

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

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33 Abstract

34 Long-term survival rates in childhood acute lymphoblastic leukemia (ALL) are 35 currently above 85% due to huge improvements in treatment. However, 15-20% of children 36 still experience relapses. Relapses can either occur in the bone marrow or at extramedullary 37 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 38 39 review, we have attempted to assemble the evidence concerning the microenvironmental 40 factors that could explain why ALL cells reside in such sites. We present criteria that make 41 extramedullary leukemia niches and solid tumor metastatic niches comparable. Indeed, 42 considering extramedullary leukemias as metastases could be a useful approach for proposing 43 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. 44

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

47 I. Introduction

48 Acute lymphoblastic leukemia (ALL) is a hematological malignancy defined by the 49 clonal expansion and accumulation of abnormal immature lymphoid precursor cells in the 50 bone-marrow (BM) compartment. This abnormal cell population replaces normal 51 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 52 most frequent malignancy found in children and accounts for approximately 80% of 53 54 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 55 56 risk-adapted groups for treatment, and improved treatments have drastically reduced the 57 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. 58

59 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 60 61 achievement of complete remission. Relapses are generally classified and stratified according 62 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 63 prognostic factor: the earlier the relapse occurs, the worse the prognosis. The definition of 64 65 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 66 67 probably the anatomical sites of relapse, which are commonly divided into bone marrow 68 (BM) alone (worst prognosis), extramedullary alone (best prognosis), and combined relapses (intermediate prognosis). Although immunophenotype T was initially associated with poor 69 70 survival and is still used in tumor classification, its prognostic value appears to be less 71 important due to better stratification [8]. Furthermore, the assessment of early minimal

5

residual disease (MRD) is also used to predict the prognosis of patients presenting a relapse,
as it is for newly-diagnosed ALL [9–12]

Given the considerable therapeutic progress that has been made, one of the main goals is to attenuate treatment-related sequelae by reducing their toxicity, while continuing to improve current survival rates. Decreasing the incidence of relapse and identifying optimal treatments and therapeutic strategies when relapse occurs, are critical goals as well. Indeed, deciphering the underlying mechanisms that lead to relapse and understanding how and why 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. Third, we will discuss the interplay between ALL cells and the microenvironments they 84 colonize. Fourth, we will discuss the potential predisposition of some ALL cells to reside in 85 86 specific niches. Finally, we will conclude with therapeutic perspectives related to microenvironment targeting. 87

88 II. Cellular origin of relapse

Multiple biological factors may contribute to the emergence of ALL relapses [13]. Cumulative evidence suggests a clonal origin of ALL [14]. It has previously been shown that clonal evolution is involved in relapse, with the possible emergence of subclones derived either from the major clone found at initial diagnosis, minor subclones, or a common ancestral pre-leukemic clone (Figure 1) [15–20]. Several studies have described chemoresistancerelated mutations in the BM samples of relapsed patients, such as those that affect the glucocorticoid response pathway (mutations of the glucocorticoid receptor gene *NR3C1* or 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 suggested for brain and testicular tissues [27,28]. Local conditions, the cell matrix, cell-cell 110 111 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 diagnosis [29,30]. Why and how they reenter the cell cycle and proliferate to induce relapse 113 114 still needs to be addressed for understanding the evolution of these dormant cells. How such cells reach and invade distant favorable microenvironments from their native marrow niche is 115 116 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, regardless of the type of relapse. However, the occurrence of relapse may not depend only on leukemic cell features, but also on the microenvironment. In the next section, we will describe the most frequently-reported sites of relapse and the concept of niches, proposing that extramedullary leukemia relapse is similar to that of solid tumor metastasis, anticipating a 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 be more common in children, they also concern adults. These sites may represent the first 132 133 extramedullary sites colonized by leukemic cells but can also be colonized secondary to other extramedullary sites that may have evaded clinical detection. Prophylactic cranial irradiation 134 was added to systemic chemotherapy treatment in the 1970s to attempt to prevent CNS 135 136 relapses [33]. Intrathecal chemotherapy and the use of high-dose methotrexate for neuromeningeal prophylaxis have progressively replaced irradiation, with good results for 137 diminishing neurological sequelae, showing that the blood-tissue barrier problem can be 138 139 bypassed [34]. Similarly, treatment intensification, with the introduction of intermediate and high-dose methotrexate, has significantly decreased the incidence of testicular relapses, thus 140 141 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 involved (approximately 65% of cases) [3,8,34-37]. There is no clear evidence for a 150 151 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 vessels close to endosteum, where they settle. From these perivascular endosteal niches, they 159 160 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 3.5-8% of ALL relapses in children (including boys and girls) followed for up to 20 years 163 [3,8,38–41,46]. Most studies mentioning testicular biopsies in the context of ALL did not 164 indicate the precise location of blasts [47–49]. Such information can be retrieved from rare 165 166 and old clinical studies and animal models [50-56]. Animal models indicate that leukemic cells infiltrate the interstitial testicular tissue mainly at the perivascular and peritubular 167 168 regions, forming small foci, notably in subcapsular areas. Despite perivascular interstitial invasion, extensive infiltration of the interstitium is rare. On the other hand, the epididymis is 169

more often massively invaded by blast cells [54,55]. Leukemic invasion seems to occur 170 171 through the hematogenous route, with possible extension to the epididymis via the lymphatic route [55]. In these models, although seminiferous tubules were degenerated or destroyed near 172 173 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 diffuse in a postmortem series of children who died of very advanced leukemia. Leukemic 175 cells were found in the tubules in half the patients with heavily infiltrated testes [57]. This 176 177 relatively old study included children with uncontrolled leukemia at a time when outcomes were poor and probably does not reflect the route of initial invasion. Thus, it seems that 178 179 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 system. Without effective treatment, seminiferous tubules may be destroyed and invaded as 181 182 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 or not, nor the treatment performed [62]. The effects of chemotherapy could explain the 189 190 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 clinically suspected with a simple physical examination, there is no overt clinical sign that 197 198 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 [63]. However, it was possible to detect leukemic cells in up to 70% of samples by 200 polymerase chain reaction of cryopreserved ovarian tissue retrieved at diagnosis (or shortly 201 202 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 do not report the precise location of leukemic cells in the ovary [58]. In their autopsy series, 205 206 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 be invaded by leukemic cells [68,69]. Indeed, Price and Johnson studied 126 brains from 215 216 children who died of ALL and developed a grading system to evaluate the severity of arachnoid involvement. They detected leukemic cells in the CNS of 55% of patients. Most 217 218 showed moderate leukemic infiltration and the authors observed that infiltration of the brain parenchyma occurred only in cases of extensive and deep leukemic arachnoid involvement 219

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 leukemic cells transit through the blood-CSF barrier to reach the leptomeninges [69,70]. 225 Although Price and Johnson only studied brain and did not consider the spinal cord or dura 226 227 matter in their study, diffusion throughout the CNS via the CSF has been confirmed by leukemic cell DNA detection, both in the lumbar spinal cord and frontal brain of rats 228 229 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 most often diffuse infiltration [71,72]. However, although much rarer and mostly represented 231 232 by case reports in the literature, CNS involvement may also consist of epidural masses that can compress the spinal cord [73]. Thus, leukemic cells do not colonize one specific 233 234 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 mouse model, has shown that meningeal invasion occurs through the diapedesis of leukemic 236 cells from cranial or spinal bone marrow of adjacent bones. The authors reported that 237 238 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 this mechanism involves an interaction between a6 integrin and laminin and that it can be 240 blocked by specific PI3Kδ inhibitors [74]. 241

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 published data consist mostly of case reports [75]. Although no site can be defined as
privileged, since ALL can settle in many tissues, breast involvement may be a privileged site
of extramedullary leukemia, mostly described by Isabel Cunningham [75–79].

248 2. Similarities between sanctuary sites

We chose not to provide an exhaustive comparison between metastatic niches but rather to focus on three representative common actors: the CXCL12/C-X-C chemokine 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 in specialized compartments, called "niches", within the BM [85]. Such niches allow the 255 maintenance and regulation of HSCs by providing specific local conditions, cytokines, 256 chemokines, and other soluble factors, as well as various types of specialized stroma cells. 257 Different types of HSC niches have been described in the literature and may be named 258 differently, depending on the authors, which can be confusing [80,81,86,87]. Our 259 understanding of the BM microenvironment is constantly evolving and is still a subject of 260 261 debate. For example, some studies have provided evidence for the existence of an osteoblastic niche [86], but this has been more recently questioned. Indeed, the existence of an authentic 262 osteoblastic HSC niche has been ruled out, even if osteoblastic lineage cells have an indirect 263 role in the regulation of HSCs and appear to be part of a specialized niche for lymphoid 264 265 progenitors [81,84]. Rather than viewing the BM as a suite of separated niches, it is more likely a unique continuous niche in which some components are shared (and some others not) 266 267 between more specialized compartments within this niche, as proposed by Hira et al. [88].

HSCs localize mainly to perivascular zones, as do leukemic cells, with most found close tosinusoids, 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 predisposed distant niches, which they can then remodel to suit their needs [94,95]. Such 273 274 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 some of these shared components that have been found to play a major role in these 276 277 microenvironments.

278

b) CXCL12/CXCR4

CXCL12, also known as stromal derived factor 1 alpha (SDF1 α), is one of the most 279 280 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 the niche. CXCR4 is a seven-transmembrane G-protein-coupled receptor that is expressed on 282 multiple cell types, including, notably, hematopoietic cells [96]. The inhibition of CXCR4 is 283 284 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 sinusoid vessels within the BM, called CXCL12 abundant reticular cells, and favors HSC 286 retention in the BM [84,97]. 287

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

the cancer cell surface. More importantly, ascites samples from patients with advanced 292 293 ovarian cancer showed significantly higher CXCL12 levels [101]. The role of the CXCL12/CXCR4 axis in the dissemination of peritoneal cancer has been clarified more 294 295 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 cells to promote spermatogonial stem-cell migration through the seminiferous tubules during 305 spermatogenesis [106–109]. Sertoli cells reside in seminiferous tubules, where leukemic cells 306 307 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 migration towards the testis might be at least partially mediated (or not exclusively) by the 311 312 CXCL12/CXCR4 axis. Moreover, McIver et al. have shown that although CXCL12 expression is restricted to Sertoli cells in the normal testis, it is expressed in the tumor stroma 313 314 of seminoma samples [107]. Pathological conditions may induce CXCL12 expression in the interstitium of the testis [111]. Thus, it is possible that leukemic cells could be attracted and 315 316 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.* away from vessel lumen) of CNS endothelial cells retains leukocytes in the perivascular 322 323 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 they could stay, possibly in a quiescent state. Indeed, recent studies in a T-ALL model have 325 326 shown that the CXCL12/CXCR4 axis plays an important role in leukemic-cell migration into the CNS [121,122]. Nevertheless, although treating xenografted mice with plerixafor (a 327 CXCR4 antagonist) reduced engraftment into the BM and liver in a B-ALL model, it failed to 328 329 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 associated with more pronounced infiltration of extramedullary tissues, such as liver and 333 334 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 chemoresistance through the CXCL12/CXCR4 axis [125]. Overall, there is evidence in the 337 338 literature showing that the CXCL12/CXCR4 axis plays a major role in ALL cell homing, both to the BM and extramedullary niches. Thus, CXCL12 represents a common feature of 339 340 hematopoietic, extramedullary, and solid tumor niches, which may reconcile ALL and solid 341 tumor models.

342

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

Low oxygen concentrations have been highlighted during cancerogenesis in solid 353 354 tumors. As reviewed elsewhere, hypoxia in cancer cells and in the tumor microenvironment 355 aberrant angiogenesis, epithelial-mesenchymal transition. radiopromotes 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].

More importantly, low oxygen concentrations are thought to be an important component of leukemic niches in the BM and recent results have shown that targeting this hypoxic microenvironment may be promising [141–148]. Indeed, hypoxia has been shown to enhance the chemoresistance of ALL cells and appears to confer stem-cell properties, such as quiescence [141,149,150]. Drugs active only under conditions of hypoxia were shown to
reduce the leukemia burden in the BM when administrated three days after ALL cell injection
into NOD/SCID mice, suggesting that hypoxia may favor leukemic-cell engraftment
[141,147].

The testes are also thought to be one of the most hypoxic tissues. Direct measurements in a rat model established the interstitial testicular oxygen concentration between 1.5 and 3.5% [151,152]. Another study using pimonidazole staining, commonly used as a hypoxic marker, confirmed these results in a mouse model and suggested an even lower intratesticular oxygen tension, especially in the seminiferous tubules, as well as the interstitium [153]. Nevertheless, there is no evidence of an association between the hypoxic features of the testis 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 evaluated the oxygen tension within the CSF, with estimated values of approximately 5 to 6% 380 381 [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 µM from the vessels, especially the narrowest ones [156]. Thus, although oxygen 383 384 concentrations have not been precisely measured in the subarachnoidal space, they are likely to be low. Recent published data have indeed suggested a role for low oxygen concentrations 385 386 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 endothelial growth factor A (VEGFA) gene, which is a principal target of the hypoxia master 389 390 transcription factor, hypoxia inducible factor 1 (HIF1). More importantly, Münch et al. further 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].

In solid tumors, SCF/c-KIT signaling is also frequently thought to participate in
tumorigenesis, tumor progression, and metastasis. Soluble SCF is produced by cancer cells

within tumor tissues in different cancer models [170]. c-KIT has been found to be 415 416 overexpressed, abnormally expressed, or associated with an aggressive phenotype in several types of tumors, including melanoma, lung cancer, renal carcinoma, and seminoma 417 418 [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 pathway may also be involved in the pathogenesis of such tumors through its effect on mast 420 421 cells [163] and the mast cell accumulation has been associated with a worse prognosis in 422 several cancer models.

The SCF/c-KIT signaling pathway has also been found to be deregulated mostly in 423 AML models rather than in ALL models. However, although c-KIT is little expressed in ALL 424 425 relative to AML, RNA-seq data indicate that some ALLs overexpress c-KIT, such as ETS variant 6-runt related transcription factor 1 (ETV6-RUNX1) B-ALL or mixed lineage 426 427 leukemia (MLL) rearranged leukemias [173]. Consistent with the expression of c-KIT in these malignancies, SCF participates in early B-cell development in association with interleukin-7 428 429 (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 pathophysiology of ALL may merit exploration, as suggested by a recent report [174]. 431

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 SCF) loci [161,163]. In the testis, SCF is produced by Sertoli cells and c-KIT is expressed on 434 435 the surface of germ cells and Leydig cells, raising the possibility that Sertoli cells secrete a soluble form of SCF [171,175,176]. Indeed, a higher percentage of mRNA for the soluble 436 437 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 adult interstitial testicular tissue has been reported both in mice and humans in several
immunohistochemistry studies, even if several others did not [178–181]. Thus, leukemia cells
may be attracted to or retained in testicular interstitial tissue at least partially through
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 development during embryogenesis [182–184]. There is no direct evidence in the literature for 445 446 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 blood-brain barrier [183]. Kallman et al. compared gene expression profiles of human 448 449 umbilical vein endothelial cells (HUVEC) and primary human cerebral endothelial cells using a cDNA array technique and found that 35 genes were differentially expressed in cerebral 450 endothelial cells, including those for interleukin-6 (IL-6), transforming growth factor $\beta 2$ 451 $(TGF-\beta 2)$, VEGFA, and SCF [183]. An enzyme-linked immunosorbent assay (ELISA) 452 453 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

The extracellular matrix protein osteopontin, which is physiologically produced in the BM, participates in the pathophysiology of various cancers, including hematological malignancies, with a potential role in leukemic cell CNS infiltration [185,186]. Colony stimulating factor 1 (CSF1), a cytokine involved in macrophage differentiation and

constitutively expressed by osteoblasts, is expressed in prepubertal and adult testicular 464 interstitial tissue, notably by macrophages, and has a role in regulating spermatogonial stem 465 cells and their niche[187-189]. CSF1 is also expressed in the CNS, where it maintains 466 microglia and participates in CNS development [190]. CSF1 deficiency impairs both 467 reproductive function and integrity of the CNS. Genetic alterations involving the CSF1 468 receptor, including the recurrent SSBP2-CSF1R fusion, have been identified in B-ALL and 469 have been found to lead to a functional fusion, suggesting that targeting CSF1 signaling could 470 be promising [191–193]. Another cytokine, transforming growth factor β 1 (TGF β 1), regulates 471 472 Leydig cell function in the testis, but is also secreted by the choroid plexus in the CSF, where it contributes to the maintenance of the choroid plexus itself and appears to be involved in the 473 474 protection and repair of nervous tissue. TGF^β1 has been shown to be involved in cancer stemness, metastasis, and epithelial-mesenchymal transition in several cancer models. 475 Recently, TGF^β1 was found to promote the escape of leukemic cells from natural killer (NK) 476 immune surveillance in an ALL model. 477

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 targets. BM and extramedullary sites may therefore contain pharmacological niches, where 480 481 leukemic cells are protected from the effects of chemotherapy. For example, mesenchymal stem cells have been shown to participate in the resistance of leukemic cells to asparaginase 482 483 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 blocking the secretion of asparagine and overcoming this resistance mechanism [195]. This 485 486 indicates that treatments may exert their effect not only by affecting leukemic cells but also their microenvironment. 487

We have shown that leukemic cells, like other cancer cells, exploit several identical features of the microenvironment to reach distant sites. We now discuss whether blast cells 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 solely by embolism, formulated his famous "seed and soil" hypothesis [196]. He compared 495 496 cancer cells to plant seeds that can only grow in favorable soil, that is, in a tissue somewhat predisposed to welcome them. At that time, he already recognized that not only cancer cells 497 498 but also their "soil" would be worth studying. Since then, a large amount of information has accumulated on cancer cell biology and the organotropism phenomenon has been 499 500 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 and different microenvironment will not perfectly match the original niche and will probably 503 504 not meet all the needs of the leukemic cells.

Indeed, there is evidence that leukemic cells can remodel their microenvironment [199]. Colmone *et al.* used an *in vivo* microscopy imaging approach and found that ALL cells remodeled the BM microenvironment they colonized, notably through SCF signaling, leading to an abnormal niche that recruited and somehow trapped normal hematopoietic progenitors [43]. More recently, Boyerinas *et al.* xenografted the leukemic Nalm6 cell line in a mouse model and showed that the leukemic cells secreted osteopontin into the invaded 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 et al. in which Nalm6 cells colonized CXCL12 niches in the BM of xenografted mice, which 520 521 subsequently resulted in marked downregulation [43,202]. A recent report showed that leukemic cells can promote osteoclast-mediated bone resorption through the production of 522 receptor activator of nuclear factor kB ligand (RANKL) [203]. This is in accordance with a 523 524 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 and endosteal lining. This report also showed that, following chemotherapy, leukemic cells 528 529 secreted cytokines, namely CCL3 and TGF^β, 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 the leukemic cells into a mature form, which led to the activation of TGF^β signaling, thereby 532 conferring chemoresistance [44]. 533

Recent studies on solid tumors have led to a better understanding of cancer-cell organotropism and metastatic niche formation. Kaplan *et al.* first demonstrated, in 2005, that 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 molecules, such as peptides or nucleic acids. Thus, exosomes are a means of intercellular 542 543 communication suitable for remote cells. Exosomes derived from different cancer cell lines 544 specifically target different tissues. An elegant experiment showed that exosomes can "educate" cancer cells to reach a specific tissue. Mice were injected with exosomes from cells 545 546 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 in lung metastases. Going further, the authors found that exosomal organotropism was 548 549 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 plasma samples of patients with pancreatic or breast cancer correlated with the location of the 553 554 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 to other ALL cells, as well as BM stromal cells, resulting in phenotypic changes in the 564 565 recipient cells [209]. The transfer of large EVs to BM stromal cells induced a metabolic change, favoring glycolysis in these cells, which increased the lactate concentration in the 566 medium, thereby providing the leukemic cells with their preferred fuel, as hypothesized by the 567 568 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 phenotypic changes in remote stromal cells to promote ALL-cell migration to the secondary 570 571 sites. Although more compelling evidence has yet to be provided, it seems likely that both exosomes and large EVs are involved in preparing a remote suitable niche for ALL cells. In 572 addition, large EVs may also contain intact organelles that could be transferred to recipient 573 cells [209]. Not all cells can generate EVs. Thus, as discussed by the authors, such 574 mechanisms may allow EV-producing resistant leukemic cells to promote chemoresistance, or 575 576 even resistance to targeted therapies, by transferring pro-resistant cellular content to other 577 sensitive cells.

The transfer of organelles, such as mitochondria, could also allow recipient cells to cope 578 with acute stress [210]. Another recent study showed that ALL cells also communicate with 579 580 mesenchymal stromal cells using tunneling nanotubes to set up their niche [211]. Tunneling nanotubes are thin plasma membrane protrusions that connect one cell to another and allow 581 582 the exchange of cellular content. This means of intercellular communication is independent of others and can promote leukemic cell survival, chemoresistance, and the release of cytokines 583 584 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 cells and *vice versa*, confirming that ALL cells establish crosstalk with the surrounding
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 cytotoxic drugs [212-215]. Such cell-cell contact was found to be mediated, notably, by the 591 592 binding of integrin $\alpha 4\beta 1$, 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 $\alpha 4\beta 1$ 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, it is likely that integrin $\alpha 4\beta 1$ binding to its receptor induces signaling cascades to promote 597 598 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– 223]. Although the mechanisms are still unclear, it appears that Cx43 gap junctions are 604 605 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 to be of crucial importance. Once in the secondary niche, leukemic cells must deal with the 613 614 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 astrocytes, choroid plexus epithelial cells, and even more strongly to meningeal cells, to 616 which they could strongly adhere. The authors showed that these cells from the CNS secreted 617 618 amounts of CXCL12 equivalent to those of BM stromal cells and osteoblasts and that CXCR4 blockade with plerixafor could inhibit ALL cell migration toward meningeal and plexus 619 620 choroid epithelial cells. They also observed that co-culture of both ALL cell lines and primary ALL cells with any of these CNS cells decreased the effects of commonly used chemotherapy 621 622 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 leukemic-supportive properties and adapt themselves to better settle into secondary niches. 627

The interconnections involved in this interplay are complex, but the methods used in 628 studies usually only focus on one aspect of one component of the leukemic 629 microenvironment. For example, cell-cell interactions have mostly been studied through co-630 culture systems consisting of only leukemic cells and one type of stromal cell, although 631 632 leukemic cells probably interact not only with supportive stromal cells but also other cells within the microenvironment. Similarly, hypoxia can induce CXCL12 and CXCR4 633 634 expression, as well as that of SCF, and SCF itself has been found to increase HIF1 α expression [226]. Leukemic cells may escape the effects of therapy by taking advantage of 635

this system, of which the complexity is even more pronounced if we consider otherparameters, such as temporality.

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

An intriguing question is whether the cells found far from their original niche are the 639 640 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 before diagnosis. Recently, Williams et al. addressed this question in the context of B-ALL 642 643 and found that most leukemic cells had the capacity to infiltrate the CNS and were not restricted to a specific population [69]. They demonstrated, in clonal tracking experiments, 644 645 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 welladapted for invading the CNS microenvironment and surviving there. They also did not 649 650 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 embryonic or fetal hematopoiesis and would stay in a dormant state in the CNS. 652

Similarly, solid tumor cells can settle at tissues distant from their original site early during the course of the disease, even before detection of the primary tumor [227,228]. Solid cancers, as well as leukemia, must be viewed as a systemic disease, in which cancer cells spread and engraft early at predisposed tissues that provide protection against insults, such as chemotherapy, notably through the induction and maintenance of a dormant state [227,229]. However, as metastasis is an inefficient process, only a few cells may have the ability to survive and grow out of their original niche [230]. Such rare cells would be able to adapt themselves to a new microenvironment, where they could become quiescent or grow slowly, explaining why some patients experience very late relapses in distant tissues. The existence of leukemic stems cells (LSCs) that would be responsible for late relapses may also be evoked. However, these LSCs are not well characterized, especially in B-ALL, and several reports indicate that ALL cells with the capacity to initiate leukemia are relatively frequent [231, Le viseur 2008]. We have therefore chosen to focus on the role of the microenvironment, which 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 xenografts to provide evidence that such dormant cells exist in ALL and provided a robust 668 669 preclinical model [45]. The authors found that these dormant cells shared a similar transcriptional program with leukemic cells found at MRD level in patients. Moreover, these 670 671 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 two principal clinically relevant implications: first, the dormant phenotype that appears to be 673 harbored by MRD cells is reversible and second, the microenvironment appears to play a 674 675 critical role, strongly suggesting that targeting the microenvironment could be a promising strategy to kill the disease and prevent relapse and treatment failure. As the authors state, it 676 may also explain why children treated for ALL benefit from standard maintenance therapy 677 678 that continues for two years from diagnosis: daily doses of low-dose chemotherapy might help to progressively eradicate all ALL cells, including the dormant cells that may reenter the cell 679 680 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 in other tissues. It would be informative to use their model to verify whether such dormant 682 683 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

extramedullary origin for combined ALL relapses, especially late relapses [59], as thedormant ALL cells were shown to have leukemia-initiating properties.

687 V. From bench to bedside

Leukemic cells interact dynamically with their microenvironment. Indeed, intravital 688 689 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 burden and induced an increase in the proportion of quiescent cells, but did not modify the 693 694 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 cells. This was recently corroborated by two independent studies [69,231,232]. In these 698 699 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 in one site over another. As the authors discussed, the specific microenvironment may confer 707 708 an advantage to arriving leukemic cells that favors settlement and/or the proliferation, 709 explaining the observed asymmetric distribution. Notwithstanding the evidence provided by

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 help predict CNS relapse at the initial diagnosis. A comparison of the gene expression profiles 716 717 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 for osteopontin) and SCD (coding for stearoyl-CoA desaturase) were found to be upregulated, 719 720 which was further confirmed both at the mRNA and protein levels. Retrospective flow cytometry analysis of BM samples from patients who experienced a CNS relapse showed that 721 722 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 Williams et al. could be partially explained by methodological discrepancies (mouse 726 xenograft model versus a human model). The idea that CNS tropism may be linked to a 727 728 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 t(1;19), leading to the TCF3-PBX1 fusion transcript, has been associated with an increased 730 731 risk of ALL relapse in the CNS [234,235]. This clinical finding has recently been supported by a biological explanation. It was shown that primary leukemic cells expressing the TCF3-732 733 PBX1 transcript expressed high levels of Mer tyrosine kinase, for which the signaling

pathway was further shown to be involved in ALL cell survival and chemoresistance in theCNS 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 found that fourteen genes were differentially expressed in this type of leukemia [237]. Among 739 740 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 pathogenesis of this particular B-ALL subtype [239]. We found that CD9 has a role in 742 743 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 [110]. Thus, ETV6-RUNX1 ALL relapses may be partially explained by enhanced CD9 746 747 expression, which favors blast dissemination and engraftment into the testis.

We postulate that ALL relapses occurring at specific sites could be explained by the association of both extrinsic and intrinsic factors, thus reconciling biological and clinical research findings. Specific genetic alterations may modify cell signaling pathways that could render the cells stochastically exposed to a specific microenvironment to take advantage of it and durably settle in it.

The role of the microenvironment in the pathogenesis of ALL has also been suggested by the good results obtained by treating or preventing extramedullary relapses with specifically directed local treatments. Indeed, aside from the introduction of high-dose methotrexate or aracytine, successful treatment of testicular or meningeal involvement has been specifically directed at the tumor: surgery/radiation therapy for the testis and intrathecal chemotherapy or radiation therapy for the meninges. This may explain the relatively good

33

759 prognosis for relapses at these sites and is another argument for an important role of the microenvironment, which is removed by surgery and radiotherapy. The role of the intrinsic 760 properties of leukemic cells concerning their interaction with their surrounding 761 762 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 to chemotherapy relative to other B-ALL subtypes. Within these subtypes, alteration of the 764 Ikaros gene is frequently found. This alteration was shown to alter the adhesive properties of 765 leukemic cells and may influence their settlement in their niche.[240]. 766

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 factors has not only improved their survival, but also allowed to efficiently treat 771 extramedullary leukemia without using local treatments. As mentioned earlier, the use of high 772 doses of certain drugs, such as methotrexate, reduced the incidence of extramedullary 773 774 involvement. Apart from these leukemic cell-oriented targeted therapies, therapeutic strategies oriented toward leukemic niches are also attracting increasing interest [241]. Figure 3 775 776 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.

First, preventing cell migration and engraftment into the niche or, conversely, the mobilization of niched cells could be attempted, for example by disruption of the CXCL12/CXCR4 axis using CXCR4 inhibitors, such as plerixafor [105,124,242,243]. 783 Targeting cell adhesion to the extracellular matrix or supportive cells may also be a useful 784 strategy, for example, by inhibiting $\alpha 4\beta 1$ integrin-mediated adhesion with a specific monoclonal antibody, such as natalizumab, or a chemical inhibitor, such as TCB3486 785 786 [244,245]. Another possible niche-oriented therapeutic strategy could be to target integrins, since exosomes home to future metastatic sites via integrins in melanoma and lung cancer 787 models. Directly targeting integrins could thus interrupt cellular communication with the 788 789 secondary niches [206]. Targeting niche features may also be a beneficial strategy, for example by using hypoxia-activated prodrugs, such as PR104 and TH-302 [141,145-790 791 147,246]. 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 healthy microenvironment, for example by using bisphosphonates that could prevent bone 793 794 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 TGF β and HIF1 α 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 anti-programmed cell death 1 (PD-1)/PD-1 ligand 1 (PD-L1) antibodies [248,249]. 800

Many challenges have yet to be addressed concerning these and other strategies that could target malignant cells in their niches in hematological and solid cancers. Given the complexity of the microenvironment, successful treatment will probably require a combinatory approach, by associating these therapies both between themselves and with conventional chemotherapies. It is likely that such treatments would exert their full potential by adding them to chemotherapy after the initial diagnosis to prevent the occurrence of relapse. They should probably be reserved for refractory patients or those with the highest riskof relapse.

809 VII. Conclusion and future directions

Leukemic niches and their interplay with leukemic cells are of tremendous complexity, 810 811 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 nichetargeted therapeutic strategies that could be considered in the near future. We chose to 813 814 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 can reach secondary sites and settle down, where they can remain dormant for years before 819 initiating a relapse. Although some intrinsic factors may predispose cells to invade one tissue 820 821 over another, the microenvironment undoubtedly plays an important role in leukemic cell 822 engraftment, making niche-based targeted therapies promising and worth developing. However, given the complexity of the niches and the dynamic interconnections between each 823 824 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.

828 VIII. Practice points

ALL cells appear to localize preferentially in perivascular regions of the tissues they
 colonize.

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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1584

1585 **Conflict of Interest Statement**

1586 All authors declare to have no conflict of interest to disclaim.

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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 alterations to give rise to leukemic "ancestral clones". Such clones may be present before 1596 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 clone, which may have evolved on their own (situations A and E). 1605





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



Figure 3. Potential therapeutic strategies to target the leukemic niche. The leukemic niche is a highly complex microenvironment made of many components or aspects that could be targeted. Potential niche-based treatments are represented by the darts, which could be used simultaneously to target different components, as symbolized by the blurry delineations on the target.

1644 TABLE LEGENDS

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 (<i>in vitro</i> 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 (<i>in vitro</i> study)	Plerixafor, TC14012	NA	Reduced chemotaxis of B-ALL cells Reduced proliferation of B-ALL cells cultured on stromal BM cells

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
					Augmentation of inhibitory effects of chemotherapy
Integrin alpha4	Hsieh et al. 2013 [245]	Preclinical (<i>in vitro</i> 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 (<i>in vitro</i> and animal study)	TBC3486	NA	Reduced chemoresistance of B- ALL cells Prolonged survival of xenografted mice Synergistic effects with chemotherapy <i>in vivo</i>
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 (<i>in vitro</i> and animal study)	TH-302 (evofosfamide)	NA	Cytotoxicity on B-ALL cells in hypoxic culture conditions Not tested in an <i>in vivo</i> 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 (<i>in vitro</i> 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 (<i>in vitro</i> 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) administrated 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

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

- 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