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Requirement for CD44 in homing and engraftment of BCR-ABL–expressing leukemic stem cells

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In individuals with chronic myeloid leukemia (CML) treated by autologous hematopoietic stem cell (HSC) transplantation, malignant progenitors in the graft contribute to leukemic relapse[1], but the mechanisms of homing and engraftment of leukemic CML stem cells are unknown. Here we show that CD44 expression is increased on mouse stem-progenitor cells expressing BCR-ABL and that CD44 contributes functional E-selectin ligands. In a mouse retroviral transplantation model of CML, *BCR-ABL1*–transduced progenitors from CD44-mutant donors are defective in homing to recipient marrow, resulting in decreased engraftment and impaired induction of CML-like myeloproliferative disease. By contrast, CD44-deficient stem cells transduced with empty retrovirus engraft as efficiently as do wild-type HSCs. CD44 is dispensable for induction of acute B-lymphoblastic leukemia by BCR-ABL, indicating that CD44 is specifically required on leukemic cells that initiate CML. The requirement for donor CD44 is bypassed by direct intrafemoral injection of *BCR-ABL1*–transduced CD44-deficient stem cells or by coexpression of human CD44. Antibody to CD44 attenuates induction of CML-like leukemia in recipients. These results show that BCR-ABL–expressing leukemic stem cells depend to a greater extent on CD44 for homing and engraftment than do normal HSCs, and argue that CD44 blockade may be beneficial in autologous transplantation in CML.

Acute and chronic myeloid leukemias are maintained by a small population of leukemic stem cells that are defined by their ability to transplant disease to a recipient and that must be eliminated for cure[2]. Studies of cells from individuals with CML[3] and mouse models[4,5] indicate that the leukemic stem cell is a multipotential progenitor similar to normal HSCs. Most individuals in cytogenetic remission after treatment with the kinase inhibitor imatinib carry residual BCR-ABL$^+$ stem cells[6], and some will develop progressive leukemia. Autologous transplantation with Philadelphia chromosome–negative (Ph$^-$) stem cells obtained at the time of minimal disease[7] could be effective salvage therapy, but efforts to purge autografts of CML stem cells, either by antisense oligodeoxynucleotides targeted to *BCR-ABL1* or c-*MYB*[8] or through *in vitro* culture[9], have had limited success. An alternative strategy is to block selectively homing and engraftment of BCR-ABL–expressing leukemic stem cells without adversely affecting repopulation by normal HSCs—a multistep process[10] of rolling on endothelial selectins[11], adhesion through β_1 and β_2 integrins[12,13], and transmigration mediated by stromal cell–derived factor-1 (SDF-1)[13] and its HSC receptor CXCR4. Because Ph$^+$ stem cells have functional defects in β_1 integrins[14] and in CXCR4 (ref. 15), they must rely on alternative adhesive pathways for engraftment.

We previously observed using a mouse model of CML that BCR-ABL–expressing leukemic stem cells rely to a greater extent on selectins and their ligands for engraftment than do normal HSCs[16]. Because a modified glycoform of the hyaluronan receptor CD44 is a source of selectin ligands on human HSCs[17] and mouse neutrophils[18], here we compared CD44 expression on normal mouse c-Kit$^+$Lin$^-$ stem-progenitor cells and their BCR-ABL$^+$ counterparts (**Fig. 1a**). We found that, similar to human CMLs[19], CD44 was increased on BCR-ABL–expressing progenitors from bone marrow of mice with CML-like leukemia, but decreased on malignant progenitors from blood or spleen, suggesting that CD44 may be involved in bone marrow retention of BCR-ABL$^+$ stem cells. We compared selectin-binding activity on transduced stem-progenitor cells from wild-type donor mice with those from mutant mice lacking all CD44 isoforms[20]. BCR-ABL increased the expression of selectin ligands on wild-type progenitors; by contrast, BCR-ABL$^+$ *Cd44*$^{-/-}$ progenitors showed both a smaller fraction of selectin ligand–positive cells (by 33%) and lower mean selectin-binding activity (by 40%), relative to BCR-ABL–expressing wild-type progenitors (**Fig. 1b**). This observation suggests that BCR-ABL increases selectin ligands on leukemic stem cells in part through upregulation of CD44.

To study the role of CD44 in engraftment of BCR-ABL$^+$ leukemia-initiating cells, we used the *Cd44*$^{-/-}$ mice as donors in a retroviral transplantation model of CML[21]. These mice are developmentally normal[20,22] and have decreased splenic and circulating myeloid progenitors[22] but a numerically and functionally normal HSC population[23]. Because the *Cd44*$^{-/-}$ mice were in a mixed B6 and 129 background, we used B6×129 F_2 mice as wild-type controls. The efficiency of induction of CML-like myeloproliferative disease (MPD) in this H-2–identical but mixed background is lower than in syngeneic B6 mice[24], but nonetheless most (90%) wild-type recipients of *BCR-ABL1*–transduced wild-type bone marrow succumbed to fatal CML-like leukemia within 2 months (**Fig. 1c**). Similar results were obtained

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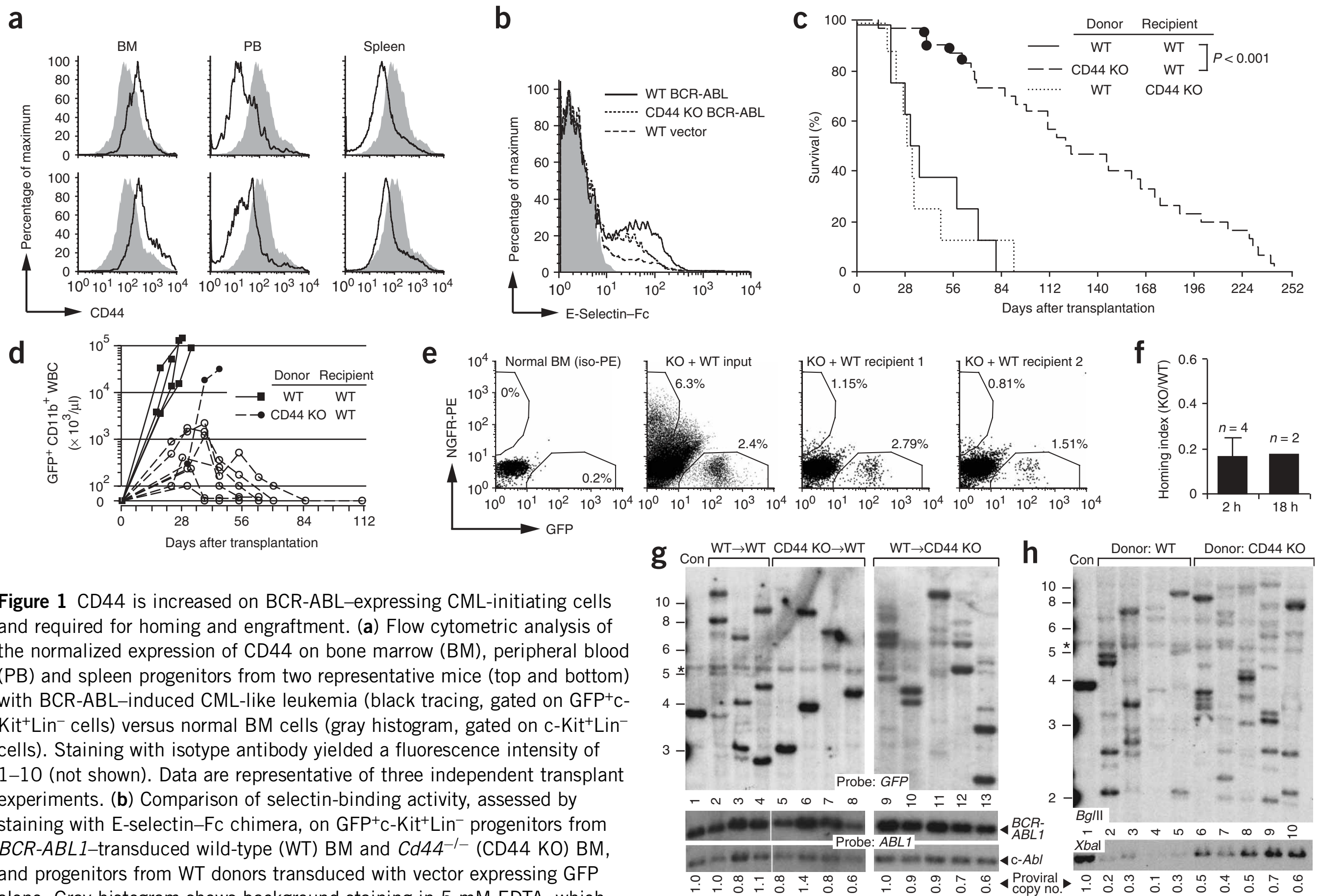

Figure 1 CD44 is increased on BCR-ABL–expressing CML-initiating cells and required for homing and engraftment. (**a**) Flow cytometric analysis of the normalized expression of CD44 on bone marrow (BM), peripheral blood (PB) and spleen progenitors from two representative mice (top and bottom) with BCR-ABL–induced CML-like leukemia (black tracing, gated on GFP$^+$c-Kit$^+$Lin$^-$ cells) versus normal BM cells (gray histogram, gated on c-Kit$^+$Lin$^-$ cells). Staining with isotype antibody yielded a fluorescence intensity of 1–10 (not shown). Data are representative of three independent transplant experiments. (**b**) Comparison of selectin-binding activity, assessed by staining with E-selectin–Fc chimera, on GFP$^+$c-Kit$^+$Lin$^-$ progenitors from *BCR-ABL1*–transduced wild-type (WT) BM and $Cd44^{-/-}$ (CD44 KO) BM, and progenitors from WT donors transduced with vector expressing GFP alone. Gray histogram shows background staining in 5 mM EDTA, which was similar for all populations. (**c**) Survival curves for WT B6×129 F_2 ($n = 24$) or $Cd44^{-/-}$ ($n = 8$) recipients of *BCR-ABL1*–transduced WT BM, and WT recipients of *BCR-ABL1*–transduced $Cd44^{-/-}$ BM ($n = 30$). Survival of recipients of $Cd44^{-/-}$ BM was significantly prolonged ($P < 0.001$). All mice that succumbed prior to day 60 developed CML-like leukemia (circles in the $Cd44^{-/-}$ donor cohort). (**d**) Time course of GFP$^+$CD11b$^+$ cells in peripheral blood of some of the recipients in **c**. Squares, recipients of transduced WT BM; open circles, recipients of transduced $Cd44^{-/-}$ BM that did not develop CML-like leukemia; filled circles, rare recipients that succumbed to CML-like disease. (**e**) Short-term competitive homing of *BCR-ABL1*–transduced WT and $Cd44^{-/-}$ progenitors. Irradiated recipient mice ($n = 4$) were injected with a mixture of *BCR-ABL1*–transduced WT BM (expressing GFP) and *BCR-ABL1*–transduced $Cd44^{-/-}$ BM (expressing NGFR) and killed 2 h later. From left to right: flow cytometric analysis of c-Kit$^+$Lin$^-$ normal BM stained with isotype-PE antibody; input mixture of transduced WT and $Cd44^{-/-}$ progenitors; and corresponding c-Kit$^+$Lin$^-$ populations isolated from BM of two representative recipients, stained with PE-conjugated antibody to human NGFR. The percentage of GFP$^+$ and NGFR$^+$ cells is indicated. (**f**) BM homing index for *BCR-ABL1*–transduced $Cd44^{-/-}$ progenitors relative to WT progenitors, corrected for the ratio of input cells (Methods). In independent experiments, similar results were obtained 2 h ($n = 4$) and 18 h ($n = 2$) after injection. (**g**) Impaired engraftment of *BCR-ABL1*–transduced $Cd44^{-/-}$ donor stem cells. Genomic DNA from spleens of leukemic mice was analyzed by Southern blot with a *GFP* probe to detect distinct proviral integration events (top), and an *ABL* probe allowing determination of the total proviral content of each sample (bottom). Control DNA (Con) was from a cell line that contains a single *BCR-ABL1*–provirus. Lanes 2–4, WT recipients of *BCR-ABL1*–transduced WT BM; lanes 5–8, WT recipients of *BCR-ABL1*–transduced $Cd44^{-/-}$ donor BM; lanes 9–13, $Cd44^{-/-}$ recipients of *BCR-ABL1*–transduced WT BM. The calculated proviral copy number is indicated. (**h**) No defect in engraftment of $Cd44^{-/-}$ HSCs transduced with empty GFP virus. BM from WT and $Cd44^{-/-}$ donors was transduced with empty retrovirus expressing GFP alone, and equivalent numbers of cells were transplanted into lethally irradiated WT recipients. After 10 weeks, recipient BM was collected, the number of engrafting proviral clones was determined by Southern blotting of *Bgl*II-digested DNA with a *GFP* probe (top), and the proviral copy number was determined on *Xba*I-digested DNA (bottom). Lanes 2–5, recipients of WT BM; lanes 6–10 recipients of $Cd44^{-/-}$ BM. In **g** and **h**, the *GFP* probe also faintly detects a common sequence (asterisk) in mouse genomic DNA.

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when $Cd44^{-/-}$ recipients were used, indicating that CD44 is not required in the bone marrow niche. By contrast, only 4 of 30 (13%) wild-type recipients of *BCR-ABL1*–transduced bone marrow from $Cd44^{-/-}$ donors developed CML-like disease (**Fig. 1c**), showing that bone marrow lacking CD44 is profoundly defective for CML leukemogenesis. Half (16/30) of the recipients eventually succumbed to BCR-ABL–induced lymphoid leukemias of B or T cell origin, whereas the rest died of nonmalignant causes. Some of these recipients had transient circulating myeloid cells positive for green fluorescent protein (GFP; **Fig. 1d** and **Supplementary Fig. 1** online), consistent with engraftment by progenitors with short-term repopulating potential. The overall survival of this cohort was significantly prolonged ($P < 0.001$, Mantel-Cox test).

To obtain direct evidence that CD44 has a role in leukemic stem cell engraftment, we used a competitive homing assay[25]. Wild-type donor bone marrow was transduced with retrovirus coexpressing BCR-ABL and GFP, whereas $Cd44^{-/-}$ bone marrow was transduced with virus of equivalent titer coexpressing BCR-ABL and a truncated human nerve growth factor receptor (NGFR) cell surface reporter. The two populations were mixed and injected into irradiated recipients, and recovery

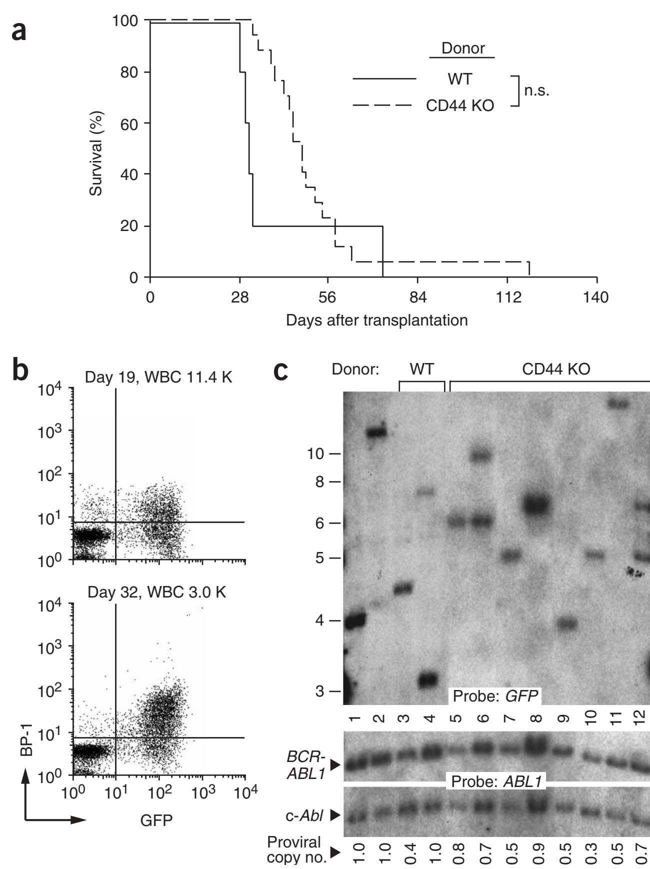

Figure 2 Donor CD44 is not required for engraftment of B-ALL–initiating cells. (**a**) Survival curve for WT recipients of *BCR-ABL1*–transduced BM from non-5-FU–treated WT donors ($n = 5$), and for WT recipients of *BCR-ABL1*–transduced *Cd44*$^{-/-}$ BM ($n = 17$). All recipients succumbed to B-ALL. There was no significant difference in the survival of the two cohorts ($P = 0.2254$). (**b**) Serial flow cytometric analysis of peripheral blood from a representative leukemic WT recipient of *BCR-ABL1*–transduced BM from non-5-FU–treated *Cd44*$^{-/-}$ donors, showing expression of GFP and BP-1, a marker of immature B-lymphoid cells. Blood sampling and analysis were done on days 19 and 32 after transplantation; the respective total blood leukocyte counts (WBCs) were 11,400 and 3,000 cells/mm^3. (**c**) Southern blot analysis of genomic DNA from leukemic spleens of recipients of *BCR-ABL1*–transduced BM from WT (lanes 3–4) or *Cd44*$^{-/-}$ (lanes 5–12) donors, using *GFP* and *ABL1* probes as in **Figure 1g**. The disease is mono- to oligoclonal in both cohorts.

from recipient bone marrow was assessed 2 h later (**Fig. 1e**). There was a marked decrease in the recovery of *BCR-ABL1*–transduced *Cd44*$^{-/-}$ progenitors (NGFR$^+$c-Kit$^+$Lin$^-$) relative to *BCR-ABL1*–transduced wild-type progenitors (GFP$^+$c-Kit$^+$Lin$^-$). When normalized for the ratio of input cells, the homing index of *BCR-ABL1*–transduced *Cd44*$^{-/-}$ progenitors was less than 0.2, indicating a more than fivefold decrease in bone marrow homing as compared with wild-type progenitors (**Fig. 1f**). Similar results were obtained when recipients were collected 18 h after injection (**Fig. 1f**). As previous studies found homing of untransduced *Cd44*$^{-/-}$ HSCs to be normal[23], these results suggest that BCR-ABL expression increases the dependence of stem cells on CD44 for engraftment.

We assessed engraftment of leukemia-initiating cells by counting the number of proviral clones in leukemic cell DNA. In wild-type or *Cd44*$^{-/-}$ recipients of *BCR-ABL1*–transduced wild-type donor bone marrow, the CML-like leukemia was oligoclonal to polyclonal, as previously reported[21], and an average of 5.8 ± 2.0 independent clones contributed to the MPD (**Fig. 1g**). In rare recipients of *BCR-ABL1*–transduced *Cd44*$^{-/-}$ bone marrow that developed CML-like disease, by contrast, the number of proviral clones was significantly lower ($P = 0.0017$, *t*-test), with engraftment of an average of 1.5 ± 0.6 BCR-ABL$^+$ stem cells per recipient (**Fig. 1g** and **Supplementary Table 1** online). To determine whether CD44 deficiency affects engraftment of normal HSCs in this model, we transduced bone marrow from wild-type and *Cd44*$^{-/-}$ donors with retrovirus expressing GFP alone and transplanted equal numbers of cells into lethally irradiated wild-type recipients (**Fig. 1h**). After 10 weeks, we observed equivalent numbers of proviral clones in bone marrow–derived myeloid cells from recipients of wild-type (6.5 ± 0.6) and *Cd44*$^{-/-}$ (8.2 ± 0.7) marrow ($P = 0.1355$), indicating that, in the absence of BCR-ABL and in agreement with previous studies[23], transduced *Cd44*$^{-/-}$ stem cells engraft as efficiently as do wild-type HSCs. These results show that CD44 is selectively required on BCR-ABL–expressing leukemic stem cells for efficient engraftment and subsequent development of CML-like MPD.

Because most recipients of *BCR-ABL1*–transduced *Cd44*$^{-/-}$ bone marrow developed lymphoblastic leukemia (**Fig. 1c**), CD44 may not be required for engraftment of this disease, which originates from committed lymphoid progenitors[4]. To test this idea, we used *Cd44*$^{-/-}$ donors in a model in which all recipients of *BCR-ABL1*–transduced bone marrow develop B-cell acute lymphoblastic leukemia (B-ALL) rather than CML[21]. There was no defect in lymphoid leukemogenesis by *BCR-ABL1*–transduced *Cd44*$^{-/-}$ donor cells (**Fig. 2a**): all recipients developed leukemia of immature (B220$^+$BP-1$^+$) B-lymphoid phenotype (**Fig. 2b**) within 5–7 weeks, and the disease was mono- to oligoclonal in both cohorts (**Fig. 2c**), as previously reported[21]. These results show that CD44 is selectively required for engraftment of BCR-ABL$^+$ CML-initiating cells, but is dispensable for engraftment of B-ALL–initiating cells.

To confirm that the leukemogenesis defect of *BCR-ABL1*–transduced *Cd44*$^{-/-}$ progenitors was due to decreased bone marrow homing, we injected the same number of *BCR-ABL1*–transduced wild-type or *Cd44*$^{-/-}$ cells directly into the femoral bone marrow cavity of irradiated wild-type recipients[26]. About 70% of recipients injected intrafemorally with *BCR-ABL1*–transduced wild-type progenitors developed CML-like leukemia within 7 weeks (**Fig. 3a**), reflecting the technical difficulty of this procedure. A similar frequency of CML-like leukemia was induced by intrafemoral transplantation of *BCR-ABL1*–transduced *Cd44*$^{-/-}$ progenitors, showing that delivery of BCR-ABL–expressing *Cd44*$^{-/-}$ leukemic stem cells directly to the marrow can bypass the requirement for CD44. Intrafemoral transplantation also significantly increased the clonality of the CML-like leukemia induced by *Cd44*$^{-/-}$ progenitors (3.9 ± 2.0 clones per recipient versus 1.5 ± 0.5 clones for the same population transplanted intravenously, $P = 0.017$; **Fig. 3b**). These results indicate that the leukemogenic defect of BCR-ABL–expressing *Cd44*$^{-/-}$ stem-progenitors cells is predominantly a consequence of impaired marrow homing and engraftment.

We also tested whether the genomic *Cd44* mutation could be complemented or 'rescued' by transduction of *Cd44*$^{-/-}$ progenitors with retrovirus coexpressing BCR-ABL and a human *CD44* cDNA, which expresses the non-spliced (standard) CD44 isoform, CD44s, in leukocytes at levels slightly higher than endogenous CD44 (**Supplementary Fig. 2** online). Coexpression of BCR-ABL and CD44s markedly increased the incidence of CML-like leukemia in recipients from 13% to more than 60% (**Fig. 3c**), but did not affect leukemogenesis by wild-type donor cells or alter the phenotype of the MPD, although myeloid cells expressed CD44s (**Fig. 3d**). There was a modest

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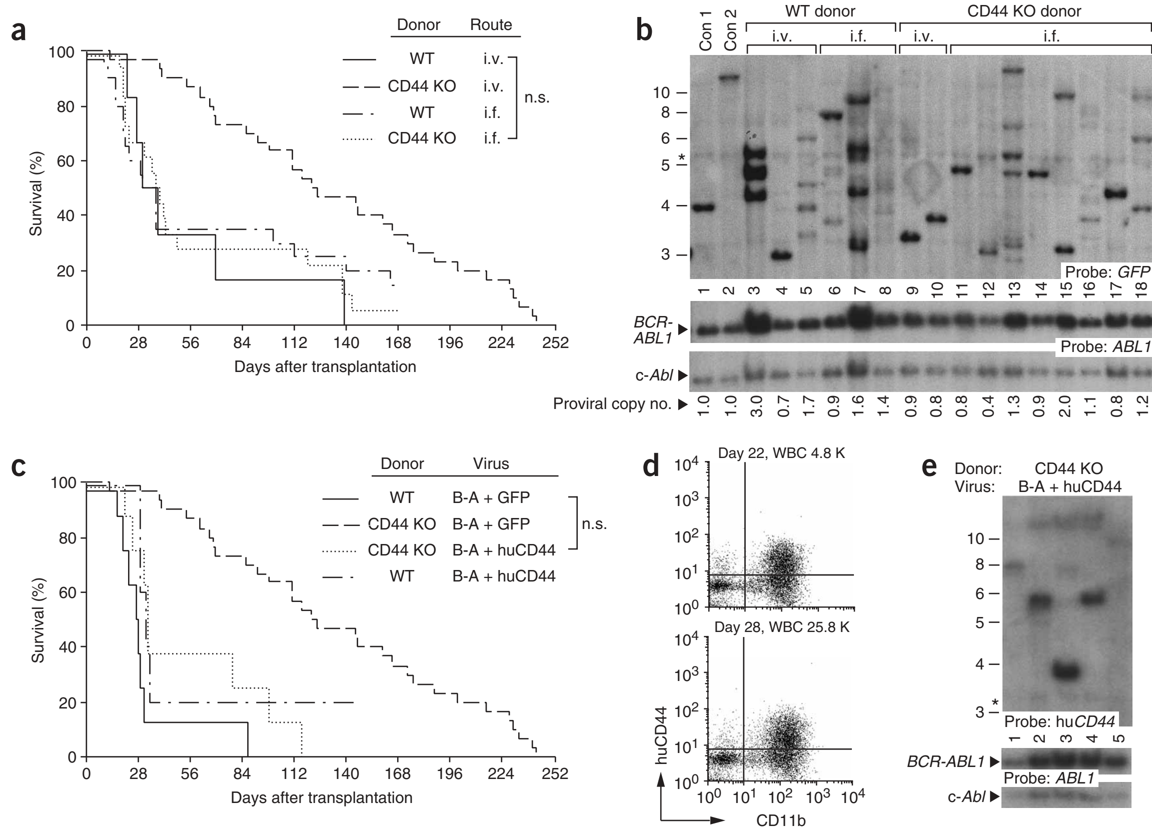

Figure 3 Engraftment defect of *BCR-ABL1*–transduced $Cd44^{-/-}$ progenitors is rescued by direct intrafemoral injection or by coexpression of human CD44s. (**a**) Survival curve for WT recipients of *BCR-ABL1*–transduced BM from WT donors transplanted either intravenously (i.v.; $n = 6$) or intrafemorally (i.f.; $n = 20$), and for WT recipients of *BCR-ABL1*–transduced BM transplanted i.f. ($n = 18$). Transplantation of transduced $Cd44^{-/-}$ BM i.v. ($n = 30$; from **Fig. 1**) is shown for comparison. All mice that succumbed before day 60 developed CML-like leukemia; mice that became ill later than 2 months after transplant died of BCR-ABL$^+$ lymphoid leukemia or histiocytic sarcoma. Overall survival of recipients of WT donor cells transplanted i.v. versus $Cd44^{-/-}$ donor cells transplanted i.f. was not significantly different ($P = 0.768$). (**b**) Southern blot analysis of genomic DNA from leukemic spleen cells from recipients in **a** using *GFP* and *ABL1* probes (see **Fig. 1g**). Leukemias induced by WT donor cells were polyclonal whether the cells were transplanted i.v. (lanes 3–5) or i.f. (lanes 6–8), but the number of engrafting $Cd44^{-/-}$ leukemic stem cells was increased by i.f. transplantation (see lanes 13, 16 and 18). The *GFP* probe also detects a common sequence (asterisk) in mouse genomic DNA. (**c**) Survival curve for recipients of WT BM transduced with retrovirus coexpressing BCR-ABL and either GFP (B-A + GFP; $n = 8$) or human CD44s (B-A + huCD44; $n = 5$), and for recipients of $Cd44^{-/-}$ BM transduced with B-A + huCD44 retrovirus ($n = 8$). Transplantation of $Cd44^{-/-}$ BM transduced with B-A + GFP retrovirus ($n = 30$; from **Fig. 1**) is shown for comparison. The overall survival of recipients of WT donor cells transduced with B-A + GFP retrovirus versus $Cd44^{-/-}$ donor cells transduced with B-A + huCD44 retrovirus was not significantly different ($P = 0.0667$). (**d**) Serial flow cytometric analysis of a representative leukemic recipient of $Cd44^{-/-}$ BM transduced with B-A + huCD44 retrovirus, showing expression of CD11b and human CD44. Blood sampling and analysis were done on days 22 and 28 after transplantation; the respective total blood leukocyte counts (WBCs) were 4,800 and 25,800 cells/mm^3. (**e**) Southern blot analysis of genomic DNA from recipients of $Cd44^{-/-}$ BM transduced with B-A + huCD44 retrovirus that developed CML-like leukemia (lanes 1–5), using a probe from the human *CD44* gene (top) to detect individual provirus integration sites, and from the *ABL1* gene to assess loading (bottom). The common band at ~3 kb (asterisk) detected with the human *CD44* probe arises from the murine *Cd44* gene.

but significant increase in the clonality of the CML-like leukemia (**Fig. 3e**) in recipients of $Cd44^{-/-}$ bone marrow transduced with CD44s virus (3.0 ± 1.2 clones versus 1.5 ± 0.5 clones for GFP virus, $P = 0.0239$), showing that rescue correlates with increased engraftment of leukemic stem cells. Complete leukemia rescue may require expression of variant CD44 isoforms found on normal HSCs[27]. Although we cannot exclude the possibility that CD44 functions in a step after engraftment, such as leukemic stem cell survival or proliferation, rescue was much more efficient when transplantation was delayed by 24 h to allow increased cell-surface expression of CD44s (data not shown), suggesting that CD44 functions immediately after injection.

As a step towards the development of clinically relevant methods to block leukemic stem cell engraftment, we treated *BCR-ABL1*–transduced BALB/c bone marrow before transplantation with antibodies to CD44 and/or P-selectin glycoprotein ligand-1 (PSGL-1), a principal selectin ligand expressed on stem-progenitor cells (**Fig. 4a**). Antibody to PSGL-1 modestly decreased engraftment (**Fig. 4b**) but did not prolong overall survival, possibly because of the more aggressive MPD induced by BCR-ABL in this strain[24]. By contrast, an antibody to CD44 (clone IM7) that blocks hyaluronan binding, either alone or in combination with anti–PSGL-1, significantly attenuated CML-like leukemia in recipients (**Fig. 4a**) and also reduced engraftment, although this reduction did not reach statistical significance. Hyaluronan binding by CD44 may also contribute to homing of normal HSCs, because IM7 partially blocks marrow homing of mouse CFU-S[28,29]. For human HSCs, SDF-1 increases CD44 binding to hyaluronan, whereas a different hyaluronan-blocking CD44 antibody reduces SDF-1–dependent homing and engraftment in

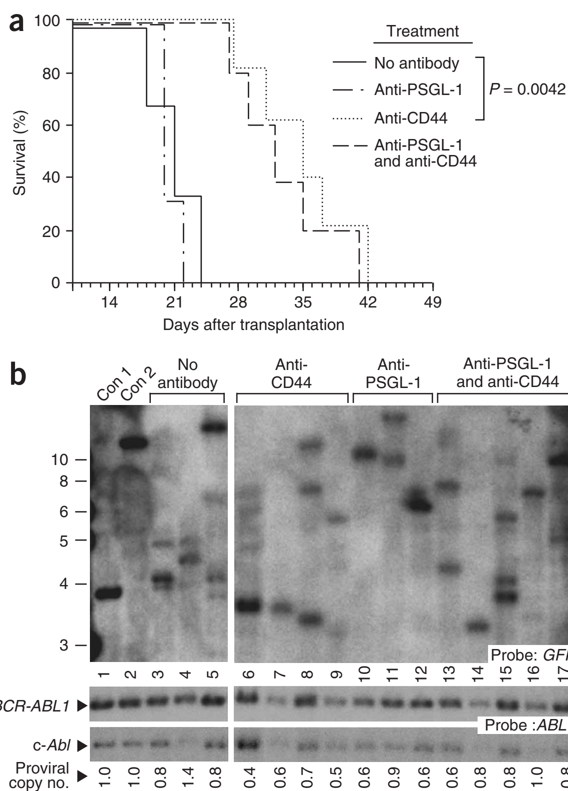

Figure 4 CD44-specific antibody treatment prolongs survival of recipients of *BCR-ABL1*–transduced progenitors. (**a**) Survival curve for WT recipients of *BCR-ABL1*–transduced WT BM either untreated ($n = 3$) or incubated with monoclonal antibodies to CD44 ($n = 5$), to PSGL-1 ($n = 3$) or to both CD44 and PSGL-1 ($n = 5$). All mice developed CML-like MPD. The prolongation in survival in the recipients of CD44-treated BM was statistically significant as compared with recipients of untreated BM ($P = 0.0042$). (**b**) Southern blot analysis of genomic DNA of leukemic spleens from the cohorts in **a** who received untreated (lanes 3–5), CD44 antibody–treated (lanes 6–9), PSGL-1 antibody–treated (lanes 10–12), or CD44 and PSGL-1 antibody–treated (lanes 13–17) *BCR-ABL1*–transduced BM, using *GFP* and *ABL1* probes (see **Fig. 1g**).

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NOD-SCID (non-obese severe combined immunodeficient) mice[30]. CML progenitors have defective CXCR4 signaling[15] and may lack both responses, however, suggesting that hyaluronan is not the predominant counter-receptor for CD44 in CML stem cell homing. As our previous results implicated recipient selectins in engraftment of BCR-ABL–expressing progenitors[16], and most CD44 antibodies (including IM7) do not block CD44-dependent selectin ligands (R. Sackstein, personal communication), testing other antibodies is warranted. Genetic analysis of CD44 ligands in leukemogenesis by expression of CD44 mutants in the leukemic stem cells is also indicated.

In conclusion, our studies show that CD44 has a key role in homing and engraftment of BCR-ABL–expressing leukemia-initiating cells in the mouse. We consider CD44 a promising target for preventing engraftment of leukemic stem cells in individuals with CML treated by autologous transplantation. Current translational studies are directed at developing protocols to achieve this goal in the clinic.

METHODS

Retroviral constructs. We ligated the cDNA encoding the human CD44s isoform (obtained from B. Seed, Massachusetts General Hospital) into the pMIGR1 vector in place of GFP. The MSCV-IRES–NGFR vector was obtained from W. Pear (University of Pennsylvania). We subsequently cloned the p210 BCR-ABL cDNA into the *Eco*RI site 5′ of the IRES in both vectors.

Bone marrow transduction and transplantation. All mouse experiments were approved by the Institutional Animal Use and Care Committee of Tufts– New England Medical Center. We produced ecotropic replication-defective retroviral stocks by co-transfection of 293 cells with the *kat* packaging plasmid[4]. We matched viral titers by flow cytometric analysis of transduced fibroblasts and primary bone marrow cells, and by Southern blot analysis of proviral copy number.

For induction of CML-like leukemia, we collected bone marrow from B6×129 F_2 wild-type or *Cd44*$^{-/-}$ donor mice[20] (Jackson Laboratory) 4 d after intravenous administration of 200 mg per kg (body weight) 5-fluorouracil (5-FU), transduced with retrovirus *in vitro* as described[4], and injected 3×10^5 cells intravenously into lethally irradiated (1,050 cGy) B6×129 F_2 recipients. For induction of B-ALL, we transduced bone marrow from untreated wild-type or *Cd44*$^{-/-}$ mice with BCR-ABL retrovirus, and injected 10^6 cells into lethally irradiated wild-type recipients[21]. Intrafemoral injection of transduced bone marrow was done by flexing the knee and by making an intrafemoral tunnel with a 27G needle just above the patellar ligament, as described[26]. A total of 3×10^5 transduced cells in a volume of 30 μl were subsequently injected with a 0.5-ml insulin syringe and a 28G needle.

For antibody blocking experiments, we incubated 2–3 × 10^6 transduced donor bone marrow cells with 10 μg of antibody to murine CD44 (clone IM7, a rat IgG2b monoclonal antibody recognizing all human and mouse isoforms of the CD44 glycoprotein expressed on hematopoietic cells), antibody to PSGL-1 (clone 2PH1), or a combination of the CD44- and PSGL-1–specific antibodies (both from Pharmingen) in 1 ml of Hank's balanced salt solution (HBSS) for 30 min at 20 °C, washed the cells once in HBSS, and injected them into the recipient mice.

Analysis of diseased mice. We diagnosed CML-like leukemia, B- or T-ALL, or histiocytic sarcoma based on clinicopathological criteria, as described[4]. For analysis of CD44 expression, we stained leukocytes from mice with established CML-like leukemia with biotinylated antibodies to lineage antigens (CD5, B220, Gr-1 and TER119), peridinin chlorophyll protein–conjugated streptavidin, allophycocyanin, conjugated antibody to c-Kit (clone 2B8), and phycoerythrin (PE)-conjugated antibody to pan CD44 (clone IM7; all from Pharmingen), and analyzed the cells by four-color flow cytometry gated on GFP^+c-$Kit^+$$Lin^-$ cells. For analysis of circulating leukemic cells, we stained GFP^+ leukocytes with PE-conjugated antibody to CD11b (CML-like leukemia) or PE-conjugated antibody 6C3 to BP-1 (B-ALL).

For analysis of human CD44 expression, we stained transformed lymphoblasts or leukocytes from mice with CML-like leukemia with fluorescein isothiocyanate (FITC)-conjugated antibody to CD11b or B220, respectively, along with PE-conjugated antibody to human CD44 (clone G44-26) or to pan-CD44 (clone IM7). For analysis of selectin ligands, cells were stained with 5 μg/ml of recombinant murine E-selectin–Fc fusion protein (R&D Systems) in HBSS containing 5% fetal calf serum and 2 mM $CaCl_2$, and counterstained with goat F(ab′)$_2$ antibody to human IgG (CalTag). As a control for specificity, parallel staining was carried out in buffer containing 5 mM EDTA.

Southern blot analysis. We digested genomic DNA from leukemic tissues with *Bgl*II, transferred DNA to nylon membranes, and hybridized the membranes with a radioactively labeled probe from the *GFP* or the human *CD44* gene to detect distinct retroviral integration events. Subsequently, the membrane was probed with a human *ABL* probe to determine the total proviral content of each sample[4].

Competitive homing analysis. The competitive *in vivo* homing assay was done as described[25]. We transduced marrow from 5-FU–treated wild-type donors with BCR-ABL plus GFP or BCR-ABL plus NGFR retrovirus, mixed equal numbers of the two cell populations, and determined the input ratio of GFP^+ to $NGFR^+$ cells in the c-$Kit^+$$Lin^-$ fraction by flow cytometry using PE-conjugated antibody to human NGFR (CD271; clone C40-1457, BD Pharmingen). We injected 1–2 × 10^7 cells from this mixture into each irradiated wild-type recipient. Between 2 and 18 h after injection, we collected

recipient bone marrow and analyzed the populations by flow cytometry for the proportions of c-Kit$^+$Lin$^-$ cells expressing GFP or NGFR. The homing index (HI) was calculated as the ratio of (c-Kit$^+$Lin$^-$GFP$^+$)$_{tissue}$/(c-Kit$^+$Lin$^-$NGFR-PE$^+$)$_{tissue}$ to (c-Kit$^+$Lin$^-$GFP$^+$)$_{input}$/(c-Kit$^+$Lin$^-$NGFR-PE$^+$)$_{input}$.

Statistical methods. We assessed differences in overall survival of transplanted cohorts by Kaplan-Meier non-parametric estimates (Mantel-Cox or log-rank tests), and confirmed the assumption of proportional hazards by analysis of log cumulative hazard functions using StatView (SAS Institute). We analyzed differences in mean provirus number between different cohorts by unpaired *t*-test, and present the data as the mean ± s.d.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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