crazzolara *et al.* found that high CXCR4 expression at the surface of ALL cells was

333   associated with more pronounced infiltration of extramedullary tissues, such as liver and

334   spleen, which was confirmed later in another study using a NOD/SCID immunodeficient

335   mouse model [123,124]. Consistent with the latter studies, Kato *et al.* demonstrated that the

336   liver constitutes a favorable niche promoting ALL cell engraftment, survival, and

337   chemoresistance through the CXCL12/CXCR4 axis [125]. Overall, there is evidence in the

338   literature showing that the CXCL12/CXCR4 axis plays a major role in ALL cell homing, both

339   to the BM and extramedullary niches. Thus, CXCL12 represents a common feature of

340   hematopoietic, extramedullary, and solid tumor niches, which may reconcile ALL and solid

341   tumor models.

16

c) Oxygen content

343   Improperly termed hypoxia, low oxygen concentrations are physiologically found in

344   specific areas in mammalian organisms [126]. Indeed, the BM is likely to be one of the tissues

345   in which the oxygen concentration is the lowest [127]. Ishikawa and Ito measured oxygen

346   tension in BM samples from 4.9% to 8.8%, but it likely poorly reflects the true oxygen level

347   found in the BM [128]. Later, mathematical modeling predicted the existence of an oxygen

348   gradient in the BM, with oxygen tension decreasing progressively, starting from the sinus

349   [129,130]. Since then, experimental evidence has confirmed very low oxygen concentrations

350   in the BM, with the lowest value measured at 0.6% [131,132]. Increasing evidence suggests

351   that the niches in which HSCs reside are hypoxic and that low oxygen concentrations may be

352   needed to maintain their stemness [132–137].

353   Low oxygen concentrations have been highlighted during cancerogenesis in solid

354   tumors. As reviewed elsewhere, hypoxia in cancer cells and in the tumor microenvironment

355   promotes   aberrant   angiogenesis,   epithelial-mesenchymal   transition,   radio-   and

356   chemoresistance, and metastasis [138]. For example, it has recently been shown in a breast

357   cancer model that hypoxia can upregulate pro-metastatic genes and promote metastasis,

358   particularly under conditions of intermittent hypoxia, [139]. Moreover, the authors found that

359   breast-cancer cells cultured under hypoxic conditions secreted pro-tumorogenic cytokines.

360   Hypoxia was similarly shown to enhance the invasiveness of melanoma cell lines *in vitro* and

361   promote metastasis *in vivo* [140].

362   More importantly, low oxygen concentrations are thought to be an important component

363   of leukemic niches in the BM and recent results have shown that targeting this hypoxic

364   microenvironment may be promising [141–148]. Indeed, hypoxia has been shown to enhance

365   the chemoresistance of ALL cells and appears to confer stem-cell properties, such as

366 quiescence [141,149,150]. Drugs active only under conditions of hypoxia were shown to

367 reduce the leukemia burden in the BM when administrated three days after ALL cell injection

368 into NOD/SCID mice, suggesting that hypoxia may favor leukemic-cell engraftment

369 [141,147].

370   The testes are also thought to be one of the most hypoxic tissues. Direct measurements

371 in a rat model established the interstitial testicular oxygen concentration between 1.5 and

372 3.5% [151,152]. Another study using pimonidazole staining, commonly used as a hypoxic

373 marker, confirmed these results in a mouse model and suggested an even lower intratesticular

374 oxygen tension, especially in the seminiferous tubules, as well as the interstitium [153].

375 Nevertheless, there is no evidence of an association between the hypoxic features of the testis

376 and testicular leukemia infiltration as it has not been directly studied.

377   Low oxygen concentrations are also found in the CNS, from 0.5% to 7%, both in

378 humans and animal models [126]. There are few clues in the literature to estimate the oxygen

379 tension of subarachnoidal tissue, where ALL cells preferentially settle. Some studies have

380 evaluated the oxygen tension within the CSF, with estimated values of approximately 5 to 6%

381 [154,155]. More recently, Sharan *et al.* showed that oxygen tension decreases rapidly from

382 pial vessels to the extravascular space in a rodent model, with oxygen levels as low as 3% 30

383 µM from the vessels, especially the narrowest ones [156]. Thus, although oxygen

384 concentrations have not been precisely measured in the subarachnoidal space, they are likely

385 to be low. Recent published data have indeed suggested a role for low oxygen concentrations

386 in leukemic cell CNS infiltration [157,158]. In these two independent studies, the authors

387 found that leukemic cells retrieved from the CNS of xenografted mice had a hypoxia-related

388 gene set profile and expressed a particularly high level of the transcript of the vascular

389 endothelial growth factor A (VEGFA) gene, which is a principal target of the hypoxia master

390 transcription factor, hypoxia inducible factor 1 (HIF1). More importantly, Münch *et al.* further

18

391 showed that VEGFA mediated the migration of ALL cells through a monolayer of a brain-

392 microvasculature cell line [158]. The results from these studies are in accordance with those

393 of a previous one showing that patients with CNS leukemia had elevated levels of VEGF in

394 the CSF [159]. Thus, the role of hypoxia in extramedullary leukemia merits exploration and

395 may offer new therapeutic perspectives, as outlined by Izraeli and Eckert [160].

396   *d) Stem cell factor*

397   Stem cell factor (SCF), also known as steel factor or mast cell growth factor, is one of

398 the main growth factors that regulate the proliferation and survival of HSCs and

399 hematopoietic progenitors in the BM, through binding to its receptor, c-KIT [161–163].

400 Indeed, insertion of the *Egfp* gene into the endogenous locus of the *Scf* gene in C57BL mice

401 results in perinatal death with severe anemia. GFP expression patterns within the BM have

402 suggested that SCF is produced in the BM by perivascular stromal and endothelial cells,

403 which was further demonstrated using a conditional deletion model in the same mice [164].

404 Moreover, Ding *et al.* demonstrated that SCF is necessary to maintain adult HSCs, using the

405 same conditional deletion model. Along with its effects on progenitor cell survival and

406 proliferation, SCF has also been shown to have a chemotactic function in these cells [162].

407 Proteolytic cleavage and alternative splicing lead to various tissue-specific isoforms of SCF,

408 which can be produced as soluble or membrane-bound forms [161–163,165,166]. The

409 membrane-bound form of SCF appears to be essential [162]. The membrane-bound form of

410 SCF has notably been shown to enhance human HSC engraftment in murine xenografts. It has

411 also been shown to promote cell adhesion *in vitro* and HSC residence in the BM niche *in vivo*

412 [162,167–169].

413   In solid tumors, SCF/c-KIT signaling is also frequently thought to participate in

414 tumorigenesis, tumor progression, and metastasis. Soluble SCF is produced by cancer cells

415 within tumor tissues in different cancer models [170]. c-KIT has been found to be

416 overexpressed, abnormally expressed, or associated with an aggressive phenotype in several

417 types of tumors, including melanoma, lung cancer, renal carcinoma, and seminoma

418 [163,171,172]. Activating mutations of c-KIT can be found in some tumor types, especially

419 gastrointestinal stromal tumors, and may be involved in their pathogenesis. The SCF/c-KIT

420 pathway may also be involved in the pathogenesis of such tumors through its effect on mast

421 cells [163] and the mast cell accumulation has been associated with a worse prognosis in

422 several cancer models.

423   The SCF/c-KIT signaling pathway has also been found to be deregulated mostly in

424 AML models rather than in ALL models. However, although c-KIT is little expressed in ALL

425 relative to AML, RNA-seq data indicate that some ALLs overexpress c-KIT, such as ETS

426 variant 6-runt related transcription factor 1 (ETV6-RUNX1) B-ALL or mixed lineage

427 leukemia (MLL) rearranged leukemias [173]. Consistent with the expression of c-KIT in these

428 malignancies, SCF participates in early B-cell development in association with interleukin-7

429 (IL7) [161]. Moreover, it was shown that ALL cells can modify their microenvironment by

430 producing SCF [43]. Thus, the involvement of SCF signaling in the homing and

431 pathophysiology of ALL may merit exploration, as suggested by a recent report [174].

432   The SCF/c-KIT axis plays also an important role in male reproduction, as suggested by

433 the infertility of mice with mutations affecting both the *W* (encoding c-KIT) or *Sl* (encoding

434 SCF) loci [161,163]. In the testis, SCF is produced by Sertoli cells and c-KIT is expressed on

435 the surface of germ cells and Leydig cells, raising the possibility that Sertoli cells secrete a

436 soluble form of SCF [171,175,176]. Indeed, a higher percentage of mRNA for the soluble

437 than membrane form of SCF correlated with higher serum testosterone levels in infertile

438 patients [177]. These results suggest that Sertoli cells may produce soluble SCF that reaches

439 Leydig cells and thus enhance testosterone production. Moreover, SCF expression in normal

20

440   adult interstitial testicular tissue has been reported both in mice and humans in several

441   immunohistochemistry studies, even if several others did not [178–181]. Thus, leukemia cells

442   may be attracted to or retained in testicular interstitial tissue at least partially through

443   activation of the SCF/c-KIT pathway.

444   SCF is also expressed in the adult CNS, as well as in CSF, and is involved in CNS

445   development during embryogenesis [182–184]. There is no direct evidence in the literature for

446   a role of the SCF/c-KIT axis in the attraction of leukemia cells to the CNS or their subsequent

447   survival. However, SCF is one of the specific cytokines expressed by endothelial cells of the

448   blood-brain barrier [183]. Kallman *et al.* compared gene expression profiles of human

449   umbilical vein endothelial cells (HUVEC) and primary human cerebral endothelial cells using

450   a cDNA array technique and found that 35 genes were differentially expressed in cerebral

451   endothelial cells, including those for interleukin-6 *(IL-6)*, transforming growth factor β2

452   *(TGF-β2)*, *VEGFA,* and *SCF* [183]. An enzyme-linked immunosorbent assay (ELISA)

453   confirmed that the supernatant from these cells contained SCF. Thus, endothelial cells of the

454   CNS can produce SCF that may attract and/or promote the survival of leukemia cells that

455   express c-KIT.

456   Overall, these examples suggest that the SCF/c-KIT axis may be an attractive

457   therapeutic target for leukemia relapse, both at medullary and extramedullary sites, as well as

458   for various solid tumors.

459   *d) Other shared factors*

460   The extracellular matrix protein osteopontin, which is physiologically produced in the

461   BM, participates in the pathophysiology of various cancers, including hematological

462   malignancies, with a potential role in leukemic cell CNS infiltration [185,186]. Colony

463   stimulating factor 1 (CSF1), a cytokine involved in macrophage differentiation and

464 constitutively expressed by osteoblasts, is expressed in prepubertal and adult testicular

465 interstitial tissue, notably by macrophages, and has a role in regulating spermatogonial stem

466 cells and their niche[187–189]. CSF1 is also expressed in the CNS, where it maintains

467 microglia and participates in CNS development [190]. CSF1 deficiency impairs both

468 reproductive function and integrity of the CNS. Genetic alterations involving the CSF1

469 receptor, including the recurrent SSBP2-CSF1R fusion, have been identified in B-ALL and

470 have been found to lead to a functional fusion, suggesting that targeting CSF1 signaling could

471 be promising [191–193]. Another cytokine, transforming growth factor β1 (TGFβ1), regulates

472 Leydig cell function in the testis, but is also secreted by the choroid plexus in the CSF, where

473 it contributes to the maintenance of the choroid plexus itself and appears to be involved in the

474 protection and repair of nervous tissue. TGFβ1 has been shown to be involved in cancer

475 stemness, metastasis, and epithelial-mesenchymal transition in several cancer models.

476 Recently, TGFβ1 was found to promote the escape of leukemic cells from natural killer (NK)

477 immune surveillance in an ALL model.

478 All these factors and other components, such as the various cell types found within the

479 niche, may also contribute to therapy resistance by preventing treatments from reaching their

480 targets. BM and extramedullary sites may therefore contain pharmacological niches, where

481 leukemic cells are protected from the effects of chemotherapy. For example, mesenchymal

482 stem cells have been shown to participate in the resistance of leukemic cells to asparaginase

483 treatment by providing them with asparagine [194]. Interestingly, it was further found that

484 vincristine treatment administrated prior to asparaginase can affect mesenchymal cells, thus

485 blocking the secretion of asparagine and overcoming this resistance mechanism [195]. This

486 indicates that treatments may exert their effect not only by affecting leukemic cells but also

487 their microenvironment.

488   We have shown that leukemic cells, like other cancer cells, exploit several identical

489 features of the microenvironment to reach distant sites. We now discuss whether blast cells

490 may prepare their distant niches for engraftment and proliferation.

491 **IV. The interplay between leukemic cells and the microenvironment**

492   1. Leukemic and cancer cells model their niche

493   More than one century ago, Stephen Paget noted that breast cancer metastases occur

494 more often in some tissues than others and, refuting that this could hardly occur by chance or

495 solely by embolism, formulated his famous "seed and soil" hypothesis [196]. He compared

496 cancer cells to plant seeds that can only grow in favorable soil, that is, in a tissue somewhat

497 predisposed to welcome them. At that time, he already recognized that not only cancer cells

498 but also their "soil" would be worth studying. Since then, a large amount of information has

499 accumulated on cancer cell biology and the organotropism phenomenon has been

500 experimentally explored and demonstrated [197,198]. We illustrated above that some

501 leukemic cells, like some cancer cells, may be prone to home to and remain in certain

502 microenvironments that resemble that from which they originated. However, such a remote

503 and different microenvironment will not perfectly match the original niche and will probably

504 not meet all the needs of the leukemic cells.

505   Indeed, there is evidence that leukemic cells can remodel their microenvironment [199].

506 Colmone *et al.* used an *in vivo* microscopy imaging approach and found that ALL cells

507 remodeled the BM microenvironment they colonized, notably through SCF signaling, leading

508 to an abnormal niche that recruited and somehow trapped normal hematopoietic progenitors

509 [43]. More recently, Boyerinas *et al.* xenografted the leukemic Nalm6 cell line in a mouse

510 model and showed that the leukemic cells secreted osteopontin into the invaded

511 microenvironment, which in turn also produced osteopontin [200]. It was also shown using *ex*

512 *vivo* co-cultures of primary mesenchymal stromal cells and either the Nalm6 cell line or

513 primary ALL cells that although the CXCL12/CXCR4 axis was involved in leukemic cell

514 migration, other chemokines, such as C-C motif chemokine ligand 2 (CCL2) and CXCL8 may

515 be involved in leukemic niche formation, independently of the CXCL12/CXCR4 axis.

516 Transwell assays also demonstrated that leukemic cells could recruit mesenchymal stromal

517 cells to initiate the leukemic niche, in which secreted chemokines might attract other leukemic

518 cells while inhibiting that of healthy hematopoietic progenitors and mesenchymal stromal

519 cells [201]. These results are consistent with those of other studies including that of Colmone

520 *et al.* in which Nalm6 cells colonized CXCL12 niches in the BM of xenografted mice, which

521 subsequently resulted in marked downregulation [43,202]. A recent report showed that

522 leukemic cells can promote osteoclast-mediated bone resorption through the production of

523 receptor activator of nuclear factor κB ligand (RANKL) [203]. This is in accordance with a

524 previous report that demonstrated in a T-ALL mouse model that ALL cell engraftment in the

525 BM of mice was associated with bone microenvironment remodeling due to a marked loss of

526 osteoblastic cells [204]. Duan *et al.* used *ex vivo* imaging of xenografted mice to confirm that

527 engrafting ALL cells alters the BM microenvironment, resulting in damage to the vasculature

528 and endosteal lining. This report also showed that, following chemotherapy, leukemic cells

529 secreted cytokines, namely CCL3 and TGFβ, inducing phenotypic changes in the surrounding

530 mesenchymal stem cells. These cells provided a protective microenvironment for the

531 leukemic cells by converting a growth differentiation factor 15 (GDF-15) proform secreted by

532 the leukemic cells into a mature form, which led to the activation of TGFβ signaling, thereby

533 conferring chemoresistance [44].

534   Recent studies on solid tumors have led to a better understanding of cancer-cell

535 organotropism and metastatic niche formation. Kaplan *et al.* first demonstrated, in 2005, that

536 cancer cells can remotely prepare an appropriate niche before reaching their metastatic site

537　[198,205]. The authors demonstrated that cancer cells secrete certain factors that may

538　predispose them to metastasize toward a specific location. They first suggested cytokines, but

539　discovered several years later that the cancer cells produced exosomes that could direct

540　metastatic cancer cells to specific tissues [198,206]. Exosomes are small extracellular vesicles

541　(30-100 nm of diameter) that can be transferred to other distant cells to deliver functional

542　molecules, such as peptides or nucleic acids. Thus, exosomes are a means of intercellular

543　communication suitable for remote cells. Exosomes derived from different cancer cell lines

544　specifically target different tissues. An elegant experiment showed that exosomes can

545　"educate" cancer cells to reach a specific tissue. Mice were injected with exosomes from cells

546　that usually metastasize to the lungs before being transplanted with cells that do not usually

547　metastasize to the lungs, but to bones. This experiment resulted in a large significant increase

548　in lung metastases. Going further, the authors found that exosomal organotropism was

549　determined by their integrins, which allowed exosome uptake by specific tissue-resident

550　stromal cells within target tissues. Exosome uptake by these cells was followed by

551　upregulation of the expression of *S100* family pro-migratory and pro-inflammatory genes,

552　which could favor tumor metastasis. Importantly, specific exosomal integrins recovered from

553　plasma samples of patients with pancreatic or breast cancer correlated with the location of the

554　metastases, and may even likely predict future metastatic sites at the time diagnosis.

555　　2. Means of communication between ALL cells and supportive cells

556　　Several means of communication are available to ALL cells to interact and

557　communicate with their surrounding microenvironment and they may be used simultaneously.

558　The role of exosomes in the pathophysiology of several cancers is becoming increasingly

559　understood but has been little studied, to date, in the context of ALL [83,207]. However,

560　recent findings have shown that large extracellular vesicles (EVs), released through plasma

561　membrane budding, may be of particular importance for the communication between ALL

25

562 cells and their microenvironment [208,209]. Indeed, the group of Vaskar Saha showed that

563 ALL cells can produce EVs of varying size, some of which could be transferred horizontally

564 to other ALL cells, as well as BM stromal cells, resulting in phenotypic changes in the

565 recipient cells [209]. The transfer of large EVs to BM stromal cells induced a metabolic

566 change, favoring glycolysis in these cells, which increased the lactate concentration in the

567 medium, thereby providing the leukemic cells with their preferred fuel, as hypothesized by the

568 authors [208]. These large EVs were released into the systemic circulation of xenografted

569 mice. It is thus possible that EVs participate in similar metabolic reprogramming and/or other

570 phenotypic changes in remote stromal cells to promote ALL-cell migration to the secondary

571 sites. Although more compelling evidence has yet to be provided, it seems likely that both

572 exosomes and large EVs are involved in preparing a remote suitable niche for ALL cells. In

573 addition, large EVs may also contain intact organelles that could be transferred to recipient

574 cells [209]. Not all cells can generate EVs. Thus, as discussed by the authors, such

575 mechanisms may allow EV-producing resistant leukemic cells to promote chemoresistance, or

576 even resistance to targeted therapies, by transferring pro-resistant cellular content to other

577 sensitive cells.

578   The transfer of organelles, such as mitochondria, could also allow recipient cells to cope

579 with acute stress [210]. Another recent study showed that ALL cells also communicate with

580 mesenchymal stromal cells using tunneling nanotubes to set up their niche [211]. Tunneling

581 nanotubes are thin plasma membrane protrusions that connect one cell to another and allow

582 the exchange of cellular content. This means of intercellular communication is independent of

583 others and can promote leukemic cell survival, chemoresistance, and the release of cytokines

584 into the microenvironment. The use of fluorescent dyes has made it possible to visualize the

585 exchange of cellular content in both directions, from leukemic cells to mesenchymal stromal

586 cells and *vice versa*, confirming that ALL cells establish crosstalk with the surrounding

587 microenvironment to protect themselves and promote their survival [211].

588   Direct contact of leukemic cells with stromal cells has also been previously

589 highlighted in the pathogenesis of leukemia. Indeed, direct contact with stromal cells favors

590 ALL cell survival, offers them a protective environment, and may minimize the effects of

591 cytotoxic drugs [212–215]. Such cell-cell contact was found to be mediated, notably, by the

592 binding of integrin α4β1, also called very late antigen-4 (VLA4), expressed on the surface of

593 ALL cells, to the vascular adhesion molecule-1 (VCAM1), expressed on the stromal cell

594 surface [216]. Integrin α4β1 can also bind to extracellular matrix proteins, such as

595 osteopontin and fibronectin, and has been shown to play an important role in ALL cell homing

596 in mice [200,217,218]. In addition to its role in adhesion to extracellular matrix and/or stroma,

597 it is likely that integrin α4β1 binding to its receptor induces signaling cascades to promote

598 leukemic cell survival, proliferation, and/or chemoresistance [218]. Gap junctions are also

599 involved in cell-cell interactions within the leukemic niche. Gap junctions allow the direct

600 exchange of small molecules between two adjacent cells by creating a channel, most often

601 through the alignment and docking of two connexons (consisting of hexamers of specialized

602 proteins called connexins) of each cell. Several studies have suggested that leukemic cells can

603 communicate with stromal cells through gap junctions formed with connexin 43 (Cx43) [219–

604 223]. Although the mechanisms are still unclear, it appears that Cx43 gap junctions are

605 dysregulated in leukemic BM. Another connexin, Cx25, has recently been thought to be

606 involved in the pathogenesis of leukemia through gap junctions [224]. The authors showed,

607 using both T-ALL and AML cells, that inhibiting gap junctions by selective Cx25 knockdown

608 sensitized the cells to chemotherapy, suggesting that concomitant Cx25-targeted treatment

609 could allow reducing the dose of cytotoxic agents in clinical practice, thereby reducing the

610 side effects of chemotherapy while conserving its efficacy [224].

611   Direct communication of leukemic cells with the surrounding supportive cells,

612   probably as well as distant communication from the original to the secondary niche, appears

613   to be of crucial importance. Once in the secondary niche, leukemic cells must deal with the

614   local cells, which must be suitable surrogates that sufficiently resemble BM stromal cells. In

615   the CNS, for example, Akers *et al.* found, *in vitro,* that ALL cell lines were attracted to

616   astrocytes, choroid plexus epithelial cells, and even more strongly to meningeal cells, to

617   which they could strongly adhere. The authors showed that these cells from the CNS secreted

618   amounts of CXCL12 equivalent to those of BM stromal cells and osteoblasts and that CXCR4

619   blockade with plerixafor could inhibit ALL cell migration toward meningeal and plexus

620   choroid epithelial cells. They also observed that co-culture of both ALL cell lines and primary

621   ALL cells with any of these CNS cells decreased the effects of commonly used chemotherapy

622   drugs on leukemic cell viability. The provided chemoprotection was likely the result of the

623   combined effects of soluble factors and cell-cell contacts between the ALL cells and the CNS

624   cells [225]. This study illustrates that ALL cells can interact with supportive cells other than

625   those which belong to the niche from which they originate. Such cells may therefore be

626   similar to those of the BM niche and ALL cells can communicate with them to enhance their

627   leukemic-supportive properties and adapt themselves to better settle into secondary niches.

628   The interconnections involved in this interplay are complex, but the methods used in

629   studies usually only focus on one aspect of one component of the leukemic

630   microenvironment. For example, cell-cell interactions have mostly been studied through co-

631   culture systems consisting of only leukemic cells and one type of stromal cell, although

632   leukemic cells probably interact not only with supportive stromal cells but also other cells

633   within the microenvironment. Similarly, hypoxia can induce CXCL12 and CXCR4

634   expression, as well as that of SCF, and SCF itself has been found to increase HIF1α

635   expression [226]. Leukemic cells may escape the effects of therapy by taking advantage of

28

636 this system, of which the complexity is even more pronounced if we consider other
637 parameters, such as temporality.

638   3. The chicken or the egg causality dilemma in ALL relapses

639   An intriguing question is whether the cells found far from their original niche are the
640 result of the development of a resistant clone that reached its secondary niche in response to
641 treatment, or whether these cells were already there as leukemic or preleukemic clones, even
642 before diagnosis. Recently, Williams *et al.* addressed this question in the context of B-ALL
643 and found that most leukemic cells had the capacity to infiltrate the CNS and were not
644 restricted to a specific population [69]. They demonstrated, in clonal tracking experiments,
645 that the populations engrafting the CNS were polyclonal, and that the clones found in the
646 CNS were also found in the spleen or BM of xenografted mice. The authors concluded that
647 CNS invasion is a "generic" capacity of leukemic cells but, as they also discussed, their
648 results do not rule out the possibility of the existence of specific clones particularly well-
649 adapted for invading the CNS microenvironment and surviving there. They also did not
650 discuss whether the cells already possessed this capacity to invade the CNS at a preleukemic
651 stage. Also, one cannot exclude that a leukemic cell may had been generated during
652 embryonic or fetal hematopoiesis and would stay in a dormant state in the CNS.

653   Similarly, solid tumor cells can settle at tissues distant from their original site early
654 during the course of the disease, even before detection of the primary tumor [227,228]. Solid
655 cancers, as well as leukemia, must be viewed as a systemic disease, in which cancer cells
656 spread and engraft early at predisposed tissues that provide protection against insults, such as
657 chemotherapy, notably through the induction and maintenance of a dormant state [227,229].
658 However, as metastasis is an inefficient process, only a few cells may have the ability to
659 survive and grow out of their original niche [230]. Such rare cells would be able to adapt

29

660 themselves to a new microenvironment, where they could become quiescent or grow slowly,

661 explaining why some patients experience very late relapses in distant tissues. The existence of

662 leukemic stems cells (LSCs) that would be responsible for late relapses may also be evoked.

663 However, these LSCs are not well characterized, especially in B-ALL, and several reports

664 indicate that ALL cells with the capacity to initiate leukemia are relatively frequent [231, Le

665 viseur 2008]. We have therefore chosen to focus on the role of the microenvironment, which

666 appears to play an important part in the leukemia-initiating ability of ALL cells.

667 Ebinger *et al.* recently used an ingenious cell labeling system in patient-derived

668 xenografts to provide evidence that such dormant cells exist in ALL and provided a robust

669 preclinical model [45]. The authors found that these dormant cells shared a similar

670 transcriptional program with leukemic cells found at MRD level in patients. Moreover, these

671 dormant cells were resistant to conventional chemotherapy *in vivo*, but became sensitive to

672 drugs when retrieved from their microenvironment and treated *ex vivo* [45]. This result has

673 two principal clinically relevant implications: first, the dormant phenotype that appears to be

674 harbored by MRD cells is reversible and second, the microenvironment appears to play a

675 critical role, strongly suggesting that targeting the microenvironment could be a promising

676 strategy to kill the disease and prevent relapse and treatment failure. As the authors state, it

677 may also explain why children treated for ALL benefit from standard maintenance therapy

678 that continues for two years from diagnosis: daily doses of low-dose chemotherapy might help

679 to progressively eradicate all ALL cells, including the dormant cells that may reenter the cell

680 cycle after the intensive phase of treatment. Although the authors showed that dormant ALL

681 cells were preferentially found close to the BM endosteum, they did not explore engraftment

682 in other tissues. It would be informative to use their model to verify whether such dormant

683 cells can also be found in the extramedullary tissues frequently invaded in clinical practice,

684 especially the perivascular areas described above. This study also argues in favor of an

685   extramedullary origin for combined ALL relapses, especially late relapses [59], as the

686   dormant ALL cells were shown to have leukemia-initiating properties.

V.   From bench to bedside

688   Leukemic cells interact dynamically with their microenvironment. Indeed, intravital

689   microscopy experiments, which allowed the high-resolution observation of leukemic cells in

690   live animals, showed the leukemic cells to be motile without any objective preference for a

691   specific location or supportive cells [204]. Treatment of the mice with drugs commonly used

692   in clinical practice (vincristine, dexamethasone, and asparaginase) reduced the leukemic

693   burden and induced an increase in the proportion of quiescent cells, but did not modify the

694   patterns of cell distribution and the cells kept moving, even faster than those of untreated mice

695   [204]. These results suggest that leukemic engraftment was randomly driven, in contrast with

696   other reports and commonly held beliefs. Thus, as stated by Williams *et al.*, engraftment

697   potential may not be restricted to a specific cell population but may be shared by most ALL

698   cells. This was recently corroborated by two independent studies [69,231,232]. In these

699   studies, the authors used a cellular barcoding strategy to track leukemic clone engraftment in

700   the BM of different bones and several extramedullary tissues of xenografted mice [231,232].

701   Both studies showed that the engrafting cell populations were polyclonal, similarly to solid

702   tumors, suggesting that all founder cells were able to reconstitute a leukemic bulk population.

703   However, the authors found that the clonal distribution differed depending on the tissues

704   analyzed: a clone could be more highly represented in one tissue, whereas another could be

705   predominant in another tissue. The authors stated that this asymmetric clonal distribution was

706   presumably stochastic and probably not due to clonal preference or a predisposition to settle

707   in one site over another. As the authors discussed, the specific microenvironment may confer

708   an advantage to arriving leukemic cells that favors settlement and/or the proliferation,

709   explaining the observed asymmetric distribution. Notwithstanding the evidence provided by

31

710   the intravital microscopy experiment of Hawkins *et al.*, some cells from the founder

711   population exposed to such a niche may become quiescent and be the ancestor of a subsequent

712   relapse [204].

713   Aside from these studies, there is evidence in the literature suggesting that leukemic

714   cells could themselves carry specific features that may direct their engraftment to one or

715   another tissue. Van der Velden *et al.* recently identified a protein profile that could potentially

716   help predict CNS relapse at the initial diagnosis. A comparison of the gene expression profiles

717   between leukemic cells from the BM and CNS of patients allowed the identification of genes

718   differentially expressed by leukemic cells from the CNS. Among these genes, *SSP1* (coding

719   for osteopontin) and *SCD* (coding for stearoyl-CoA desaturase) were found to be upregulated,

720   which was further confirmed both at the mRNA and protein levels. Retrospective flow

721   cytometry analysis of BM samples from patients who experienced a CNS relapse showed that

722   both SPP1 and SCD proteins were elevated in a small subpopulation of cells, suggesting that

723   this expression profile might be used in a clinical setting to predict CNS relapse [233]. As

724   Frishman-Levy and Izraeli discussed in a recent review, in which they summarized current

725   knowledge concerning CNS leukemia, the contradictory nature of these results with those of

726   Williams *et al.* could be partially explained by methodological discrepancies (mouse

727   xenograft model *versus* a human model). The idea that CNS tropism may be linked to a

728   specific ALL population is also supported by the association of several recurrent genetic

729   alterations with the incidence of CNS relapse. Indeed, the chromosomal rearrangement

730   t(1;19), leading to the *TCF3-PBX1* fusion transcript, has been associated with an increased

731   risk of ALL relapse in the CNS [234,235]. This clinical finding has recently been supported

732   by a biological explanation. It was shown that primary leukemic cells expressing the *TCF3-*

733   *PBX1* transcript expressed high levels of Mer tyrosine kinase, for which the signaling

734 pathway was further shown to be involved in ALL cell survival and chemoresistance in the

735 CNS microenvironment [236].

736 Leukemia with the t(12;21) chromosomal rearrangement, which leads to the ETV6-

737 RUNX1 fusion protein, is associated with an increased risk of testicular relapse [4]. We

738 previously compared the expression profiles of ETV6-RUNX1 with those of other B-ALL and

739 found that fourteen genes were differentially expressed in this type of leukemia [237]. Among

740 these genes, *CD9* (tetraspanin) was found to be underexpressed, which was further confirmed

741 by flow cytometry of patient samples [238]. We reasoned that CD9 may be involved in the

742 pathogenesis of this particular B-ALL subtype [239]. We found that CD9 has a role in

743 leukemic cell dissemination through the Ras-related C3 botulinum toxin substrate 1 (RAC1)

744 signaling pathway. Importantly, we showed that the more CD9 was expressed at the surface of

745 ALL cells, the more cells were able to migrate into the testes in a mouse xenograft model

746 [110]. Thus, ETV6-RUNX1 ALL relapses may be partially explained by enhanced CD9

747 expression, which favors blast dissemination and engraftment into the testis.

748 We postulate that ALL relapses occurring at specific sites could be explained by the

749 association of both extrinsic and intrinsic factors, thus reconciling biological and clinical

750 research findings. Specific genetic alterations may modify cell signaling pathways that could

751 render the cells stochastically exposed to a specific microenvironment to take advantage of it

752 and durably settle in it.

753 The role of the microenvironment in the pathogenesis of ALL has also been suggested

754 by the good results obtained by treating or preventing extramedullary relapses with

755 specifically directed local treatments. Indeed, aside from the introduction of high-dose

756 methotrexate or aracytine, successful treatment of testicular or meningeal involvement has

757 been specifically directed at the tumor: surgery/radiation therapy for the testis and intrathecal

758 chemotherapy or radiation therapy for the meninges. This may explain the relatively good

759 prognosis for relapses at these sites and is another argument for an important role of the

760 microenvironment, which is removed by surgery and radiotherapy. The role of the intrinsic

761 properties of leukemic cells concerning their interaction with their surrounding

762 microenvironment may be illustrated by the worse prognosis seen in Ph-like and Ph+ B-

763 ALLs. Ph+ B-ALL and Ph-like B-ALLs share similar genetic alterations and a poor response

764 to chemotherapy relative to other B-ALL subtypes. Within these subtypes, alteration of the

765 Ikaros gene is frequently found. This alteration was shown to alter the adhesive properties of

766 leukemic cells and may influence their settlement in their niche.[240].

767 **VI. Therapeutic challenges**

768   Therapeutic efforts in the past 15 years have mainly focused on the development of

769 new molecules and immunological approaches directed against specific targets expressed on

770 leukemic cells. Treatment intensification in patients with the most unfavorable prognostic

771 factors has not only improved their survival, but also allowed to efficiently treat

772 extramedullary leukemia without using local treatments. As mentioned earlier, the use of high

773 doses of certain drugs, such as methotrexate, reduced the incidence of extramedullary

774 involvement. Apart from these leukemic cell-oriented targeted therapies, therapeutic strategies

775 oriented toward leukemic niches are also attracting increasing interest [241]. Figure 3

776 illustrates a few examples of strategies that could be used to target leukemic cells in their

777 microenvironment and Table 1 shows several examples of molecules that could be used or

778 tested in clinical practice during the coming years. These strategies may also be applied to

779 reach cancer cells in solid tumor metastatic niches.

780   First, preventing cell migration and engraftment into the niche or, conversely, the

781 mobilization of niched cells could be attempted, for example by disruption of the

782 CXCL12/CXCR4 axis using CXCR4 inhibitors, such as plerixafor [105,124,242,243].

34

783 Targeting cell adhesion to the extracellular matrix or supportive cells may also be a useful

784 strategy, for example, by inhibiting α4β1 integrin-mediated adhesion with a specific

785 monoclonal antibody, such as natalizumab, or a chemical inhibitor, such as TCB3486

786 [244,245]. Another possible niche-oriented therapeutic strategy could be to target integrins,

787 since exosomes home to future metastatic sites via integrins in melanoma and lung cancer

788 models. Directly targeting integrins could thus interrupt cellular communication with the

789 secondary niches [206]. Targeting niche features may also be a beneficial strategy, for

790 example by using hypoxia-activated prodrugs, such as PR104 and TH-302 [141,145–

791 147,246]. In ALL, modifying the leukemic microenvironment to reverse the phenotype of

792 dormant blast cells could be also a viable option [45]. This could be achieved by restoring a

793 healthy microenvironment, for example by using bisphosphonates that could prevent bone

794 degradation, which is observed in leukemia pathogenesis [203,204]. Another possible strategy

795 could target signaling pathways that are upregulated upon the interaction of malignant cells

796 with their microenvironment, such as the TGFβ and HIF1α pathways, for which several

797 preclinical or early clinical studies have been published [246,247]. The immune system could

798 also be manipulated, as seen in Hodgkin lymphoma, a hematological malignancy with a rich

799 microenvironment, with impressive results from recent early clinical trials testing specific

800 anti-programmed cell death 1 (PD-1)/PD-1 ligand 1 (PD-L1) antibodies [248,249].

801 Many challenges have yet to be addressed concerning these and other strategies that

802 could target malignant cells in their niches in hematological and solid cancers. Given the

803 complexity of the microenvironment, successful treatment will probably require a

804 combinatory approach, by associating these therapies both between themselves and with

805 conventional chemotherapies. It is likely that such treatments would exert their full potential

806 by adding them to chemotherapy after the initial diagnosis to prevent the occurrence of

807 relapse. They should probably be reserved for refractory patients or those with the highest risk

808 of relapse.

VII. Conclusion and future directions

810   Leukemic niches and their interplay with leukemic cells are of tremendous complexity,

811 for which we have only limited information. The aim of this review was to illustrate this

812 concept by giving a few examples, rather than being exhaustive, to describe potential niche-

813 targeted therapeutic strategies that could be considered in the near future. We chose to

814 consider leukemia relapses like solid tumor metastases. We described several features of the

815 microenvironments of medullary, extramedullary, and metastatic niches, focusing on their

816 similarities, suggesting that ALL relapses might be considered more like leukemic metastases.

817 We provide lines of evidence showing that ALL cells, as well as other cancer cells, can

818 communicate with and remodel their microenvironment. Similar to solid tumors, ALL cells

819 can reach secondary sites and settle down, where they can remain dormant for years before

820 initiating a relapse. Although some intrinsic factors may predispose cells to invade one tissue

821 over another, the microenvironment undoubtedly plays an important role in leukemic cell

822 engraftment, making niche-based targeted therapies promising and worth developing.

823 However, given the complexity of the niches and the dynamic interconnections between each

824 microenvironmental actor, it will be a considerable challenge to elaborate therapies that can

825 efficiently and durably overcome resistance. Determining the appropriate placement of such

826 therapies in current treatment strategies is also an open question that will need to be

827 addressed.

VIII. Practice points

829 • ALL cells appear to localize preferentially in perivascular regions of the tissues they

830   colonize.

36

831 • Testicular, CNS and BM leukemic niches and solid tumor metastatic niches display

832 similarities potentially accounting for the residence of leukemic cells in these

833 preferred microenvironments.

834 • The interplay between leukemic niches and ALL cells is complex, dynamic and

835 bidirectional.

836 • Relapses in specific tissues can be explained by both the microenvironment in these

837 tissues and the intrinsic properties of ALL cells.

838 • Niche-based targeted therapies are currently being developed and may effectively

839 prevent ALL relapses.

840 • Systemic treatments such as chemotherapy remain the best option to successfully treat

841 extramedullary leukemia.

842

843 **IX. Research agenda**

844 • Further efforts are required to determine why leukemic cells settle preferentially in

845 specific niches, to identify the most relevant microenvironmental features for

846 therapeutic targeting.

847 • The combination of niche-based targeted treatments and conventional chemotherapy

848 may prevent relapse.

849 • Clinicians should define the categories of patients most likely to benefit from such

850 treatments.

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FIGURE LEGENDS
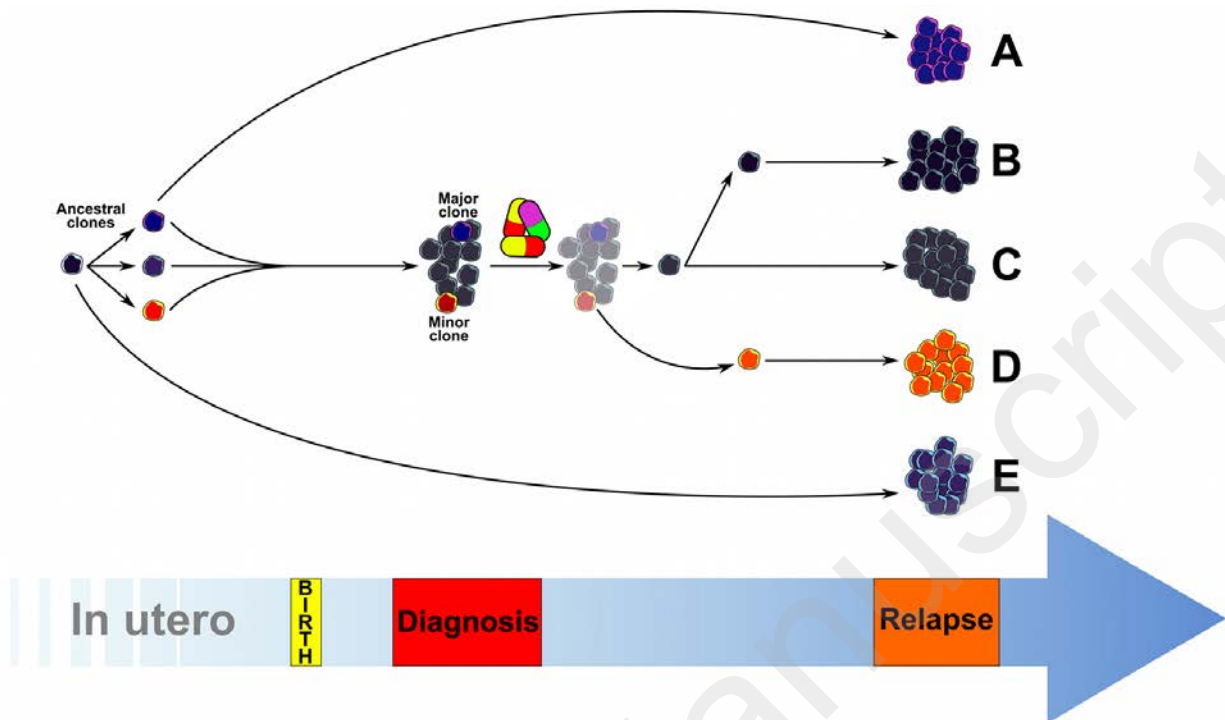
1593 **Figure 1. Clonal evolution of leukemic cells from preleukemic stage to relapse.** A first

1594 genetic hit, such as an ETV6-RUNX1 rearrangement, in a hematopoietic progenitor leads to

1595 the apparition of a preleukemic clone, which can subsequently accumulate other genetic

1596 alterations to give rise to leukemic "ancestral clones". Such clones may be present before

1597 birth, as notably shown for ETV6-RUNX1. Further genetic hits favor the emergence of clones

1598 that give rise to an overt acute lymphoblastic leukemia (ALL), which may be composed of a

1599 major clone and minor clones. After diagnosis, treatment of ALL with chemotherapy can

1600 eradicate all or almost all leukemic cells. Some cells from the bulk population may survive

1601 and give rise to a relapse with the same major clone found at diagnosis (situation C).

1602 Alternatively, cells derived from either major or minor clones may undergo other genetic

1603 alterations, leading to a different type of relapse, as shown in situations B and D. Relapsed

1604 leukemia may also derive from another ancestral clone or even directly from the preleukemic

1605 clone, which may have evolved on their own (situations A and E).
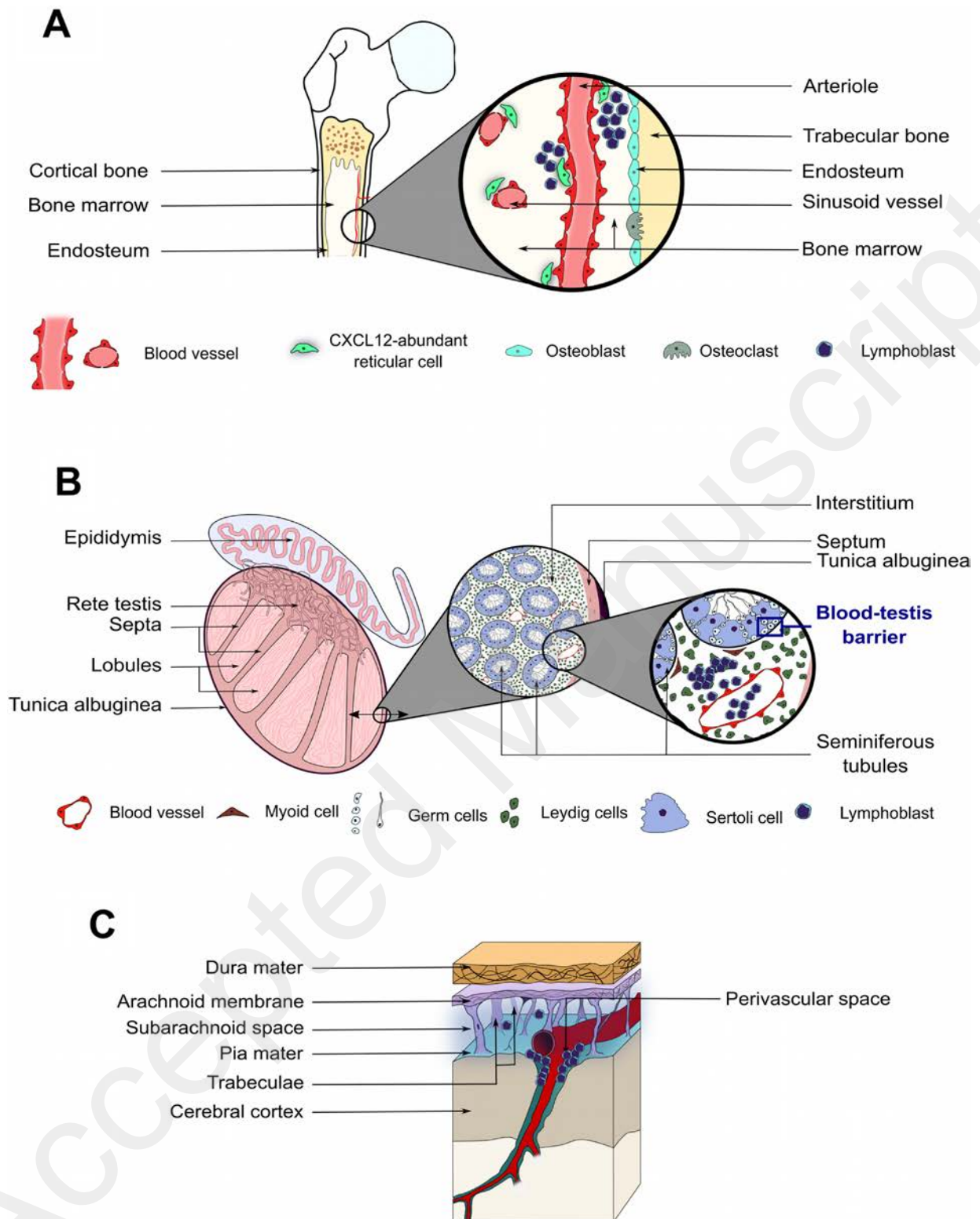
1606

1607 **Figure 2. Main sites of acute lymphoblastic leukemia (ALL) relapse.** *(A) Bone marrow.*

1608 This extensively simplified schematic view of the bone marrow (BM) illustrates where ALL

1609 cells are mainly found in the perivascular spaces close to the endosteum. The endosteum

1610   corresponds to a thin layer of conjunctive tissue delineating BM and covered with osteoblasts

1611   and osteoclasts, which participate in balancing the renewal and remodeling of bone tissue.

1612   CXCL12-abundant reticular cells, specialized stromal cells near vessels, produce CXCL12,

1613   which appears to be necessary for ALL cell engraftment in BM. *(B) Testis.* Testis is the organ

1614   in which male gametes are produced. Germ-cell maturation takes place in specialized

1615   structures called seminiferous tubules, which are surrounded by an interstitial tissue and

1616   regrouped into lobules. The lobules are separated by fibrous septa which are in continuity

1617   with the capsular region. The testis is encapsulated in a fibrous covering called the tunica

1618   albuginea. The production of germ cells begins at the basement membrane, which surrounds

1619   the seminiferous tubule and where germ cells are the most immature. The germ cells mature

1620   as they progress from the basal part of the tubule to the lumen, where fully mature sperm cells

1621   are found. Throughout the maturation process, germ cells are enclosed by Sertoli cells, which

1622   form tight junctions between themselves to protect the germ cells and compartmentalize the

1623   maturation steps. The role of the blood-testis barrier is to protect germ cells from potentially

1624   deleterious molecules or cells. The blood-testis barrier is anatomically composed of the

1625   basement membrane, Sertoli cells, and their tight junctions, but does not encompass blood

1626   vessels, making the term "blood-testis barrier" inaccurate. Between the seminiferous tubules,

1627   the interstitial tissue, or interstitium, is composed mainly of supportive cells, called Leydig

1628   cells, of which one of the main roles is to produce testosterone. During testicular leukemia,

1629   ALL cells are mainly found in the interstitial perivascular spaces, and occasionally in

1630   seminiferous tubules, but only at very advanced stages of the disease, following inappropriate

1631   treatment. *(C) Central nervous system.* The central nervous system (CNS) consists of the

1632   brain, cerebellum, and spine. CNS tissue is surrounded by three meningeal layers: the thick

1633   dura mater, the arachnoid membrane, from which trabeculae fibers cross the subarachnoid

1634   space to reach the third thin layer, the pia mater, which is in direct contact with CNS tissue.

1635 Cerebrospinal fluid circulates in the subarachnoid space, where blood vessels are found.

1636 When the CNS is involved, ALL cells localize mainly in the subarachnoid space, close to

1637 vessels, whereas some cells may circulate freely in the cerebrospinal fluid.
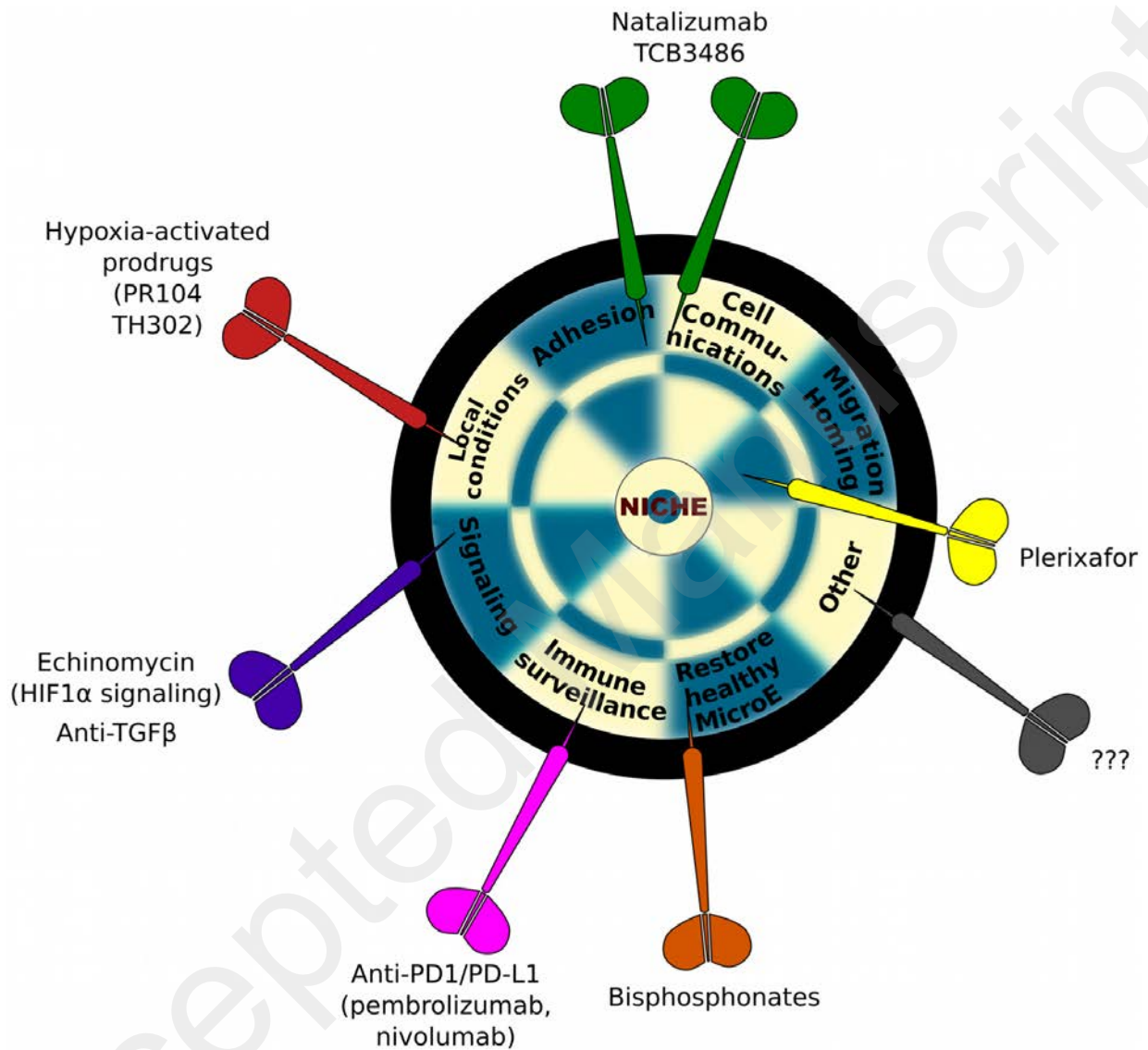
1638

1639 **Figure 3. Potential therapeutic strategies to target the leukemic niche.** The leukemic

1640 niche is a highly complex microenvironment made of many components or aspects that could

1641 be targeted. Potential niche-based treatments are represented by the darts, which could be

1642 used simultaneously to target different components, as symbolized by the blurry delineations

1643 on the target.

1644 **TABLE LEGENDS**

1645 Table 1. Examples of studies using molecules that target components of the leukemic niche.

Target	Study [ref]	Type of study	Tested molecule	Number of patients	Main results/comments
CXCL12/CXR4 axis	Cooper et al., 2017 [243]	Phase I	Plerixafor	20 (5 ALL, 12 AML, 1 AML/MDS)	Maximum dose 15 g/m^2 without DLT Heavily pretreated patients Plerixafor administrated prior to chemotherapy (HD-araC + VP16) Mobilization of lymphoblasts achieved No clinical response in ALL patients (4 SD, 1 PD)
	Juarez et al., 2007 [124]	Preclinical (animal study)	Plerixafor, TC14012, AMD3465	NA	Reduced extramedullary engraftment of ALL cells in xenografted mice Mobilization of ALL cells in PB and spleens of xenografted mice Continuous administration equivalent to intermittent administration
	Ranhawa et al, 2016 [244]	Preclinical (*in vitro* and animal study)	Plerixafor, BKT140, CXCR4 genetic deletion	NA	Reduced chemotaxis of B-ALL cells Restoration of drug sensitivity of resistant B-ALL cells co-cultured with BMSCs Delayed engraftment and prolonged survival of mice xenografted with CXCR4-deleted B-ALL cells
	Juarez et al. 2003, [105]	Preclinical (*in vitro* study)	Plerixafor, TC14012	NA	Reduced chemotaxis of B-ALL cells Reduced proliferation of B-ALL cells cultured on stromal BM cells

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Target	Study [ref]	Type of study	Tested molecule	Number of patients	Main results/comments
					Augmentation of inhibitory effects of chemotherapy
Integrin alpha4	Hsieh et al. 2013 [245]	Preclinical (*in vitro* and animal study)	Natalizumab	NA	Prolonged survival of xenografted mice Synergistic effect with chemotherapy (marked prolonged survival) Reduced engraftment in liver, spleen, and BM of xenografted mice and enhanced engraftment in lungs.
	Hsieh et al. 2014 [246]	Preclinical (*in vitro* and animal study)	TBC3486	NA	Reduced chemoresistance of B-ALL cells Prolonged survival of xenografted mice Synergistic effects with chemotherapy *in vivo*
HIF1a	Badar et al., 2016 [146]	Phase I	TH-302 (evofosfamide)	49 (39 AML, 9 ALL, 1 CML)	Heavily pretreated patients 5 days of administration over 21-day cycles Maximum tolerated dose 460 mg/m^2 as intermittent administration and 330 mg/m^2 as continuous infusion. 1 patient with T-ALL with PR
	Benito et al. 2016 [147]	Preclinical (*in vitro* and animal study)	TH-302 (evofosfamide)	NA	Cytotoxicity on B-ALL cells in hypoxic culture conditions Not tested in an *in vivo* ALL model
	Konopleva et al. 2015 [145]	Phase I/II	PR104	50 (40 AML, 10 ALL[3 T-ALL, 7 B-ALL])	Heavily pretreated patients Chosen doses for phase II: 3 g/m^2 and 4g/m^2 2 responses (1 CR and 1 MLFS) in 7 B-ALL
	Benito et al. 2011 [141]	Preclinical (*in vitro* and animal	PR104	NA	Cytotoxicity on B-ALL cells in hypoxic culture conditions

Target	Study [ref]	Type of study	Tested molecule	Number of patients	Main results/comments
		study)			Delayed engraftment of mice xenografted with B-ALL cells Prolonged survival of xenografted mice with maintained complete response at 550 mg/kg
Restoring normal microenvironment	Cheung et al, 2018 [203]	Preclinical (*in vitro* and animal study)	Zolendronic acid	NA	Reduced leukemia burden in xenografted mice with B-ALL cells Prolonged survival of mice xenografted with B-ALL cells
Immune system	Chen et al. 2017, [250]	Case report	Nivolumab	2	Heavily pretreated patients with one or two prior HSCTs Off-label use Low-dose nivolumab (40-80 mg/course) administered to prevent GVHD Clinical response with the 2 patients alive 13 months and 4 months after relapse, but with persistent disease
	Boekstegers et al. 2017 [251]	Case report	Pembrolizumab	1	Refractory T-ALL treated with HSCT followed by 4 DLIs Fast clinical response with MRD decrease Severe acute GVHD, leading to immunosuppressive treatment with subsequent re-increase of MRD, multi-organ failure, and death

1646

1647 **Table 1. Examples of studies using molecules that target components of the leukemic**

1648 **niche.** The apparently disappointing results from clinical studies may be explained by the use

1649 of new treatments mostly alone in heavily pretreated patients. The association of each of these

63

1650   molecules with each other and/or conventional chemotherapy or immunotherapy might lead to

1651   better outcomes.

1652   Abbreviations: AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; BM:
1653   bone marrow; BMSC: bone marrow stem-cell; CML: chronic myeloid leukemia; CR:
1654   complete remission; DLI: donor lymphocyte infusion; DLT: dose-limiting toxicity; GVHD:
1655   graft versus host disease; HD-AraC: high-dose aracytine; HSCT: hematopoietic stem cell
1656   transplantation; MLFS: morphological leukemia-free state; MRD: minimal residual disease;
1657   NA: not applicable; PB: peripheral blood; PD: progressive disease; PR: partial response; SD:
1658   stable disease