## Acute lymphoblastic leukemia cells are able to infiltrate the brain subventricular zone stem cell niche and impair neurogenesis

In pediatric acute lymphoblastic leukemia (ALL), the most common hematologic malignancy in childhood, central nervous system (CNS) relapse is a major clinical problem, accounting for about one-third of the relapses.[1] Since the early autopsy studies, CNS leukemia has been described primarily as a leptomeningeal disease which can be accompanied by infiltration of different brain parenchyma areas by ALL cells.[2,3] The CNS is therefore considered to act as a sanctuary for ALL, but the specific neural microenvironments in which leukemic cells can stay for prolonged periods of time as extramedullary minimal residual disease and be responsible for CNS relapses are still poorly defined. Recently, we and others have reported that the choroid plexus stroma and the leptomeningeal stromal cell network are two of those neural microenvironments able to lodge ALL cells and promote their survival and acquisition of quiescence and chemoresistance.[4,5]

Neurogenic niches, such as the subventricular zone (SVZ) and the subgranular zone, are areas of the brain in which neurogenesis takes place throughout life. Concretely, the SVZ is located along the walls of the lateral brain ventricles and represents the largest neurogenic niche in the postnatal and adult mammalian brain. Neural stem cells residing in the SVZ divide slowly and give rise to rapidly proliferating cells, called transit amplifying progenitors, which then differentiate to neuroblasts. These immature neuronal progenitors further migrate along a pathway, called the rostral migratory stream, towards the olfactory bulb where they differentiate into olfactory interneurons and integrate in the existing neuronal circuitry involved in odor discrimination.[6] Nevertheless, the neurogenic niches, apart from their ability to support and maintain neural stem cells, can also serve as refuge for neoplastic cells, and the migratory mechanisms of neural stem cells can be utilized by tumor cells;[7] therefore, in this study we investigated whether the SVZ can also harbor ALL cells.

A xenograft model of non-obese diabetic/severe combined immunodeficiency/IL-2R$\gamma^{null}$ (NSG) mice injected with the Nalm-6 human pre-B ALL cell line was used to analyze the presence of leukemia cells in the brain SVZ. When symptoms of CNS involvement (such as hind limb paralysis) appeared, animals were deeply anesthetized and transcardially perfused immediately before the SVZ were carefully dissected and dissociated. Flow cytometry analysis of cells recovered from dissociated SVZ revealed that all mice with leukemic infiltration in the CNS showed CD19$^+$ human blasts in this brain location (Figure 1A), and these ALL cells could represent up to 30% of total leukemic cells invading the nervous parenchyma (Figure 1B). Similar results were obtained

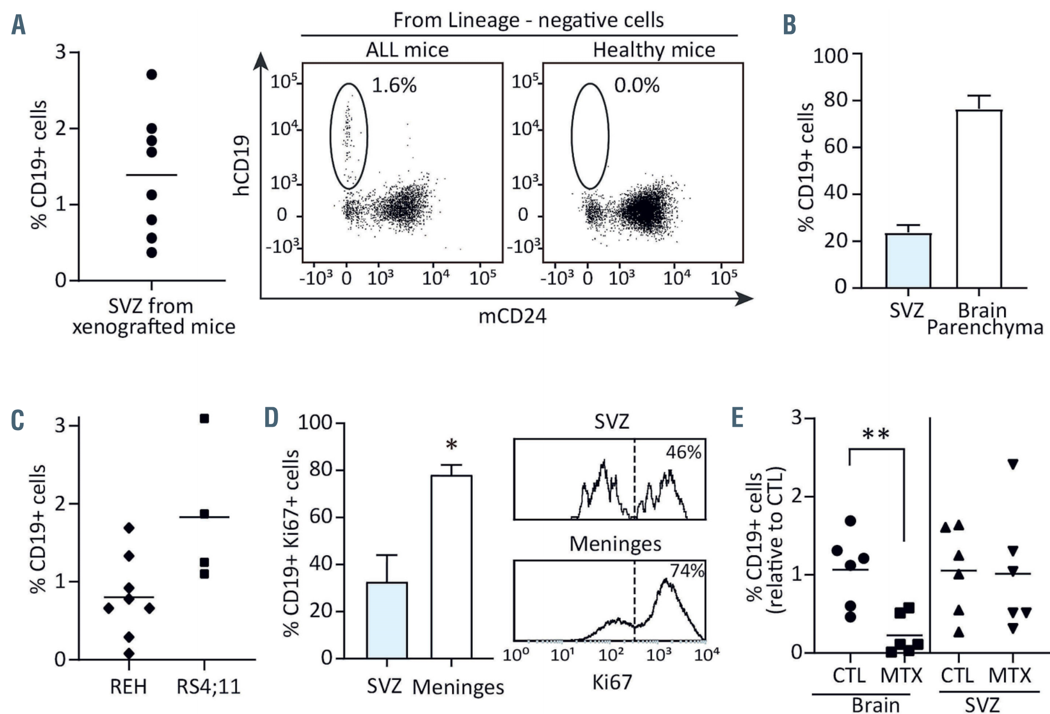


Figure 1. Acute lymphoblastic leukemia cells infiltrate the subventricular zone neurogenic niche. (A) Percentage of CD19$^+$ leukemia cells detected in subventricular zones (SVZ) from mice xenografted with pre-B acute lymphoblastic leukemia (ALL) (n=8). When disease symptoms were evident, mice were deeply anesthetized and transcardially perfused with cold 0.1 M phosphate-buffered saline (pH 7.4) to clear circulating leukemia cells prior to euthanasia. Brains were then removed, washed several times with cold Dulbecco phosphate-buffered saline and SVZ were carefully microdissected, dissociated and processed for flow cytometry. Representative dot plots show, after gating out the non-neurogenic cell populations, the expression of human CD19 (hCD19) *versus* murine CD24 (mCD24), a neuroblast cell marker, in SVZ from leukemic and healthy mice. (B) Percentages (mean ± standard deviation [SD]) represent the leukemic cells present in the SVZ or the brain parenchyma out of total CD19$^+$ cells infiltrated into the brain. (C) Proportion of CD19$^+$ leukemia cells detected by flow cytometry in SVZ from mice xenografted with ALL cell lines REH and RS4;11 (n=4-8). (D) Percentages of CD19$^+$ leukemia cells expressing Ki-67 in the SVZ and meninges from xenografted mice (n=3). Representative histograms show the expression of Ki-67 antigen in CD19$^+$ leukemic cells found in the SVZ and the meninges (**P*≤0.05; *t*-test). (E) Mice were injected with Nalm-6 cells and after successful engraftment and randomization, the leukemic mice were intraperitoneally treated with methotrexate (5 mg/kg) or saline twice a week for 4 weeks (n=6). Percentages of CD19$^+$ cells present in the SVZ and the rest of the brain, including the meninges, were determined by flow cytometry (***P*≤0.01; *t*-test).

with mice xenografted with other ALL cell lines, REH and RS4;11 (Figure 1C). However, no correlation was seen between the degree of leukemic infiltration in brain parenchyma and the proportion of ALL cells present in the SVZ. Immunofluorescence studies in brain cryosections from xenografted mice showed that leukemic cells could be seen, apart from in the SVZ niche, also along the rostral migratory stream (*Online Supplementary Figure S1*). These data indicate that the SVZ can provide a favorable microenvironment in which ALL cells can survive and be maintained over time. Supporting this, the study of the leukemia proliferation rate using Ki-67 staining showed that ALL cells found in the SVZ niche exhibit a much lower proliferative activity than those leukemic cells isolated from the meninges (Figure 1D). Furthermore, leukemic cells infiltrating the SVZ niche were shown to have higher chemoresistance after methotrexate treatment of xenografted mice (Figure 1E).

The above results showed that leukemic invasion of the SVZ neurogenic niche is a common event in the xenograft model of ALL, so we analyzed the effects of this infiltration on the differentiation of neural stem cells. The proportion of the different SVZ populations was determined by flow cytometry using a combination of multiple specific cell markers, as we previously described.[8] Non-neurogenic cells were first discarded from the study, and the remaining neurogenic lineage cell pool was subdivided according to the expression of the glial marker GLAST, the neuroblast marker CD24, the tetraspanin CD9 and the proliferation-associated receptor EGFR. The population of neural stem cells was defined as $GLAST^{+}CD24^{-/low}CD9^{high}$ and further classified by EGFR expression and GLAST intensity into quiescent, primed quiescent and activated neural stem cells. Transit amplifying progenitors were defined as $GLAST^{-}CD24^{-/low}EGFR^{+}$ cells, and the $GLAST^{-}CD24^{high}$ population included $EGFR^{+}$ proliferating (NB1 or early) and $EGFR^{-/low}$ migrating (NB2 or late) neuroblasts. As can be seen in Figure 2A, the percentage of total neural stem cells was notably increased in xenografted mice, with quiescent neural stem cells being the main subset responsible for this rise. Concomitantly, the proportion of transit amplifying progenitors and late neuro-blasts was reduced in these animals. These results suggest that SVZ neurogenesis is impaired in leukemia-bearing mice at the expense of an increase in quiescence, and the effect appears to be a direct consequence of the leukemic cell infiltration in the SVZ since the most affected animals were those showing the highest numbers of $CD19^{+}$ cells in the neurogenic niche. Figure 2B shows that the percentages of $CD19^{+}$ leukemia cells correlated directly with the accumulation of quiescent neural stem cells, and inversely with the proportions of late neuroblasts (Figure 2C). In line with these data, and since the generation of new olfactory bulb neurons from the SVZ is required for novel odor discrimination,[9] xenografted mice displayed altered olfactory discrimination capacities (Figure 2D).

To analyze the effects of leukemia on neural precursors directly, we first generated SVZ neurospheres, floating cellular aggregates clonally derived from neurosphere-ini-

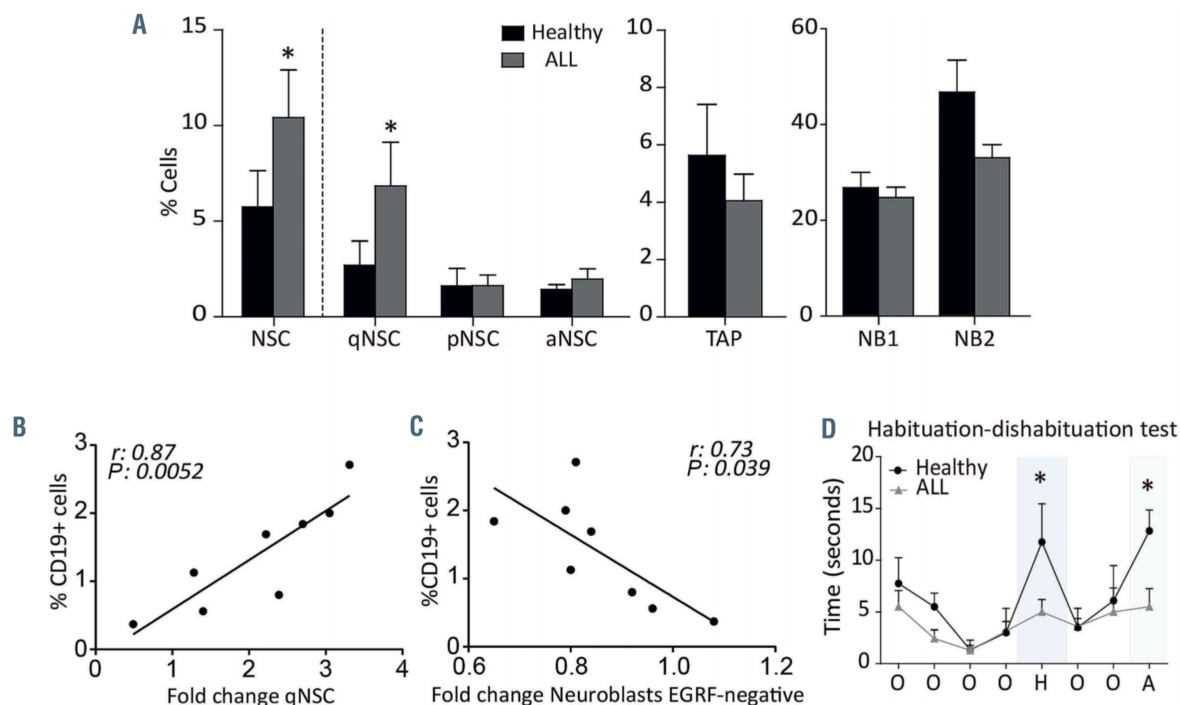


Figure 2. Subventricular zone cell populations are affected by the presence of acute lymphoblastic leukemia cells. (A) Bars represent the percentages (mean ± standard deviation [SD]) of total, quiescent (q), primed quiescent (p) and activated (a) neural stem cells (NSC), as well as transit amplifying progenitors (TAP) and proliferating (NB1) and migrating (NB2) neuroblasts present in the subventricular zones (SVZ) from leukemic (gray) and healthy (black) mice (n=8). All these neurogenic cell populations were defined according to the expression of the glial marker GLAST, the neuroblast marker CD24, the tetraspanin CD9 and the proliferation-associated receptor EGFR (*$P \leq 0.05$; *t*-test). (B,C) The percentages of $CD19^{+}$ cells found in the SVZ are represented as a function of the corresponding (B) increases in the proportion of quiescent NSC and (C) decreases in the proportion of migrating EGFR- neuroblasts. *P* values of the Pearson correlation are provided. (D) Olfactory habituation-dishabituation tests of healthy (black circles) and leukemic (gray triangles) mice were performed at week 3, before the typical disease symptoms (including rough hair, lethargy, hunched-back posture, loss of motor functions and hind limb paralysis) were observed. Exploration time (in seconds) of successive cotton swabs soaked in octanal (O), heptanal (H), or anisole (A) is shown. After exposure to octanal-soaked swabs, healthy mice reacted to heptanal- and anisole-soaked swabs; however leukemic mice displayed lower olfactory exploration and no reaction to the new odor stimuli. Asterisks represent statistically significant differences (*$P \leq 0.05$; *t*-test). ALL: acute lymphoblastic leukemia.

tiating neural stem cells which constitute an ideal system to evaluate modifications in proliferation and self-renewal. Single cells dissociated from neurospheres were either cultured with medium conditioned by leukemic cells or co-cultured with ALL cells using transwell inserts. In both cases, although no change in the number of new neurospheres was found after 10 days (Figure 3A), a significant reduction in neurosphere sizes could be clearly observed (Figure 3B), suggesting that leukemia-derived factors limit growth but not survival of neurosphere cells. In agreement, the inhibition of the expansion potential of neurospheres in the presence of leukemia cells was detected throughout the culture period using MTS proliferation assays (Figure 3C). To analyze whether ALL-mediated effects included effects on self-renewal, cells obtained from neurospheres that had been grown in the presence of soluble factors secreted by leukemic blasts were re-plated in fresh growth medium without leukemia-derived factors and neurosphere formation was evaluated. In these cultures, the numbers of secondary neurospheres were not altered but significant changes in sphere diameters could be newly detected, indicating that leukemia cells can reduce activation without altering self-renewal (Figure 3D, E).

ALL cells have been reported to be able to induce a pro-inflammatory microenvironment in different locations.[4,10] The expression of pro-inflammatory factors was therefore analyzed in the SVZ of healthy and leukemic mice. As shown in Figure 3F, the levels of IL-1β, IL-6 and TNF-α cytokines as well as CCL2 and CXCL10 chemokines were notably upregulated in the leukemia-invaded SVZ niches. All these inflammatory mediators have been described as negative regulators of neurogenesis.[11] However, we have recently reported that TNF-α, which

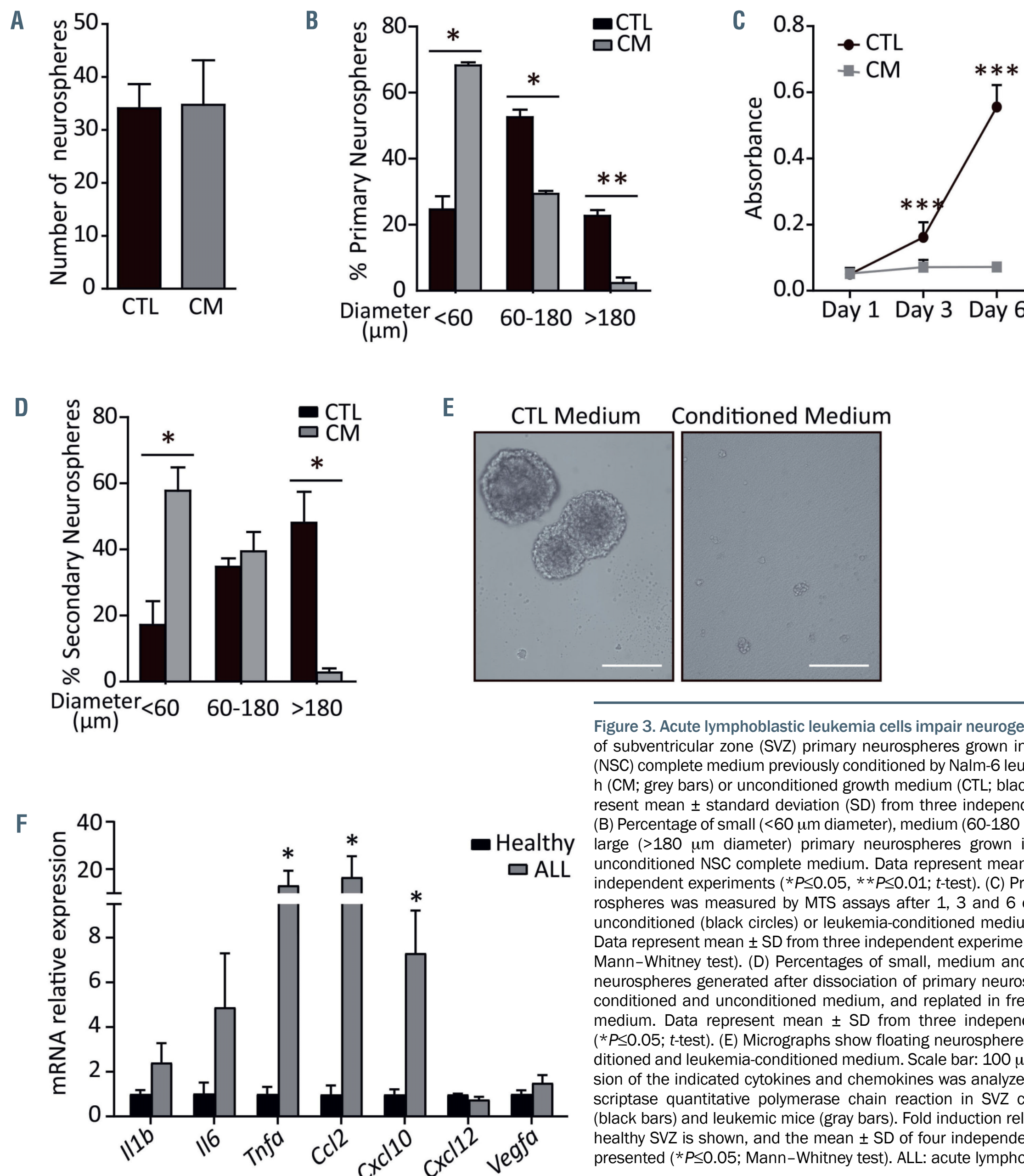


**Figure 3. Acute lymphoblastic leukemia cells impair neurogenesis.** (A) Number of subventricular zone (SVZ) primary neurospheres grown in neural stem cell (NSC) complete medium previously conditioned by Nalm-6 leukemic cells for 48 h (CM; grey bars) or unconditioned growth medium (CTL; black bars). Data represent mean ± standard deviation (SD) from three independent experiments. (B) Percentage of small (<60 μm diameter), medium (60-180 μm diameter) and large (>180 μm diameter) primary neurospheres grown in conditioned or unconditioned NSC complete medium. Data represent mean ± SD from three independent experiments (*$P \leq 0.05$, **$P \leq 0.01$; *t*-test). (C) Proliferation of neurospheres was measured by MTS assays after 1, 3 and 6 days of culture in unconditioned (black circles) or leukemia-conditioned medium (gray squares). Data represent mean ± SD from three independent experiments (***$P \leq 0.001$; Mann–Whitney test). (D) Percentages of small, medium and large secondary neurospheres generated after dissociation of primary neurospheres, grown in conditioned and unconditioned medium, and replated in fresh NSC complete medium. Data represent mean ± SD from three independent experiments (*$P \leq 0.05$; *t*-test). (E) Micrographs show floating neurospheres grown in unconditioned and leukemia-conditioned medium. Scale bar: 100 μm. (F) The expression of the indicated cytokines and chemokines was analyzed by reverse transcriptase quantitative polymerase chain reaction in SVZ cells from healthy (black bars) and leukemic mice (gray bars). Fold induction relative to cells from healthy SVZ is shown, and the mean ± SD of four independent experiments is presented (*$P \leq 0.05$; Mann–Whitney test). ALL: acute lymphoblastic leukemia.

underwent one of the highest increases in expression, reduces neuroblast generation because it induces a transient activation of neural stem cells followed by their entry into quiescence.[8] Transgenic mice overexpressing IL-6 in astrocytes exhibit reduced cycling of neural stem cells in the subgranular zone niche, suggesting that it acts as a negative regulator of proliferation,[12] and IL-1β is reportedly secreted by choroid plexus cells to the cerebrospinal fluid and induces the upregulation of VCAM-1 levels in SVZ neural stem cells, reducing their proliferation and preventing lineage progression.[13] Importantly, the levels of IL-1β, IL-6, TNF-α, CCL2 and CXCL10 have also been described to be increased in blood and cerebrospinal fluid of ALL patients, promoting the survival and quiescence of leukemic cells.[14,15]

Taken together, the results of the present study show that infiltration of the SVZ may be a common event in childhood ALL with CNS involvement, suggesting that SVZ is a sanctuary in which ALL cells could lodge, survive for prolonged periods of time and be responsible for future CNS relapses. Our results also show that leukemic infiltration of the SVZ neurogenic niche impairs neurogenesis, which likely leads to deleterious effects on brain functions. It is important to note that in human infants and young children not all neuroblasts born in the SVZ migrate to the olfactory bulb, but many of them migrate into the ventromedial prefrontal cortex as well as multiple regions of the frontal cortex, such as the cingulate gyrus.[16,17] The late incorporation of inhibitory interneurons into those regions of the developing human brain has been proposed to constitute a mechanism of delayed postnatal plasticity and, therefore, injuries affecting neuronal recruitment during this period could contribute to neurocognitive deficits and sensorimotor disturbances,[16,17] such as those reported in ALL patients at diagnosis, before treatment initiation.[18]

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