Role of CXCR4-mediated bone marrow colonization in CNS infiltration by T-cell acute lymphoblastic leukemia

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ABSTRACT

Infiltration of the central nervous system is a severe trait of T-cell acute lymphoblastic leukemia. Inhibition of CXC chemokine receptor 4 significantly ameliorates T-cell acute lymphoblastic leukemia in murine models of the disease; however, signaling by CXC chemokine receptor 4 is important in limiting the divagation of peripheral blood mononuclear cells out of the perivascular space into the central nervous system parenchyma. Therefore, inhibition of CXC chemokine receptor 4 potentially may untangle T-cell acute lymphoblastic leukemia cells from retention outside the brain. Here, we show that leukemic lymphoblasts massively infiltrate cranial bone marrow, with diffusion to the meninges without invasion of the brain parenchyma, in mice that underwent xenotransplantation with human T-cell acute lymphoblastic leukemia cells or that developed leukemia from transformed thymocytes. We tested the hypothesis that T-cell acute lymphoblastic leukemia leukemogenesis results from meningeval infiltration through CXC chemokine receptor 4-mediated bone marrow colonization. Inhibition of leukemia engraftment in the bone marrow by pharmacologic CXC chemokine receptor 4 antagonism significantly ameliorated neuropathologic aspects of the disease. Genetic deletion of CXCR4 in murine hematopoietic progenitors abrogated leukemogenesis induced by constitutively active Notch1, whereas lack of CCR6 and CCR7, which have been shown to be involved in T-cell and leukemia extravasation into the central nervous system, respectively, did not influence T-cell acute lymphoblastic leukemia development. We hypothesize that lymphoblastic meningeval infiltration as a result of bone marrow colonization is responsible for the degenerative alterations of the neuroparenchyma as well as the alteration of cerebrospinal fluid drainage in T-cell acute lymphoblastic leukemia xenografts. Therefore, CXC chemokine receptor 4 may constitute a pharmacologic target for T-cell acute lymphoblastic leukemia neuropathology. J. Leukoc. Biol. 99: 000–000; 2016.

Introduction

T-ALL arises from the malignant transformation of thymocytes, and it accounts for 10–15% of childhood and 25% of adult acute lymphoblastic leukemia cases. At diagnosis, patients with T-ALL display such clinical signs as high WBC counts, mediastinal masses, hepatosplenomegaly, and CNS involvement. Abrupt activation of transcription factors involved in T-cell differentiation is a critical transforming event in human T-ALL. This results in interference with transcriptional programs that control T-lymphocyte development, which leads to stage-specific leukemic arrest [1]. Transcriptional regulation by Notch1 has an essential role in T-cell development in lymphoid progenitors in the thymus [2]. The majority of human T-ALLs has activating mutations in the NOTCH1 gene [3]. In mice, ligand-independent activation of Notch signaling in lymphoid progenitors promotes the development of immature T-lymphoblastic neoplasia that has the characteristics of human T-ALL [4]. In recent years, a number of factors that contribute to the malignant phenotype of transformed thymocytes has been defined [5, 6].

The propensity of leukemic cells to invade the CNS is a characteristic feature that severely affects the prognosis of patients with T-ALL. In fact, CNS-directed therapy, including cranial irradiation, intrathecal chemotherapy, and systemic administration of CNS-penetrating chemotherapeutics, has

Abbreviations: BBB = blood-brain barrier, BM = bone marrow, CD = cluster of differentiation, EGFP = enhanced GFP, FLP = fetal liver progenitor, ICN1 = intracellular Notch1, NSG = nonobese diabetic/severe combined immunodeficiency/Ly-5c chain null mice, T-ALL = T-cell acute lymphoblastic leukemia, WT = wild-type

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reduced the frequency of disease recurrence that originates in the CNS from 50 to 5% of patients [7-10] and dramatically improved patient survival. This evidence suggests that occult CNS involvement could occur in the majority of patients, and factors that contribute to neural tropism of leukemic cells represent important therapeutic targets. The mechanisms of CNS infiltration by T-ALL cells are not completely understood.

Leukemic cells may enter the subarachnoid space from the BM of the skull via the bridging veins or from the cerebrospinal fluid via the choroid plexus; may invade cerebral parenchyma via brain capillaries; or may infiltrate the meninges via bony lesions of the skull. Moreover, T-ALL cells can be seeded into the CNS by traumatic lumbar puncture [11].

Xenotransplantation of human T-ALL cells in immunodeficient NSG mice provokes a disease with neurologic symptoms determined by the infiltration of the meninges by leukemic cells. In this experimental model, we observed massive colonization of the skull BM by leukemic cells, with secondary extension and infiltration of the adjacent soft tissues that appeared to be almost entirely restricted to meninges without any direct involvement or infiltration of the underlying nervous tissues. In the BM, the stimulation of CXCR4 by CXCL12 produced by stromal cells is important for the maintenance of hematopoietic progenitors [12] as well as of leukemic cells [13-15]. Therefore, to address whether BM infiltration might constitute a prerequisite for CNS pathology in T-ALL, we targeted CXCR4 in adoptive transfer experiments of T-ALL in NSG mice. This article shows that CXCR4 activity is crucial for the homing of T-ALL to the BM, disease development, and meningeal infiltration by leukemic cells.

MATERIALS AND METHODS

Cell lines

The PHOENIX cell line was cultured in DMEM 4.5% glucose, 10% FBS, MEM nonessential amino acids, 20 μM 2-ME, penicillin-streptomycin, and Glutamax (Gibco, Grand Island, NY, USA). Jurkat and CEM cells were cultured in RPMI 1640 with HEPES, 10% FBS, MEM nonessential amino acids, Glutamax (Gibco, Grand Island, NY, USA). Jurkat and CEM cells were transduced by luciferase-expressing Jurkat cells i.p. injected with 100 μl Luciferin (PerkinElmer), and after 5 min, outcome images were captured with the In Vivo Imaging System. FITC and biotin-labeled anti-human CD45 mab (clone HI30; Invitrogen, Carlsbad, CA, USA) were used for flow cytometry. Fluorochrome-conjugated streptavidin (eBioscience, San Diego, CA, USA) revealed biotin-conjugated mAbs. Samples were acquired and analyzed as described above.

Retroviral transduction of hematopoietic progenitors

The recombinant retrovirus MigRI that expressed constitutively active Notch1, together with EGFP and the EGFP-only vector, were provided by Warren S. Pear (University of Pennsylvania, Philadelphia, PA, USA). Bacterial strain JM109 was used for plasmid amplification. Retroviral supernatants were produced by transient transfection of the PHOENIX cell line and were concentrated by ultracentrifugation (16 h at 4°C at 6000 g). Targeted disruption of either CXCR4 or its ligand CXCL12 is embryonically lethal; therefore, we transduced FLPs from E14 WT mice with the Spectrum Preclinical In Vivo Imaging System. FITC and biotin-labeled anti-human CD45 mab (clone HI30; Invitrogen, Carlsbad, CA, USA) were used for flow cytometry. Fluorochrome-conjugated streptavidin (eBioscience, San Diego, CA, USA) revealed biotin-conjugated mAbs. Samples were acquired and analyzed as described above.

Patient samples and xenografts

We analyzed 18 primary human BM cell specimens in 32 xenografts. Samples were obtained from pediatric patients with newly diagnosed T-ALL (Table 1), with written informed consent obtained from the parents or legal guardians in accordance with the Declaration of Helsinki and the approval of the institutional ethics committee. For establishment of xenografts at Istituto Oncologico Veneto, 6- to 9-wk-old NSG mice were i.v. or intratibially injected with 10^7 T-ALL cells in 300 μl Dulbecco PBS. Procedures involving animals and their care were performed with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, December 12, 1987). T-ALL engraftment was monitored with periodic blood draws and flow cytometric analysis for CD5 and CD7 markers during a 3-mo period. Mice were sacrificed when > 40% of cells in peripheral blood tested positive for human CD7 or CD5 (humane end point) [17].
Histochemistry and histopathologic scoring

The entire mouse head was collected and postfixed in 4% paraformaldehyde for 1 wk at 4°C. After complete fixation, samples were decalcified in 14% tetrasodium EDTA for 10 d before processing and paraffin embedding (Thermo Scientific Excelsior AS Tissue Processor and HistoStar Embedding Workstation; Thermo Fisher Scientific, Waltham, MA, USA). Five-micrometer-thick serial coronal sections were obtained from paraffin-embedded heads, and cervical and thoracolumbar segments of the spine were sectioned sagittally (Thermo Scientific Microm HM555S microtome; Thermo Fisher Scientific). Comparable anatomic landmarks (i.e., lower incisor teeth, eyes, and external ear canal) were used as references for coronal head sectioning. Sections were mounted on Superfrost Plus Adhesion slides (Thermo Fisher Scientific) and routinely stained with H&E (Diapath, Martinengo, BG, Italy) or immunostained with antibodies raised against GFP (ab5456; Abcam, Cambridge, MA, USA) by using an automated Ventana Discovery Ultra platform (Ventana Medical Systems, Tucson, AZ, USA). The extent and the distribution of lymphomatous infiltrates in different regions of the head and spine were scored by using the histopathologic criteria listed below. Microscopic assessment was performed in a blind fashion, and details concerning the experimental design were revealed only at the end of the analysis.

The following values were used to establish the infiltration score: BM: 0 = no invasion/colonization, 1 = focal or multifocal invasion/colonization with partial effacement of preexisting hematopoietic population, 2 = diffuse invasion with almost complete or complete effacement of preexisting hematopoietic population and focal disturbance of bony encasement, and 3 = diffuse invasion with extensive effacement of bony encasement; dental alveoli: 0 = no invasion, 1 = focal or multifocal invasion with partial effacement of periodontal ligament and dental pulp, 2 = diffuse invasion with almost complete or complete effacement of periodontal ligament and dental pulp, and 3 = diffuse invasion with complete effacement and disruption of the alveolar socket; oral and rhinopharyngeal mucosa: 0 = no invasion, 1 = focal or multifocal infiltration and expansion of lamina propria and submucosa, 2 = diffuse infiltration and expansion of lamina propria and submucosa, and 3 = diffuse infiltration and expansion of lamina propria and submucosa with disruption of mucosal architecture and multiple infiltrative extension into the surrounding soft tissues; middle ear: 0 = no invasion, 1 = focal or multifocal infiltration of dura mater with partial expansion and obliteration of arachnoid space, 2 = segmental to diffuse infiltration of dura mater with extensive expansion and obliteration of arachnoid space, and 3 = diffuse infiltration of dura mater with extensive expansion and obliteration of arachnoid space and focal/multifocal compression and degeneration of the subjacent neuroparenchyma.

Statistical analysis

All experiments have been repeated ≥ 2 times with ≥ 4 mice per experimental condition (the number of mice used in the representative experiments is indicated in figure legends). Student’s t test and ANOVA were used to determine the significance of difference between mean values. Data are displayed with standard deviations. Prism 5 (GraphPad Software, La Jolla, CA, USA) was used to perform Pearson correlation coefficient and Mantel-Cox log-rank test. Values of P < 0.05 were considered significant.
assess the relevance of BM infiltration by T-ALL cells as a prerequisite for meningeal infiltration.

**Pharmacologic antagonism of CXCR4 ameliorates the neurologic phenotype of NSG mice engrafted with T-ALL cell lines**

We implanted osmotic pumps that delivered CXCR4 antagonist AMD-3100 into the peritoneum of NSG mice, which concomitantly underwent i.v. transplantation with Jurkat cells. The results of this experiment were quite impressive. We observed significant differences in body weight, a dramatic amelioration of neurologic symptoms (Fig. 3A), and a reduction of BM colonization both in flow cytometry and in live animal bioluminescent imaging (Fig. 3B and C). Leukemic cells that infiltrated the BM and meninges in the head were significantly reduced by the administration of AMD-3100 (Fig. 3D). Analogous results were obtained with CEM cells (data not shown).

Histopathologic analyses of mice that were injected with Jurkat cells revealed the relative persistence of BM encasement in mice treated with AMD-3100, with few clusters of leukemic cells evident in the BM without invasion of the meninges. In contrast, PBS-treated mice showed massive BM colonization by leukemic cells, with diffuse infiltrates, disruption of the bony encasement, and invasion of the meninges (Fig. 3E). These results suggest that pharmacologic CXCR4 antagonism preserves the integrity of skull bones and obviates meninges from leukemic infiltration. To see whether inhibition of T-ALL homing to the BM by CXCR4 antagonism could also be effective in CNS disease after disease engraftment, we implanted osmotic pumps that delivered the drug one week after T-ALL cells were injected, when leukemia was undetectable in the blood and represented 0.5% of BM cells. In this experimental setting, we also observed a dramatic amelioration of clinical parameters, including weight loss and neurologic score, as well as neuropathology by AMD-3100 administration (data not shown) [14].

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**Figure 1. Leukemia development in NSG mice injected with Jurkat cells.** (A) Weight variation over time and clinical scores (Materials and Methods) in NSG mice injected with saline or Jurkat cells (mock, n = 4; Jurkat, n = 7). (B) Statistical analysis of the recovery of T-ALL cells from different organs by using FACS in the same mice. (C) Histopathology (H&E staining) of the head showing dense infiltrates of leukemic cells with disruption of frontal bony encasement and invasion of the meninges covering the olfactory bulbs (right). (D) Histopathology (H&E staining) of the head showing dense infiltrates of leukemic cells that, from the BM of the intraparietal bone, disrupt the bony encasement and invade the meninges covering the cerebellum (right). (E) Histopathology (H&E staining) of the head showing dense infiltrates of leukemic cells that, from the BM of the hyoid bone, disrupt the bony encasement and invade the peripharyngeal soft tissues (right). Left panels show comparable regions from unaffected mice (MOCK). cf = cerebellar folia, fb = frontal bone, hb = hyoid bone, ib = intraparietal bone, li = leukemic infiltrates, ob = olfactory bulb, ph = pharyngeal cavity, sg = salivary gland, sm = skeletal muscle. *P < 0.05; ***P < 0.001; ****P < 0.0001. Scale bar = 120 μm (C), 60 μm (D), and 60 μm (E).
Intra-BM engraftment does not rescue leukemia by ICN1-transformed CXCR4<sup>−/−</sup> hematopoietic progenitors

We infected hematopoietic FLPs of WT and CXCR4<sup>−/−</sup> embryos with a retrovirus that encoded ICN1, then i.v. injected the transduced cells into NSG mice. These experiments showed a dramatic infiltration of the BM, spinal cord, and spleen by engraftment of T-ALL cells that were generated by transduction of WT progenitors. In contrast, ICN1-transduced CXCR4-deficient cells were in no case detectable (Fig. 4A). To address whether the defective leukemogenesis observed with ICN1-transduced CXCR4<sup>−/−</sup> cells was dependent on impaired homing to the BM, we injected ICN1-transduced FLPs directly into the tibia. Whereas we observed extensive infiltration of the BM, spinal cord, and spleen by WT T-ALL cells in all reconstituted hosts, ICN1-transduced CXCR4-deficient cells were not detectable (Fig. 4A). Histologic analysis of the skull did not reveal leukemic infiltrates, either in the BM or meninges by transformed CXCR4-deficient cells that were i.v. or intratibially injected. In contrast, leukemic infiltrates largely replaced hematopoietic BM in the cranial diploe and expanded into the underlying meninges in mice engrafted with ICN1-transduced WT FLPs (Fig. 4B). Further analysis with anti-GFP antibodies to punctiliously reveal ICN1-transduced cells in intratibially injected mice failed to reveal leukemia in mice transplanted with CXCR4<sup>−/−</sup>-transduced cells, whereas leukemic infiltrates that expressed GFP were detected in dura/arachnoid and pia mater in animals that were colonized by transformed WT FLPs (Fig. 4C). No signs of disease were detected in mice transferred with CXCR4<sup>−/−</sup>-transduced progenitors, regardless of the site of injection. These experiments indicate that CXCR4 is required both for BM engraftment and for CNS infiltration by T-ALL cells. Compared with i.v. injection, intratibial injection significantly anticipated disease development by WT ICN1-transduced cells (Fig. 4D). To further address whether T-ALL engraftment into the BM could be an essential step in leukemia development, we analyzed the localization of ICN1-transduced cells that were isolated after 2 passages into NSG mice, which more rapidly develop the disease, in the early phase after injection. Of note, after the initial detection in peripheral blood that was attributed to i.v. injection, the expansion of leukemic cells was first detectable in the BM (Fig. 4E). Altogether, these results suggest that BM colonization is prodromic to the development of leukemia and that CXCR4 is crucial for BM engraftment.

Chemokine receptors CCR6 and CCR7 are not required for meningeal infiltration by T-ALL cells

The chemokine receptors CCR6 and CCR7 play key roles in shaping T-cell distribution in both physiologic and pathologic conditions. In particular, the CCR6–CCL20 axis in the choroid plexus was hypothesized to control T-cell migration into the CNS [19]. Conversely, CCL19 produced by endothelial cells was hypothesized to promote CNS infiltration in mice that were engrafted with murine hematopoietic progenitors transduced with ICN1 or human T-ALL cells via CCR7 expressed in leukemic cells [20]. We analyzed CXCR4, CCR7, and CCR6 expression in the BM of human T-ALL patients. As shown in Fig. 5, high expression of CXCR4 contrasted with lower and barely, if at all, detectable expression of CCR7 and CCR6, respectively. To see whether CCR6 and CCR7 might have a role in T-ALL development, we transduced FLPs from CCR6<sup>−/−</sup> and CCR7<sup>−/−</sup> embryos with ICN1-EGFP<sup>+</sup> retrovirus and transplanted transformed cells into NSG mice. These mice revealed infiltration of target organs analogous to mice that underwent transplantation with transduced WT hematopoietic progenitors (Fig. 6A). Furthermore, the disease developed with similar kinetics (Fig. 6B). Leukemic infiltrates that replaced hematopoietic BM in the cranial diploe and invasion of underlying meninges were detected in mice that were injected with transformed CCR7<sup>−/−</sup> and CCR6<sup>−/−</sup> FLPs (Fig. 6C and D), which was analogous to mice that were injected with WT cells. These results contrast with that lack of meningeal infiltration by ICN1-induced T-cell malignancy with CCR7-deficient hematopoietic progenitors [20]. This discrepancy could be a result of the delayed leukemia development that was observed in the previous report limiting CNS invasion and/or difference in the antileukemia response of different animal strains.

Legend for Figure 2: Chemokine receptor expression in Jurkat and CEM cells. FACS analysis for chemokine receptors in Jurkat and CEM cells. Red traces represent staining with chemokine receptor–specific antibody and blue lines are isotype-matched antibodies.

Figure 2. Chemokine receptor expression in Jurkat and CEM cells. FACS analysis for chemokine receptors in Jurkat and CEM cells. Red traces represent staining with chemokine receptor–specific antibody and blue lines are isotype-matched antibodies.
houses. Recent findings suggest the possible relevance of CXCR4 as a pharmacologic target for T-ALL therapy [13, 14]. Our results are in line with this hypothesis and further indicate the importance of CXCR4, but not CCR6 or CCR7, in CNS involvement in disease.

**CXCR4-mediated infiltration of BM in xenotransplanted NSG mice by T-ALL cells from patients has a prominent role in meningeal invasion**

In NSG mice that were injected with human patient T-ALL cells that were isolated from mice that underwent previous transplantation, leukemic cells were detectable in the blood at day 2 after injection and diminished significantly on day 3 (1.41 ± 0.26 vs. 0.39 ± 0.31; n = 18; t test: P = 0.012). Subsequently, T-ALL cells expanded in the BM (Fig. 7A), as observed with FLPs transduced with ICN1 (Fig. 4E). Histopathologic and FACS analyses of a representative animal injected with cells from PDTALL10 patient show no sign of leukemic infiltration of the CNS and selective confinement of T-ALL cells to the BM at 2 wk after injection (Fig. 7B). However, at the humane end point, bone integrity was lost and leukemic cells extensively infiltrated the BM, with diffusion to meninges with no involvement of the brain parenchyma (Fig. 7D). We performed a histologic assessment of the extent and distribution of leukemic infiltrates in different regions of the

Figure 3. Amelioration of leukemia by AMD-3100. (A) Weight variation over time and clinical scores (Materials and Methods) in NSG mice injected with Jurkat cells and implanted with osmotic pumps that delivered either PBS or AMD-3100 (PBS, n = 4; AMD-3100, n = 4). (B) Jurkat cell recoveries in BM, spleen, and blood by FACS in the same mice. (C) Luciferase detection with the In Vivo Imaging System at 3 wk from injection of Jurkat cells. (D) Statistical analysis of histologic infiltration scores (Materials and Methods) in BM, dental alveoli, oral and rhinopharyngeal mucosa, middle ear, and meninges of the head in the same mice. (E) Histopathology (H&E staining) of the head. MOCK: unaffected mouse. Note the normal hematopoietic tissue (*) and the meninges devoid of meningeal infiltrates (arrows). PBS: interparietal bone, massive BM colonization by leukemic cells (**) with diffuse infiltrates that disrupt the bony encasement and invade the meninges (*) covering the inferior colliculi (arrows). AMD-3100: interparietal bone, only few clusters of leukemic cells (**) are evident in the BM without invasion of the meninges (arrows). Note the normal hematopoietic tissue (*) surrounding the leukemic infiltrates. Ic = inferior colliculi, ns = nonsignificant. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Scale bar = 110 μm.
Figure 4. Intra-BM engraftment does not rescue leukemia by ICN1-transformed CXCR4−/− hematopoietic progenitors. (A) Representative analysis of ICN1 expression by using FACS in cells from the indicated organs of mice that underwent i.v. or intratibial transplantation with WT or CXCR4−/− FLPs transduced with ICN1. Numbers indicate percentage of positive cells. (B) Histopathology (H&E staining) of the head in mice that underwent i.v. transplantation with ICN1-transduced FLPs. Left panel shows leukemic infiltrates replacing the hematopoietic BM in the cranial diploe (*) with (continued on next page)
skull in a cohort of 32 mice xenografted with primary human pediatric T-ALL cells at the humane end point (Materials and Methods). The main characteristics for the 18 human T-ALL that were used to perform the set of 32 xenografted murine models are shown in Table 1. All mice showed extensive BM colonization, and meningeal infiltration was detected in 81.25% of the xenograft cohort. Despite the detection of meningeal infiltration in 26 samples, no involvement of the brain parenchyma was apparent. We also observed a significant positive correlation of BM and meningeal infiltration scores, which supported the hypothesis that leukemia expansion in the BM is instrumental to CNS invasion (Fig. 7E). Finally, as shown with T-ALL cell lines, the administration of AMD-3100 significantly ameliorated CNS infiltration (Fig. 7E and G). These data support the hypothesis that CXCR4-mediated BM engraftment is a prerequisite for invasion of the meninges by T-ALL cells.

**DISCUSSION**

The infiltration of the CNS is an important trait of T-ALL pathophysiology. The detection of leukemia in the CNS at diagnosis [22] and the abundance of lymphoblasts in cerebrospinal fluid [23] are significant predictors of an increased risk of death. The mechanisms that T-ALL cells employ to invade the CNS are not clearly defined. The CNS is an immune-privileged site in which the infiltration of the brain parenchyma by potentially harmful cells and macromolecules is controlled by the BBB. Nevertheless, immunosurveillance of the CNS is operated by effector T cells, which migrate across the BBB. Access to the brain parenchyma is regulated by cytokines, proteases, chemokines, and chemokine receptors as well as activation markers, which act on glia limitans that is composed of parenchymal basement membrane and astrocyte foot processes [24, 25]. In our experiments, the lack of detection in the brain parenchyma of leukemic cells, which are always found confined into the meninges, is a striking feature of CNS infiltration by T-ALL cells. Extensive postmortem histopathologic analysis of human brain before the advent of CNS-specific therapy revealed a lack of leukemic cell infiltration in the brain in the presence of widespread arachnoid invasion and perivascular cuffing [26].

Abdominal expression of CXCL12 in the spinal cord microvasculature prevents migration of CXCR4-expressing cells into the brain parenchyma [27], and, in inflammatory conditions, leukocyte migration is enhanced by CXCR4 antagonism [27, 28]; therefore, CXCR4 antagonism may unleash T-ALL cells from this retention mechanism and contribute to neuropathology. However, we have shown that the administration of AMD-3100 significantly ameliorates CNS invasion and maintains bone integrity. BBB permeability may depend on regional differences in neural activation around vessels that can regulate blood cell accumulation in the CNS. In experimental autoimmune encephalomyelitis, a model of multiple sclerosis, soleus muscle–mediated sensory nerve activation enhances the expression of CCL20 in dorsal blood vessels of the fifth lumbar cord and enables entry of pathogenic T cells into the CNS [29]. In contrast to hematogenous invasion observed in the adoptive transfer of encephalitogenic T cells, histologic analysis of thoracocervical and lumbar spine in NSG mice that underwent transplantation with Jurkat cells (data not shown) suggests that spine infiltration by leukemic cells occurs by anatomic contiguity. Our observations are consistent with the hypothesis that leukemic cells expand from the cranial diploe through the foramina of nutrient vessels. Uncontrolled lymphoblast proliferation would result in bone disruption, with an accumulation of cells in the epidural space, permeation of the dura mater, and infiltration of the leptomeninges [30].

Specialized endothelium in the BM expresses CXCL12, which influences the homing of acute lymphoblastic leukemia cells via CXCR4 [15]. More recently, CXCR4 expression and signaling
were shown to influence both T-ALL cell localization in the BM and survival [13, 14]. We inhibited leukemic lymphoblast homing to the BM by pharmacologic antagonism and by using CXCR4-deficient, transformed, hematopoietic progenitors to observe whether this event might constitute a prerequisite for CNS infiltration. Our results show that CXCR4 inhibition dramatically impacted BM grafting of T-ALL cells (also upon orthotopic injection of CXCR4-deficient, ICN1-transduced, hematopoietic progenitors), leukemia development, and CNS infiltration. Because AMD-3100 is currently approved for the mobilization of hematopoietic stem cells from the BM for transplantation, our study suggests that the same pharmacologic approach, together with chemotherapeutic agents, might be exploited in patients with T-ALL to prevent or cure CNS disease. Extensive histopathologic analyses of neurologic specimens from mice that underwent transplantation with patient-derived cells, human cell lines, or murine hematopoietic progenitors that were transformed by ICN1 showed leukemic infiltrates to be almost entirely restricted to meninges. Although a direct colonization of the neuroparenchyma was not detected, meningeal infiltrates were likely responsible for a series of degenerative changes, such as the compression of the underlying neuroparenchyma and the alteration of the normal mechanism of cerebrospinal fluid drainage, with secondary hydrocephalus. The extension of leukemic cells to soft tissues, including spinal meninges, facial sense organs (in particular, the middle and inner ear), and peripheral nerve sheaths with correlated ganglia appeared secondary to BM colonization.

The observations of the current study suggest that T-ALL cells are not able to infiltrate the neuroparenchyma by breaching the BBB. It was hypothesized 60 y ago that both the BBB and the choroid plexus could be effective barriers against circulating leukemic cells [26]. Astrocytes are pivotal elements in the regulation of BBB permeability, and they express ICAM-1 (CD54) and VCAM-1 (CD105), which are ligands for LFA-1 (CD11a/CD18) and VLA-4 (α4β1) integrins, respectively. These integrins, which are expressed at the cell surface in T-ALL cells [13], mediate the adherence of activated CD4 cells to astrocytes as well as the bidirectional signaling that affects CD4 cell function and BBB permeability [31]. It could be hypothesized that integrin-mediated interaction of T-ALL cells with astrocytes at the BBB might limit their potential to infiltrate the neuroparenchyma. T-ALL relapses are frequently resistant to therapy, and the identification of new therapeutic targets is needed to improve the outcome of patients who experience relapse. The leptomeningeal space may be an important microenvironment in the maintenance of residual disease. Involved in T-ALL is a large spectrum of mutations that can be independent of CXCR4 in disease pathogenesis; however, because CXCR4 seems to be important not only for the homing and survival of leukemic cells in the BM but also for CNS infiltration, our study suggests that inhibition of CXCR4 activity or of T-ALL-specific downstream targets might help in avoiding this devastating feature of the disease.

**Authorship**

T.R.J. performed most experiments; C.B., A.R., and L.P. performed experiments; E.R. performed, analyzed, and interpreted all
Figure 7. BM and meningeal infiltration in NSG mice injected with T-ALL cells from patients. (A) Leukemic cell recovery over time by FACS from blood and BM (n = 5/d). (B) Histopathology (H&E staining) of cranial diploe showing lack of meningeal infiltration from a representative mouse injected with relapsed T-ALL cells from patient PDTALL10 at 2 wk after injection. FACS histograms for human CD45 on the indicated organs from the same mouse. Numbers indicate percentage of positive cells. (C) Statistical analysis of head infiltration scores (Materials and Methods) in mice that underwent transplantation with T-ALL cells at the indicated time points (2 wk, n = 10; end point, n = 32). (D) Histopathology (H&E staining) of the head showing extensive BM infiltration by leukemic cells (*) with diffusion to meninges (in particular, dura and arachnoid; arrows) but without infiltration of the underlying frontal lobe cortex from a representative mouse injected with PDTALL10 cells at the humane end point. (E) Linear regression of infiltration scores in the BM and meninges at end point. For each dot, the corresponding number of samples is indicated. Pearson correlation coefficient analysis is shown. (F) Statistical analysis of infiltration scores (Materials and Methods) in BM, dental alveoli, oral and rhinopharyngeal mucosa, middle ear, and meninges of the heads of mice treated with AMD-3100 or saline at 3 wk after transplantation with relapsed PDTALL10 cells (PBS, n = 5; AMD-3100, n = 4). (G, Left) Histopathology (H&E staining) of the head of normal hematopoietic BM without leukemic component in the cranial diploe (*). The meninges (arrow) are free from any leukemic infiltrate in mock-injected mice. (Middle) Leukemic infiltrates replacing the hematopoietic BM in the cranial diploe (*) with invasion into the underlying meninges (arrows) in mice treated with PBS. (Right) Leukemic infiltrates in AMD-3100-treated mice are confined to the hematopoietic BM in the cranial diploe (*) without invasion into the underlying meninges (arrow). The expansion of the arachnoid space with separation of skull and neuroparenchyma represents a common artifact associated with the preparation of paraffin sections from the whole head. cf = cerebellar folia, fb = frontal bone, fl = frontal lobe, ib = intraparietal bone. **P < 0.01; ****P < 0.0001. Scale bars = 200 μm (B), 200 μm (D), and 160 μm (G).

histopathologic findings of the work; L.O. contributed to histopathology; G.C., A.B., and S.I. provided samples from human patients; C.B. and G.t.K. provided transcriptional analysis of chemokine receptors; T.R.J., C.B., M.T., G.t.K., and F.G. analyzed data; and T.R.J. and F.G. designed the study and wrote the paper.

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