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Supplemental information

Targeted blockade of immune mechanisms inhibit B precursor acute lymphoblastic leukemia cell invasion of the central nervous system

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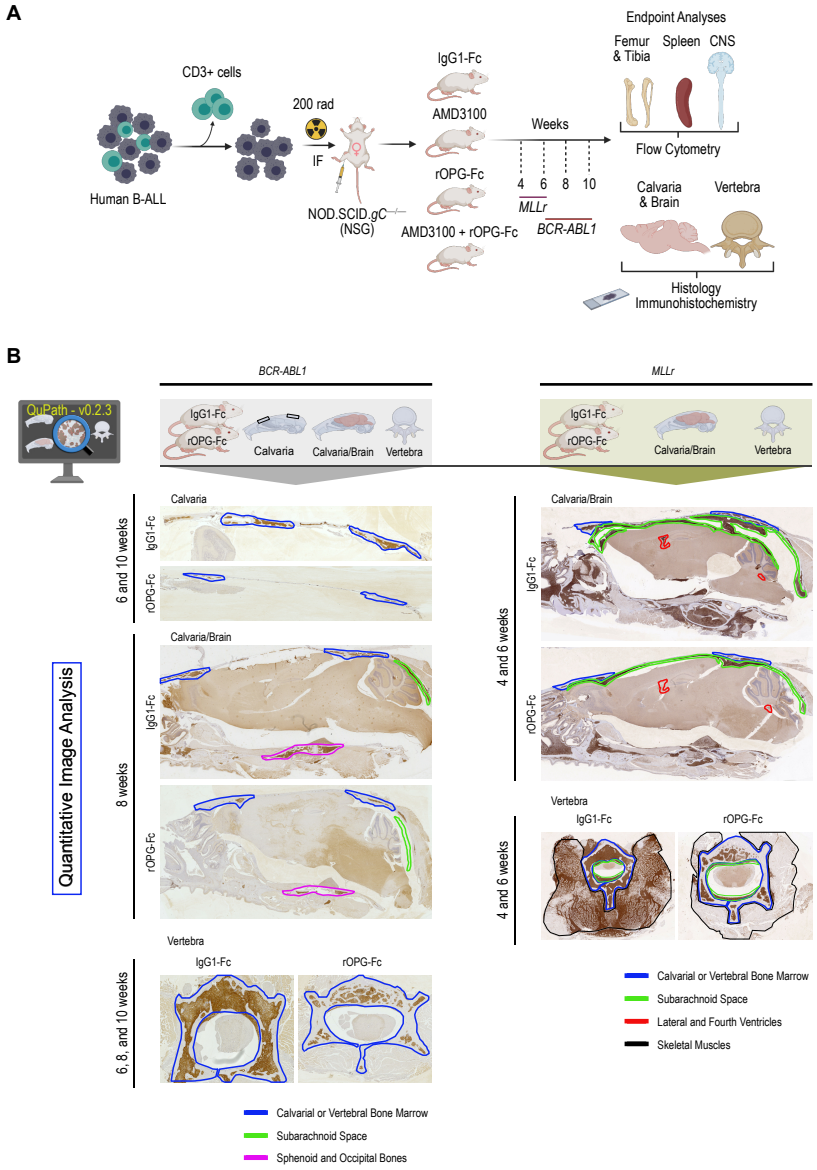

Figure S1. Schematic representation of treatment regimens and quantitative image analyses in PDX mice, Related to Figures 1, 3 to 7. (**A**) Human *BCR-ABL1* B-ALL blasts (ID 090233) after CD3 depletion or NSG-passaged human *MLLr* cells (ID 9037) were injected orthotopically (intra-femoral injection; I.F.) into the bone marrow niche of the right femur of eight weeks old, irradiated (200 rad) NSG female mice. Intrafemorally injected PDX mice were treated with IgG1-Fc control or AMD3100 or rOPG-Fc or combined AMD3100 and rOPG-Fc. In all PDX groups, drug or control treatments were initiated concurrent with leukemic blast I.F. injections and were continued until animals were euthanized. Reflecting the distinct kinetics of primary human B-ALL tumor engraftment, *MLLr* PDX mice were euthanized at 4 or 6 weeks after I.F. injection, and *BCR-ABL1* PDX mice were euthanized 6 or 8 or 10 weeks after I.F. injection. Flow cytometric analysis was performed using anti-human CD45 and CD19 antibodies to assess leukemic blast engraftment in the injected right femur, non-injected right tibia, spleen, and CNS. The brain with an intact skull and vertebral bones with an entire spinal cord was used for histological analysis and immunohistochemical staining in experiments where CNS single-cell suspensions were not performed. (**B**) Representative CD19-stained calvaria, brain with calvaria, and vertebra with spinal cord sections from *BCR-ABL1* and *MLLr* PDX mice. QuPath (v.0.2.3) software [57] was used to evaluate the invasion of $CD19^+$ B-ALL blasts. The colored lines indicate the multiple regions of interest drawn to assess $CD19^+$ B-ALL blast invasion. Refer to the Methods section for the detailed analysis.

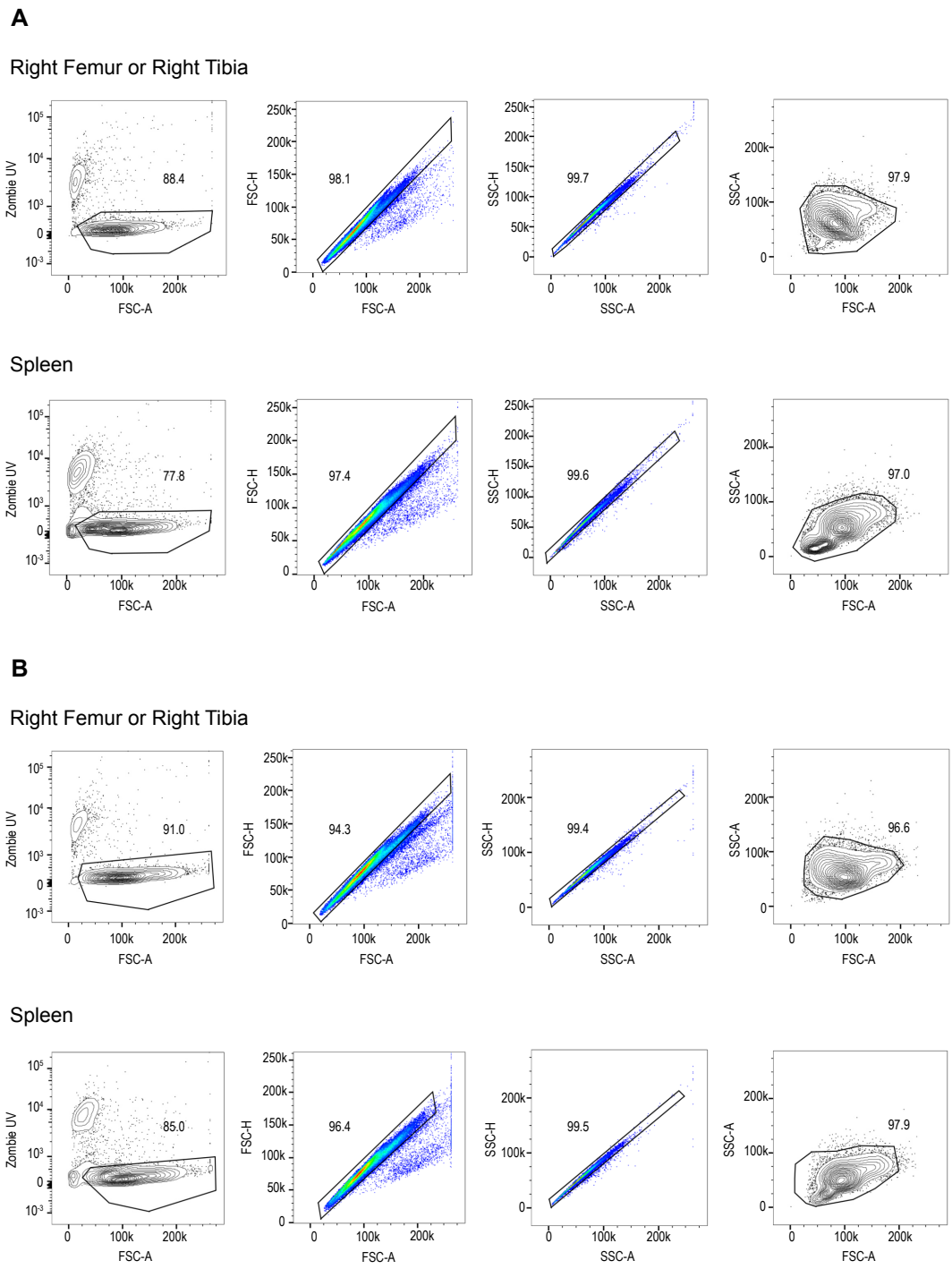

Figure S2. Gating strategy used to assess leukemic blast engraftment in *BCR-ABL1* PDX mice, Related to Figures 1 and 3. Primary *BCR-ABL1* B-ALL blasts (ID 090233) were injected orthotopically into the right femur of NSG mice and treated with either IgG1-Fc control or rOPG-Fc fusion protein. (**A**) Six weeks or (**B**) ten weeks after leukemic blast injection and treatment, mice were euthanized, and flow cytometric analyses were performed. Representative flow cytometric gating strategy of ex vivo cells from the right femur or tibia and spleen of IgG1-Fc control-treated PDX mice. Gates used to exclude dead cells (Zombie UV vs. FSC-A), and then debris and doublets (columns 2-4) are shown.

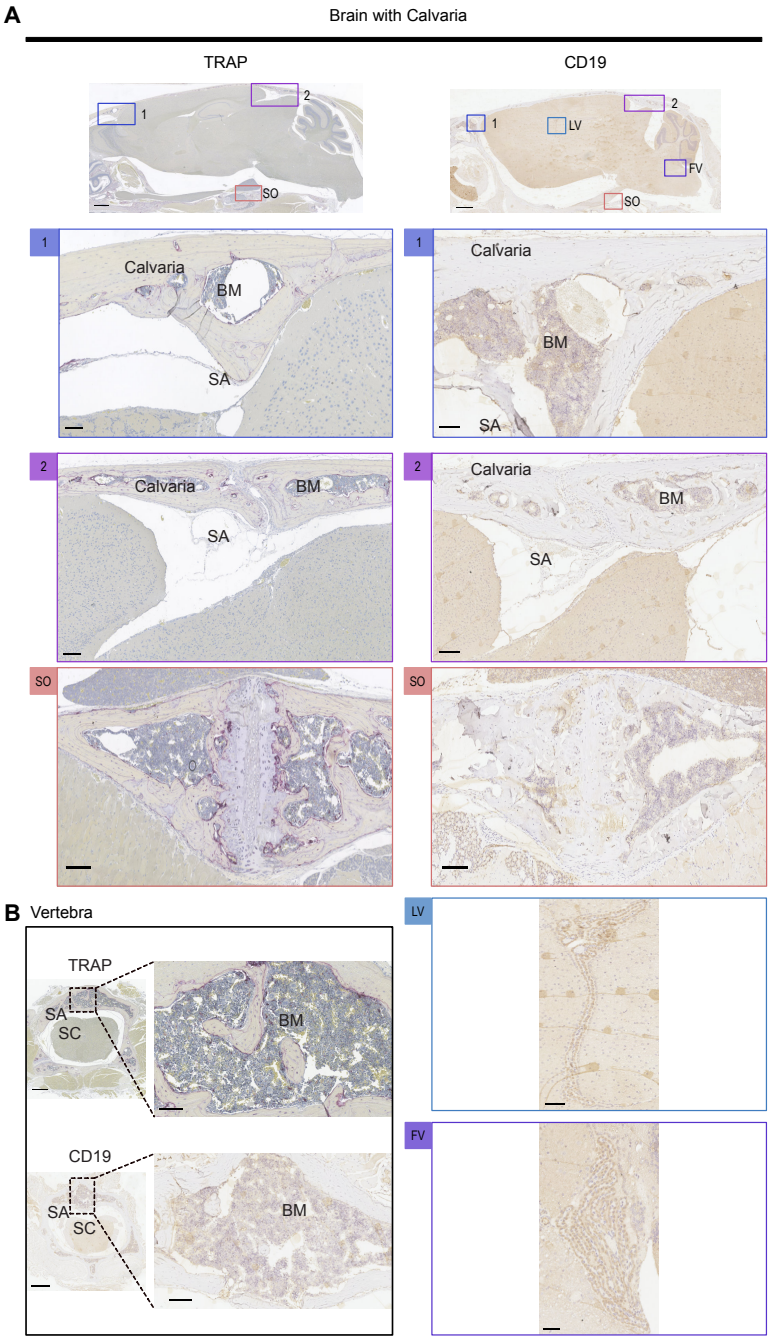

Figure S3. Irradiated non-engrafted NSG mice show normal bone architecture, Related to Figures 1 and 4. NSG female mice (n = 3 mice) at eight weeks of age were irradiated at 200 rad. Mice were euthanized at ten weeks after irradiation and assessed as a control. The whole brain with an intact skull and vertebral bones with an intact spinal cord were dissected, fixed in 10% phosphate-buffered formalin, decalcified in PBS+14% EDTA, paraffin-embedded, sectioned, and stained with TRAP or anti-human CD19 antibody. (**A**) Representative sagittal sections of the brain and skull showing calvarial bone marrow and brain subarachnoid space in the (1) frontal and (2) caudal region, sphenoid and occipital bones at the cranial base (SO), and lateral ventricle (LV) or fourth ventricle (FV). (**B**) Representative cross-sections of a vertebra showing the bone marrow, subarachnoid space, and spinal cord. Purple staining in TRAP images indicates multinucleated osteoclasts. The dotted box represents a magnified view. Abbreviations: BM, bone marrow; SA, subarachnoid space; SC, spinal cord. Scale bars, 100μm.

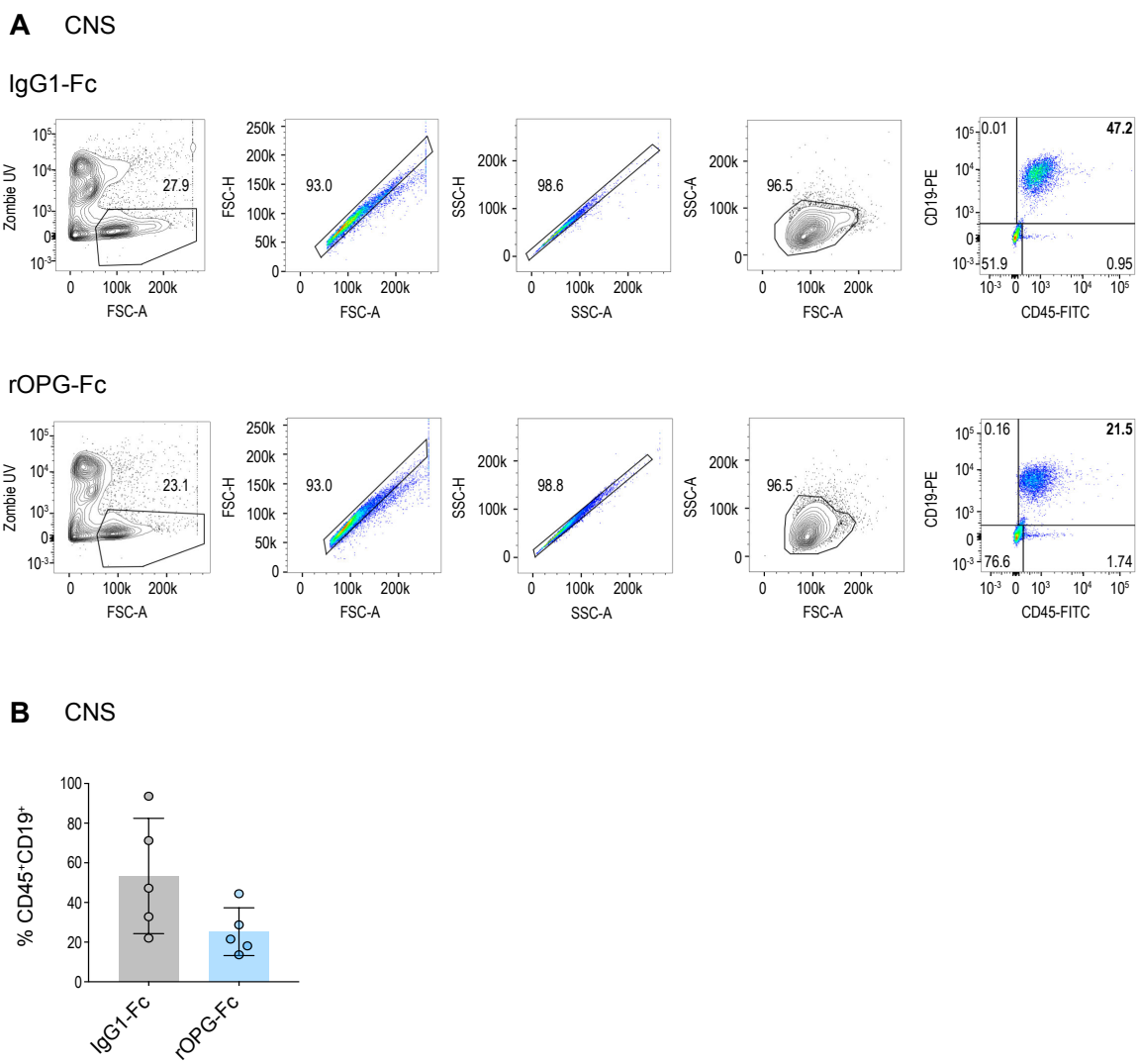

Figure S4. Gating strategy and effect of rOPG-Fc in leukemic blast engraftment in the CNS of *BCR-ABL1* PDX mice, Related to Figure 3. Primary *BCR-ABL1* B-ALL blasts (ID 090233) were injected orthotopically into the right femur of NSG mice and treated with either IgG1-Fc control or rOPG-Fc fusion protein. Ten weeks after leukemic blast injection and treatment, mice were euthanized, and flow cytometric analyses were performed. (**A**) Representative flow cytometric gating strategy of ex vivo cells from the brain and spinal cord including the subarachnoid space (CNS) of IgG1-Fc control or rOPG-Fc treated PDX mice. Gates used to exclude dead cells (Zombie UV vs. FSC-A), and then debris and doublets (columns 2-4) are shown. Leukemic blast engraftment was assessed from cells of the CNS using antibodies specific for human CD45 and CD19 (column 5). (**B**) The percentage of $CD45^{+}CD19^{+}$ leukemic blasts gated on live singlets is shown. Data are mean ± SD. A two-tailed unpaired *t*-test with Welch's correction was performed between the two groups.

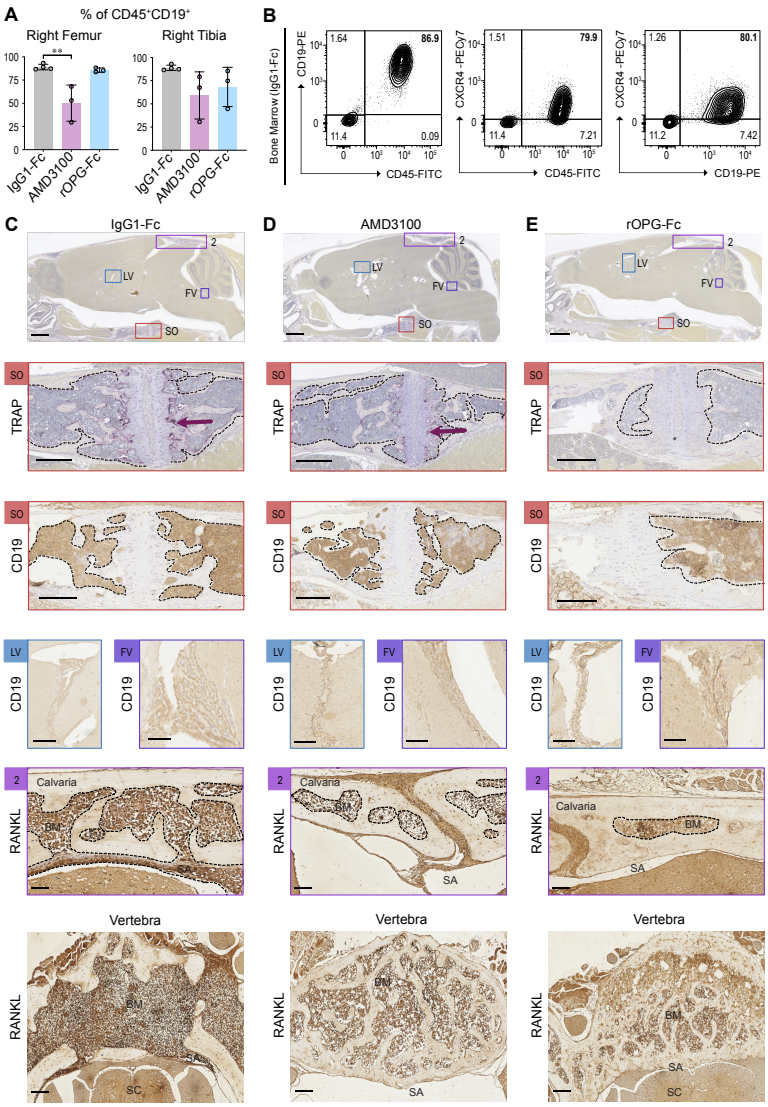

Figure S5. Effects of CXCR4 and RANKL antagonism on *BCR-ABL1* PDX mice, Related to Figure 4. Human *BCR-ABL1* leukemic blasts were injected orthotopically into the right femur of NSG mice and treated with IgG1-Fc control or AMD3100 or rOPG-Fc at the time of leukemic blast injections (n = 3 or 4 mice per group). Mice were euthanized eight weeks later. (**A**) After eight weeks of treatment, ex vivo cells from the injected right femur and non-injected right tibia after were assessed for leukemic blast engraftment using antibodies specific for human CD45 and CD19 by flow cytometry. The percentage of $CD45^{+}CD19^{+}$ leukemic blasts gated on live singlets is shown. Data are mean ± SD. Data were analyzed using one-way ANOVA (95% confidence interval) among the three groups; *P* values represent Bonferroni's multiple comparison test, $^{**}P < 0.01$. (**B**) Representative flow cytometric analysis of ex vivo cells from the bone marrow of IgG1-Fc control-treated PDX mice. CXCR4 cell-surface expression on leukemic blasts was assessed using antibodies specific for human CD45, CD19, and CXCR4 antibodies. (**C** to **E**) The whole brain with an intact skull and vertebra with an intact spinal cord was fixed in 10% phosphate-buffered formalin, decalcified in PBS+14% EDTA, paraffin-embedded, sectioned, and stained with TRAP or anti-human CD19 or anti-RANKL antibody. Representative sagittal sections of the brain and skull showing sphenoid and occipital bones at the cranial base (SO), lateral ventricle (LV) or fourth ventricle (FV), calvarial bone marrow and brain subarachnoid space, and vertebra of the IgG1-Fc or AMD3100 or rOPG-Fc treated mice. The dotted outline and brown color on CD19-stained images indicate leukemic blasts. The purple staining on TRAP images (purple arrows) indicates multinucleated osteoclasts. Abbreviations: BM, bone marrow; SA, subarachnoid space; SC, spinal cord. Scale bars, 100 µm.

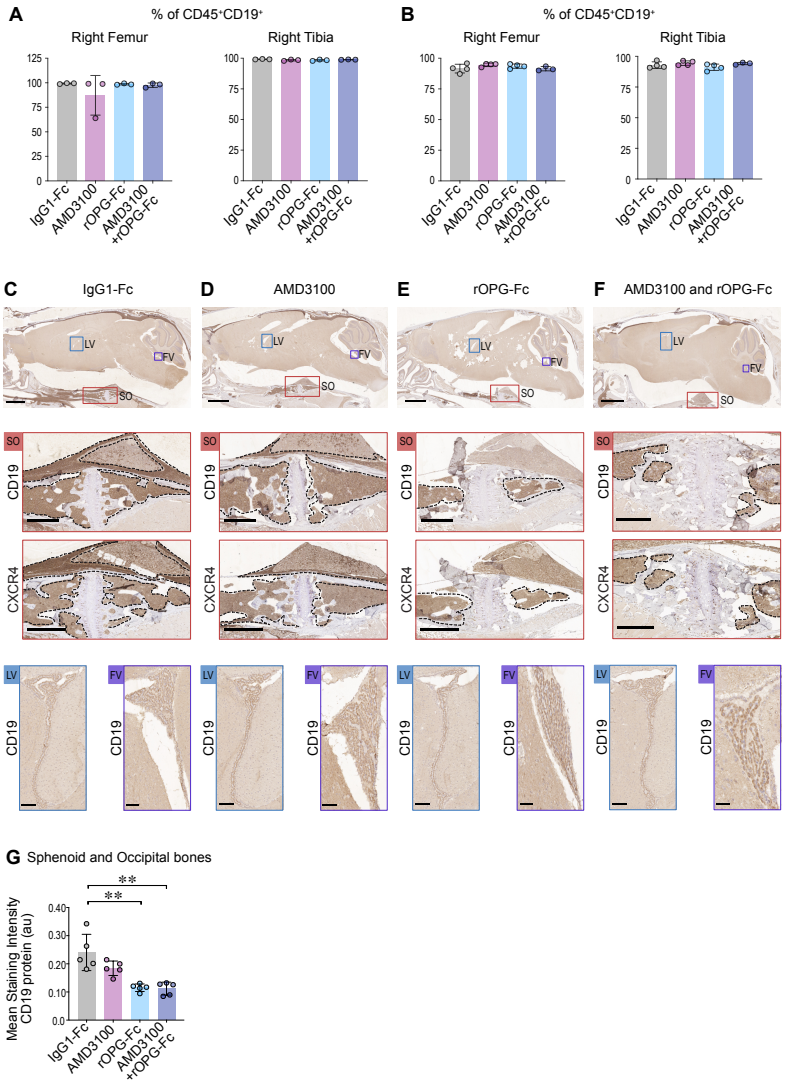

Figure S6. Effects of CXCR4 and RANKL antagonism in *MLLr* PDX mice four or six weeks after leukemic blast engraftment, Related to Figures 5 and 6. Human *MLLr* NSG-passaged leukemic blasts were injected orthotopically into the right femur of NSG mice and treated with IgG1-Fc control or AMD3100 or rOPG-Fc or combined AMD3100 and rOPG-Fc during the time of leukemic blast injections (n = 3 or 4 mice per group). Four or six weeks after leukemic blast injection and treatment, mice were euthanized. After (**A**) four or (**B**) six weeks of treatment, ex vivo cells from the injected right femur and non-injected right tibia after were assessed for leukemic blast engraftment using antibodies specific for human CD45 and CD19 by flow cytometry. The percentage of $CD45^{+}CD19^{+}$ leukemic blasts gated on live singlets is shown. After 4 weeks of treatment, the whole brain with an intact skull was fixed in 10% phosphate-buffered formalin, decalcified in PBS+14% EDTA, paraffin-embedded, sectioned, and stained with anti-human CD19 or CXCR4 antibody. (**C** to **F**), Representative sagittal sections of the brain and skull showing sphenoid and occipital bones at the cranial base (SO) and lateral ventricle (LV) or fourth ventricle (FV) of the IgG1-Fc or AMD3100 or rOPG-Fc or combined AMD3100 and rOPG-Fc treated mice. The dotted outline and brown color on CD19- and CXCR4-stained images indicate leukemic blasts. Scale bars, 100 µm. (**G**) CD19 protein (*y* axis) was quantified in regions of interest (ROIs; 25µm) from the sphenoid and occipital bones of the IgG1-Fc treated, AMD3100 treated, rOPG-Fc treated or AMD3100 + rOPG-Fc treated mice (*x* axis). The graph shows the mean staining intensity (2 µm per pixel) of CD19 protein [arbitrary units (au)]. Each dot represents multiple ROI from three biological replicates and one or two technical replicates (n = 3 mice per group). Data are mean ± SD. All data were analyzed using non-parametric Kruskal-Wallis one-way analysis of variance (95% confidence interval) among the three groups; *P* values represent post-hoc Dunn's test, where $^{**}P < 0.01$.

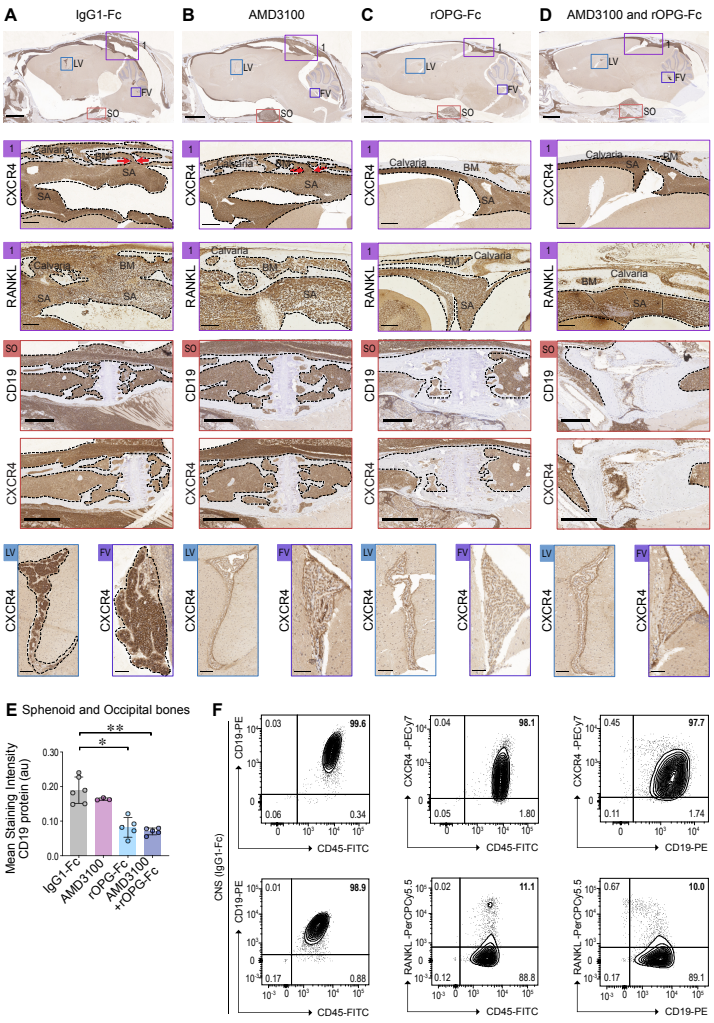

Figure S7. Effects of CXCR4 and RANKL antagonism in *MLLr* PDX mice six weeks after leukemic blast engraftment, Related to Figures 5 and 6. Human *MLLr* NSG-passaged leukemic blasts were injected orthotopically into the right femur of NSG mice and treated with IgG1-Fc control or AMD3100 or rOPG-Fc or combined AMD3100 and rOPG-Fc during the time of leukemic blast injections. Six weeks after leukemic blast injection and treatment, mice were euthanized. The whole brain with an intact skull was fixed in 10% phosphate-buffered formalin, decalcified in PBS+14% EDTA, paraffin-embedded, sectioned, and stained with anti-human CD19 or CXCR4 antibody. (**A** to **D**), Representative sagittal sections of the brain and skull showing calvarial bone marrow and subarachnoid space in the (1) caudal region, sphenoid and occipital bone at the cranial base (SO), and lateral ventricle (LV) or fourth ventricle (FV) of the IgG1-Fc or AMD3100 or rOPG-Fc or combined AMD3100 and rOPG-Fc treated mice. The dotted outline and brown color on CD19, RANKL, and CXCR4-stained images indicate leukemic blasts. Scale bars, 100 µm. (**E**) CD19 protein (*y* axis) was quantified in regions of interest (ROIs; 25µm) from the sphenoid and occipital bones of the IgG1-Fc treated, AMD3100 treated, rOPG-Fc treated or AMD3100 + rOPG-Fc treated mice (*x* axis). The graph shows the mean staining intensity (2 µm per pixel) of CD19 protein [arbitrary units (au)]. Each dot represents multiple ROI from four biological replicates and one or two technical replicates (n = 4 mice per group). Data are mean ± SD. All data were analyzed using non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) (95% confidence interval) among the three groups; *P* values represent post-hoc Dunn's test, where $*P < 0.05$, and $**P < 0.01$. (**F**) Representative flow cytometric analysis of ex vivo cells from the brain and spinal cord including the subarachnoid space (CNS) of IgG1-Fc control-treated PDX mice. CXCR4 and RANKL cell-surface expression on leukemic blasts was assessed using antibodies specific for human CD45, CD19, and CXCR4. To detect RANKL, cells were stained with biotinylated rOPG-Fc and Streptavidin-PerCP-Cy5.5.

Sample ID	Age at Diagnosis (years)	Gender	Cytogenetics	Risk Group
090233	54	M	*BCR-ABL1*	HR
9037	0.25	F	*MLL/KMT2A*-rearranged	HR

Table S1. Clinical characteristics of B-ALL (peripheral blood) samples used in the xenograft studies, Related to Figures 1, 3 to 7. M, male; F, female; HR, high-risk group. *HR group criteria: age < 1 or ≥ 10 years and white blood cell count 50×10^9/L. [S1]

Supplemental reference

S1. Smith, M., Arthur, D., Camitta, B., Carroll, A.J., Crist, W., Gaynon, P., Gelber, R., Heerema, N., Korn, E.L., Link, M., et al. (1996). Uniform approach to risk classification and treatment assignment for children with acute lymphoblastic leukemia. J Clin Oncol *14*, 18-24. 10.1200/JCO.1996.14.1.18.