Acute myeloid leukemia is propagated by a leukemic stem cell with lymphoid characteristics in a mouse model of *CALM/AF10*-positive leukemia

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Summary

A challenge for the development of therapies selectively targeting leukemic stem cells in acute myeloid leukemia (AML) is their similarity to normal hematopoietic stem cells (HSCs). Here we demonstrate that the leukemia-propagating cell in murine *CALM/AF10*-positive AML differs from normal HSCs by B220 surface expression and immunoglobulin heavy chain rearrangement. Furthermore, depletion of $B220^+$ cells in leukemic transplants impaired development of leukemia in recipients. As in the murine model, human *CALM/AF10*-positive AML was characterized by CD45RA (B220)-positive, *IG* DH-JH rearranged leukemic cells. These data demonstrate in a murine leukemia model that AML can be propagated by a transformed progenitor with lymphoid characteristics, which can be targeted by antibodies that do not crossreact with normal HSCs.

Introduction

For most cancers, the cell that propagates tumor growth and is thought to play a key role in recurrence of disease is unknown. The study of normal and malignant hematopoiesis has formed a roadmap for the detailed analysis of the concept of the tumor-initiating cell and has demonstrated that, in leukemia, the malignant clone is organized in a hierarchy in which only a small subpopulation of cells—the leukemic stem cells (LSCs)—are able to initiate and propagate the disease (Hope et al., 2004). In AML most studies indicate that malignant transformation targets cells within the hematopoietic stem cell compartment. However, more recent data demonstrated that multipotential progenitors or more committed myeloid progenitors can also acquire leukemic stem cell properties when transformed by appropriate oncogenes (Cozzio et al., 2003; So et al., 2003). These reports clearly demonstrated that cells other than hematopoietic stem cells can be the target of fully transforming oncogenes, which are characterized by their ability to transfer self-renewal potential to committed stages of differentiation (Huntly et al., 2004; Jamieson et al., 2004). The identification of leukemic stem cell properties is a key step for an improved understanding of the biology of acute leukemias and might also have clinical implications: because of its essential role for the initiation and maintenance of the leukemic clone, a curative therapeutic strategy should be able to eradicate the LSC in the diseased patients.

SIGNIFICANCE

The identification of tumor stem cells is one of the major goals of cancer research. We report on a murine model demonstrating that myeloid malignancies such as acute myeloid leukemia can be propagated by a transformed progenitor with lymphoid characteristics. These data extend our previous knowledge about leukemia stem cell candidates in acute leukemia and indicate that leukemia-propagating cells in AML might differ from the myeloid blast population as well as from the normal hematopoietic stem cell in their surface antigen profile. These findings suggest that such leukemic stem cell candidates can be targeted with antibodies that spare the normal stem cell pool. This may pave the way for innovative antibody-based therapeutic strategies in this disease.

At the same time, such a strategy should spare normal hematopoietic stem cells (HSCs) to avoid intolerable therapy-associated stem cell toxicity. However, the major challenge for the development of such therapeutic approaches is the often striking similarity between leukemic and normal HSC, e.g., with regard to their cell surface markers (Reya et al., 2001; Warner et al., 2004). The limitations of the therapeutic tools available today are well known: although standard chemotherapy is highly effective in inducing remissions in patients with AML, the majority of patients finally relapse and succumb to this disease (Hiddemann et al., 2005). One of the reasons for treatment failure is that even dose-intensive conventional chemotherapy is not able to efficiently target the LSC, demonstrated, for example, by the observation that patients in morphological complete remission harbor $CD34^+/CD38^-$ progenitor cells with the leukemia-specific genetic alteration in their bone marrow (Feuring-Buske et al., 1999). A major step toward the development of stem cell-targeted AML therapies would be the identification of key differences between normal HSCs and the LSC. It has been reported that the LSC differs from its normal counterpart by the expression of CD90 or the interleukin-3α receptor, and in a preclinical NOD/SCID mouse model a diphtheria toxin (DT) human interleukin 3 fusion protein (DT388IL3) was able to selectively kill LSCs in patients with different subtypes of AML (Blair et al., 1997; Feuring-Buske et al., 2002; Jordan et al., 2000).

The recent reports demonstrating that acute leukemias can also arise from transformed committed progenitor cells might improve the chances to define biological differences between the LSC and the HSC (Huntly et al., 2004; So et al., 2003). However, leukemic stem cell candidates such as multipotent progenitor cells (MPPs) might be difficult to distinguish from normal HSCs, although first strategies were established in mice to discriminate MPPs from long-term repopulating stem cells by immunophenotype (Forsberg et al., 2005; Morrison et al., 1997). In this report we present experimental evidence that acute myeloid leukemia can be propagated by a transformed progenitor, which differs from normal hematopoietic stem cells by surface expression of B220 and immunoglobulin heavy chain (*IG* DH-JH) rearrangement. We established a murine model of *CALM/AF10* (C/A)-positive acute myeloid leukemia using the bone marrow transplantation model and retroviral gene transfer to constitutively express the fusion gene in hematopoietic progenitor cells. The established model well predicted our findings in patients with *C/A*-positive AML, who showed a cell population expressing CD45RA, the human homolog of B220. This human $CD45RA^+$ cell population displayed *IG* DH-JH rearrangements, was positive for the fusion gene as detected by fluorescence in situ hybridization (FISH), and was able to form CFU-blast colonies in vitro. In these patients the fusion gene is formed by the translocation t(10;11) (p13;q14), which involves the *CALM* gene (associated with clathrin-mediated endocytosis) and *AF10* (a putative transcription factor). This fusion gene is found in a variety of leukemias, which are all characterized by a very poor prognosis (Dreyling et al., 1998). Of note, the malignant phenotypes associated with C/A span AML, undifferentiated leukemia, acute lymphoblastic leukemia, and T cell lymphoma, thus making it appealing to determine the nature of the leukemic stem cell candidate(s) in *C/A*-positive disease. The results of this study demonstrate in a murine leukemia model that a transformed progenitor cell population with lymphoid characteristics can play a key role in propagating *C/A*-positive AML.

Results

CALM/AF10 induces acute myeloid leukemia in mice

To test the impact of constitutive expression of *C/A* on murine hematopoietic development, we employed the murine bone marrow (BM) transplantation model. The fusion gene was expressed in a murine stem cell virus (MSCV)-based retroviral construct carrying the *C/A* cDNA upstream of an internal ribosomal entry site (IRES)-green fluorescence protein (GFP) cassette that has been shown to efficiently transduce hematopoietic stem and progenitor cells (Figures S1A–S1C in the Supplemental Data available with this article online).

All mice (n = 13) transplanted with highly purified GFP^+ *C/A* transduced cells (median retroviral transduction efficiency of 5.7, range 1.2%–16.4%) with or without nontransduced helper cells developed a disease after a median of 110 days posttransplantation (range 46–366) (Figure 1A) and suffered from cachexia, lethargy, and shortness of breath. Detailed analyses of the hematopoietic compartments showed a more than 10-fold elevated white blood cell count in the peripheral blood (PB) of all animals with a median of 4.9×10^7 cells/ml (range 3.2–8.5×10^7) compared to the GFP control (median 0.48, range 0.32–0.8×10^7) ($p < 0.002$). Furthermore, mice were anemic, with a 7.3-fold reduced median red blood cell count of 0.75×10^9 cells (range 0.6–1.25×10^9) versus 5.5×10^9 (range 4.8–7×10^9) in the control mice ($p < 0.0001$), and suffered from splenomegaly (Table 1). Panoptic staining of PB smears and cytospin preparations of BM and spleen cells revealed an accumulation of blasts accounting for medians of 32%, 40%, and 39%, respectively, of all cells (Table 1; Figure 1B). The spleen showed a massive infiltration with myeloid cells (median of 86.9% $Mac1^+$ cells [range 72.8%–91.1%] versus 12% in the control [range 7%–26.5%] and 62.5% $Gr1^+$ cells [range 40%–76.2%] compared to 9.5% in the control [range 7.5%–11.3%]) (Figure 1B). Histological sections demonstrated infiltration of blasts in multiple nonhematopoietic organs, including the brain (Figure 1C). Immunohistochemistry showed positivity of the blasts for myeloperoxidase (*MPO*) and chloracetate esterase and negativity for B220 and CD3, indicating the myeloid nature of the population (Figure 1D). Taken together, the mice suffered from acute myeloid leukemia according to the Bethesda proposal for classification of nonlymyphoid and lymphoid hematopoietic neoplasms in mice (Kogan et al., 2002; Morse et al., 2002).

Transplantation of BM cells of leukemic mice into secondary recipients (n = 22) rapidly induced leukemia with the same characteristics after a median of 15 days (range 14–21 days) posttransplantation (Figure 1A). Bone marrow aspirations of anesthetized primary recipients 8 weeks posttransplantation did not show any myeloproliferative or lymphoproliferative syndrome before the onset of leukemia (n = 5). Analysis of proviral integration sites demonstrated oligoclonal disease in primary and secondary mice (Figure S2). Sequencing of the retroviral integration sites (n = 18) in the diseased mice showed 10 out of 18 sites in or near regions described as common integration sites (CIS) in the RTCGD database (http://rtcgd.ncifcrf.gov/) (Akagi et al., 2004). No recurrent integration sites or any association of the leukemic phenotype with individual proviral integrations were observed (Table S1). In summary, these data demonstrated that constitutive expression of *CALM/AF10* induced short-latency AML in transplanted animals.

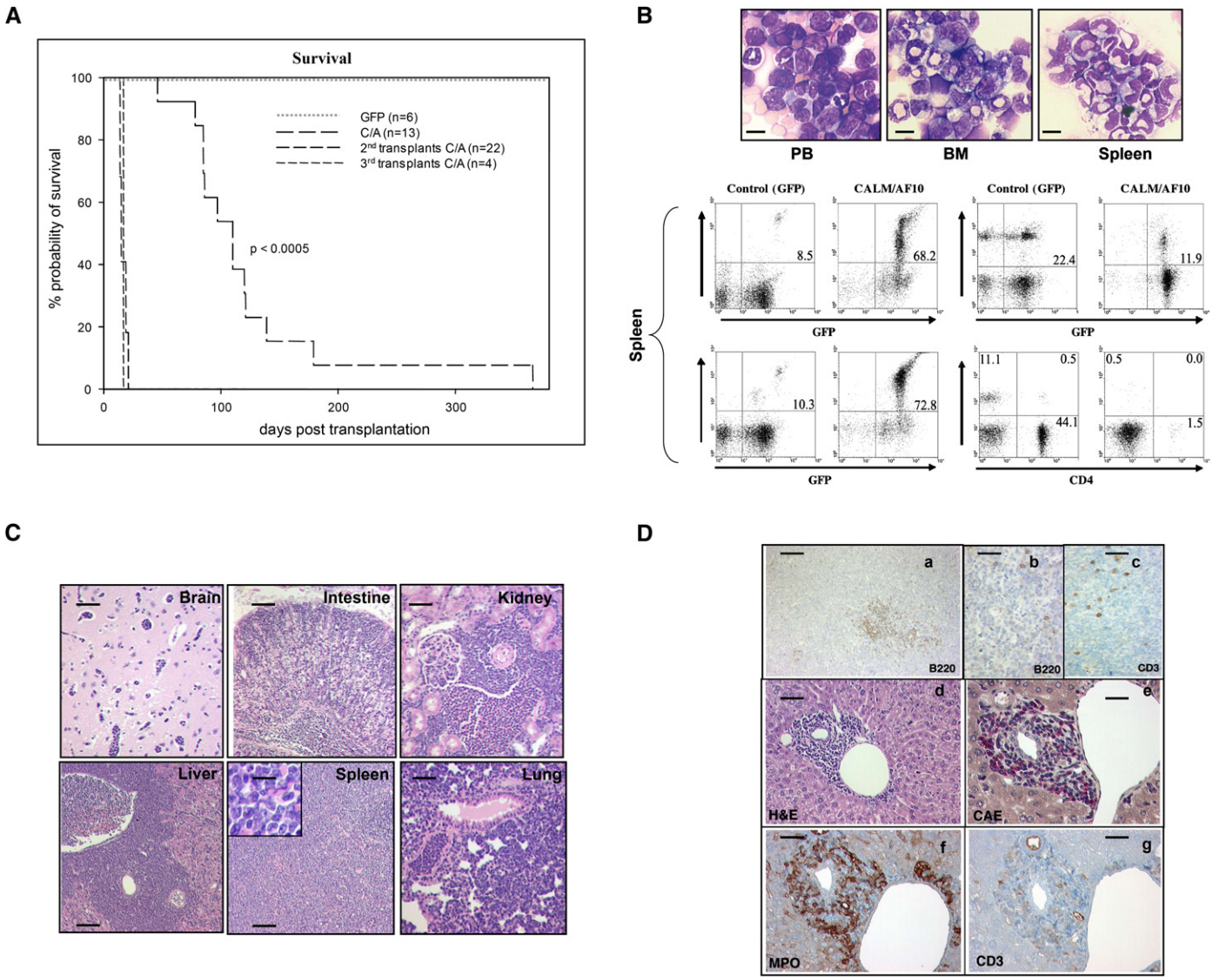

Figure 1. CALM/AF10 induces transplantable AML

A: Survival curve of mice transplanted with BM cells expressing *CALM/AF10* (n = 13, originating from five independent sets of transduction experiments). The control group was transplanted with BM infected with the GFP empty retrovirus (n = 6). The survival time of secondary recipient mice, transplanted with BM from diseased primary *CALM/AF10* mice or tertiary recipient mice injected with BM from leukemic secondary mice, is indicated. C/A, CALM/AF10.

B: Cytospin preparations from PB, BM, and spleen and immunophenotype of the spleen of a representative leukemic *CALM/AF10* mouse in comparison to a GFP control animal. Cells were stained for the myeloid markers Gr1 and Mac1 and the lymphoid markers B220, CD4, and CD8. The proportion of positive cells within the GFP^+ compartment is indicated; the CD4/CD8 costaining was gated for GFP^+ cells. BM, bone marrow; PB, peripheral blood. Scale bar, 10 μm.

C: Histological analysis of diseased *CALM/AF10* mice. Blast infiltration in the different organs is shown (brain, intestine, glomerular and tubular areas of the kidney, sinusoidal and portal areas of the liver, spleen, and lungs) Scale bar, 100 μm; scale bar in inset picture of spleen, 25 μm.

D: Histochemical and immunohistological analysis of the spleen and liver of a diseased *CALM/AF10* mouse. Anti-B220 (**Da** and **Db**) and anti-CD3 staining (**Dc**) of spleen sections and hematoxylin-eosin (**Dd**), CAE (**De**), MPO (**Df**), and anti-CD3 staining (**Dg**) of liver sections. MPO, myeloperoxidase; CAE, N-acetyl-chloroacetate esterase. Scale bars, 100 μm in **Db**–**Dg**, 25 μm in **Da**, 50 μm in **Dd**.

CALM/AF10-induced leukemia is propagated by a transformed $B220^+$, immunoglobulin DH-JH rearranged progenitor cell

More detailed analyses of the leukemic BM population (GFP^+ cells) revealed that, in addition to a majority of cells expressing myeloid markers (MM) ($Mac1^+$ cells, 82.9% [±8.6]; $Gr1^+$ cells, 86.4% [±3.7]), there was a small $B220^+/MM^-$ cell population in all animals obtained from five independent sets of experiments, which accounted on average for 6.7% (±2.1%) of the cells, comparable to the proportion of these cells in control mice (on average 9.4% ± 3%). An average of 26.0% (±8.6) and 32.5% (±13.2) of the BM cells coexpressed B220 with Mac1 or B220 with Gr1, respectively (n = 4) ($B220^+/MM^+$). Highly purified cells of the $B220^+/MM^-$ population had an immature, blast-like appearance, whereas the $B220^-/MM^+$ population included blasts as well as terminally differentiated myeloid cells (Figure 2A). Interestingly, all three subpopulations, including the cells expressing solely the MMs Mac1 or Gr1, showed DJ

Table 1. Hematological parameters in representative experimental mice

Mouse no.	Retroviral construct	Day of sacrifice	RBCs/ml × 10^9	WBCs/ml × 10^7	Spleen weight (mg)	Percent BM blasts	Percent spleen blasts	Percent PB blasts	IgH DJ rearrangement
1	C/A	10	0.75	3.5	700	30	32	27	ND
2	C/A	78	0.75	5.0	400	40	30	33	+
3	C/A	85	0.6	8.5	250	65	60	61	+
4	C/A	121	0.82	5.2	300	38	38	31	+
5	C/A	85	0.6	4.8	200	78	40	ND	+
6	C/A	86	1.25	3.2	400	ND	42	32	+
7[a]	C/A	67	4	2.5	240	32	29	33	+
8[a]	C/A	98	2.65	ND	350	36	ND	28	–
9	GFP	90	6	0.45	150	0	0	0	ND
10	GFP	157	4.8	0.32	200	0	0	0	ND
11	GFP	85	5	0.36	200	0	0	0	ND
12[b]	GFP	ND	4.8	0.76	ND	1	0	0	ND
13[b]	GFP	ND	6.4	0.8	ND	0	0	0	ND
14	GFP	90	7	0.5	51	2	0	0	ND

C/A, CALM/AF10; ND, not determined; RBC, red blood cell; WBC, white blood cell; BM, bone marrow; PB, peripheral blood.
[a]Mice were injected with highly purified B220-negative progenitor cells with IgH in germline configuration.
[b]Determined by bone marrow aspiration and bleeding of the mouse.

rearrangements of the heavy chain of the IgH locus, indicating that the myeloid population originated from DJ rearranged cells (Figure 2A). The bulk leukemic population isolated from diseased mice showed unlimited IL3-dependent growth in vitro and retained its characteristic phenotype with appearance of the $B220^+/MM^-$ as well as the $B220^+/MM^+$ and the $B220^-/MM^+$ population (n = 3).

To determine whether the growth potential differed between the three subpopulations, single cells of the $B220^+/MM^-$, $B220^+/MM^+$, and the $B220^-/MM^+$ population were sorted and tested for their proliferative capacity: strikingly, only the $B220^+/MM^-$ population displayed high clonal proliferative potential at the single-cell level, with a mean seeding efficiency of 29.3% compared to 1% in the other two populations (Figure 2B). When competitive transcriptional profiling of highly purified $B220^+/Mac1^-$ and $B220^-/Mac1^+$ cells was performed by cDNA microarray analysis, 944 genes out of a total of 3643 present genes were differentially expressed between the two populations. The gene categories, which were significantly overrepresented in the $B220^+/Mac1^-$ cells, comprised genes linked to mitosis and S phase, to response to DNA damage, to translation, and to DNA repair and replication. In contrast, the $B220^-/Mac1^+$ cells showed overrepresentation of genes belonging to the categories cell death, cell communication, and actin cytoskeleton organization, reflecting the profound differences in the transcriptome and the proliferative status between the two populations (http://www.ncbi.nlm.nih.gov/geo/; GEO accession number GSE5030) (Figure 2C). Importantly, a single $B220^+/MM^-$ cell isolated from leukemic mice could give rise to the $B220^+/MM^+$ and the $B220^-/MM^+$ populations in vitro, further indicating the hierarchical organization of the leukemic population (Figure 2D). These $B220^-/MM^+$ cells were functionally intact myeloid cells and were able to actively phagocytose yeast cells (Figure 2D). Furthermore, all the $B220^+/MM^+$ and the $B220^-/MM^+$ cells derived from a single $B220^+/Mac1^-$ cell in vitro displayed identical genomic DJ rearrangements like the original progenitor (Figure 2E). In conclusion, these data demonstrated that the $B220^+/MM^-$ cells isolated from leukemic mice are able to give rise to DJ rearranged myeloid cells and have the highest proliferative potential at the single-cell level compared to the MM^+ subpopulations.

Next it was determined whether highly purified $B220^+/MM^-$ cells generated from a single cell in vitro would be able to cause acute myeloid leukemia in vivo: all animals (n = 3) succumbed to acute myeloid leukemia after a short latency time of 35 days posttransplantation when 1×10^6 cells per mouse were injected. Thus, the data demonstrated that single $B220^+/MM^-$ cells isolated from a leukemic mouse can initiate the leukemia of the same phenotype as bone marrow cells freshly transduced with CALM/AF10 retrovirus. In order to test the hypothesis that the leukemia-propagating cell resides in the $B220^+/MM^-$ compartment, we purified the three distinct populations from leukemic primary recipients and performed limiting dilution transplantation assays that allowed us to determine the frequency of the leukemia-propagating cell in the three different subpopulations as previously described (Kroon et al., 1998). Importantly, the frequency of the leukemia-propagating cell was 1 in 36 cells in the $B220^+/MM^-$ population as opposed to 1 in 437 cells in the $B220^+/MM^+$ and 1 in 19,717 cells in the $B220^-/MM^+$ compartment. Thus, the frequency of the leukemia-propagating cell was over 548-fold higher in the $B220^+/MM^-$ population compared to the $B220^-/MM^+$ population (Figure 3A; Table S2). Strikingly, in our model removal of the $B220^+$ cells from the leukemic transplant prevented the development of leukemia in mice, in contrast to the animals, which were transplanted with leukemic $B220^+/MM^-$ cells and died after a median of 34 days posttransplantation (Figure 3B).

To test the possibility that the leukemic stem cells would also reside in the cell compartment lacking expression of B220 and the MMs Gr1 and Mac1 ($B220^-/Gr1^-/Mac1^-$), bone marrow cells of leukemic mice were costained with B220-APC, Gr1-PE, and Mac1-PE: the proportion of this cell population in the bone marrow was very small, with a median of 0.03% (range 0.01%–0.05%) in four mice tested. Furthermore, only very few cells expressed the stem cell-associated markers Sca1 and c-Kit (Figure S3A). To test this population functionally, we highly purified this subpopulation from leukemic mice and compared the clonogenic potential of these cells with the $B220^+/MM^-$ subpopulation: while the $B220^-/Gr1^-/Mac1^-$ population was not able to form any colonies in vitro, an equal number of $B220^+/MM^-$ cells generated replatable colonies with a frequency of 85 ± 13 blast colonies per 1000 cells (data from three mice

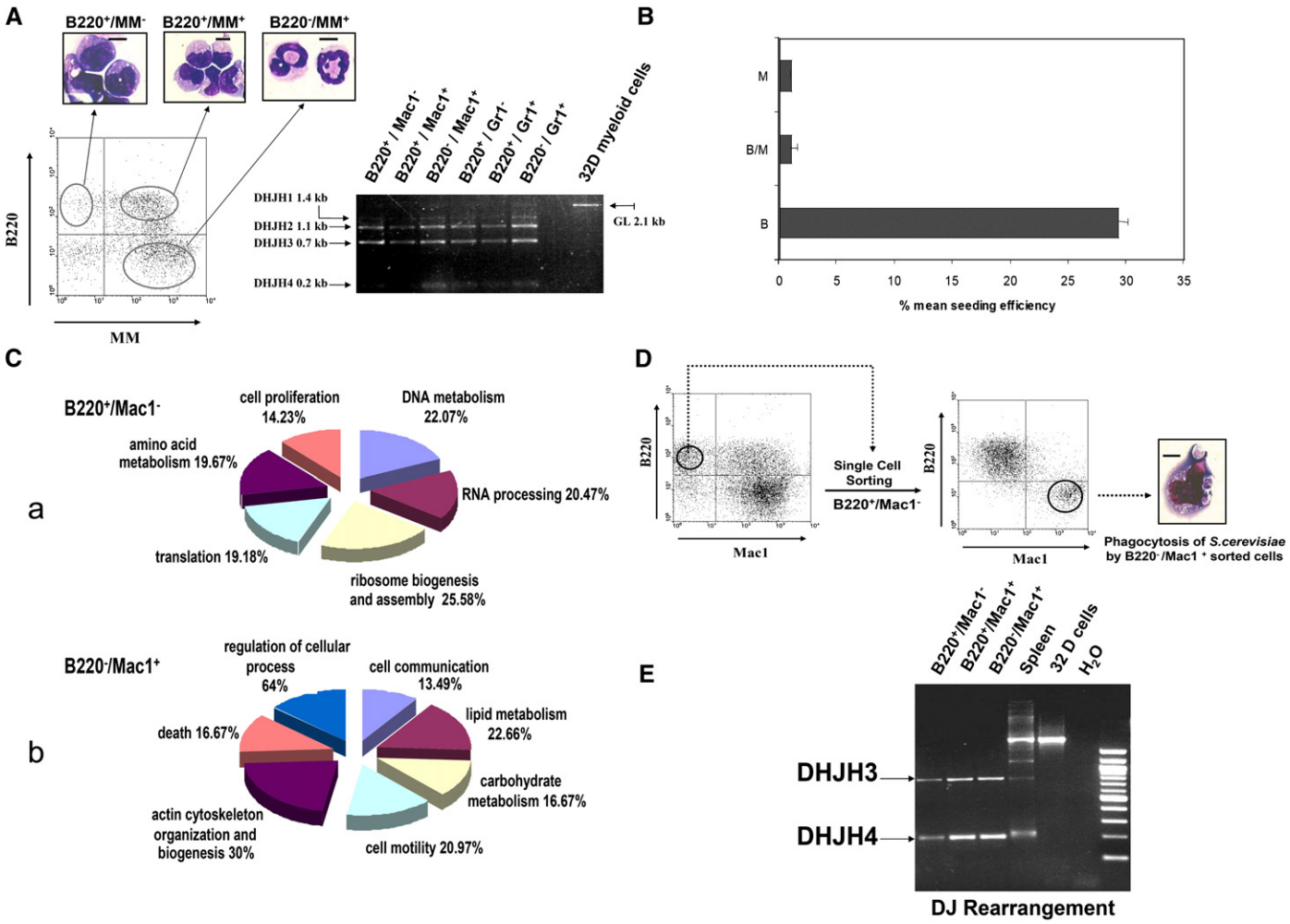

Figure 2. Transformed B220$^+$/MM$^-$ cells are IgH DH-JH positive and can generate IgH DH-JH rearranged myeloid cells at the single-cell level

A: Morphology and genomic DH-JH recombination of the immunoglobulin locus in different populations. Wright-Giemsa-stained cytospins of highly purified B220$^+$/MM$^-$, B220$^+$/MM$^+$, and B220$^-$/MM$^+$ cells and the genomic DH-JH status in different highly purified subpopulations from a representative leukemic *CALM/AF10* mouse. DH-JH status in B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, B220$^-$/Mac1$^+$, B220$^+$/Gr1$^-$, B220$^+$/Gr1$^+$, and B220$^-$/Gr1$^+$ cells is indicated; the myeloid cell line 32D was used as a DH-JH naive germline control. GL, germline; MM, myeloid marker. Scale bar, 10 μm.

B: Seeding efficiency of the different populations. Single-cell sorting of the three subpopulations, B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, and B220$^-$/Mac1$^+$, isolated from a leukemic bulk cell population was performed in 96-well plates, and single-cell growth was determined after culture for 4–5 weeks in IL3-supplemented medium. The mean seeding efficiency for each population is indicated (n = 3, plotted as mean values ± SD).

C: Distribution of gene categories significantly overrepresented in highly purified B220$^+$/Mac1$^-$ compared to B220$^-$/Mac1$^+$ cells (**Ca**) and in B220$^-$/Mac1$^+$ versus B220$^+$/Mac1$^-$ cells (**Cb**). Analysis was performed by DNA microarray-based expression profiling using a chip with 20,172 PCR-amplified, sequence-verified, gene-specific DNA fragments (LION Biosciences).

D: Phagocytosis by B220$^-$/Mac1$^+$ cells derived from a single-cell-sorted B220$^+$/Mac1$^-$ cell. Single-cell-sorted B220$^+$/Mac1$^-$ cells were expanded in vitro, and the B220$^-$/Mac1$^+$ cells generated from these cells were tested for phagocytosis of the yeast *S. cerevisiae*. A representative picture of a B220$^-$/Mac1$^+$ phagocytosing cell is shown. Scale bar, 5 μm.

E: Clonal DH-JH rearrangements in the three subpopulations. B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, and B220$^-$/Mac1$^+$ cells generated from a single B220$^+$/Mac1$^-$ sorted cell all showed the identical clonal DH-JH3 and DH-JH4 biallelic rearrangements. Genomic DNA from splenic cells from a nontransplanted mouse and the myeloid cell line 32D were used as positive and negative controls for recombination, respectively. B, B220$^+$/Mac1$^-$; B/M, B220$^+$/Mac1$^+$; M, B220$^-$/Mac1$^+$.

transplanted initially with independently transduced bone marrow; input number 100–1000 cells/dish in both arms). Importantly, the B220$^-$/Gr1$^-$/Mac1$^-$ cells did not show leukemic engraftment into secondary recipients or induced leukemia, whereas as few as 25 B220$^+$/MM$^-$ cells caused leukemic engraftment of the same phenotype as described before (input number between 25 and 113 cells for the B220$^-$/Gr1$^-$/Mac1$^-$ cells [n = 5] and an equal number of cells per mouse for the B220$^+$/MM$^-$ population [n = 5]) (Table S3). Taken together, the high frequency of the leukemia-propagating cell in the B220$^+$/MM$^-$ population, together with its ability to proliferate and to give rise to a myeloid DJ rearranged cell population, demonstrated that the leukemia-propagating cell resided in the B220$^+$/Mac1$^-$ cell compartment in this murine model.

More detailed analysis of the cell surface phenotype of the B220$^+$/MM$^-$ cells characterized them as CD43$^+$/AA4.1$^+$/CD24$^{low\text{-}pos}$/FLT3^{med}/IL-7R$^{low\text{-}neg}$, c-Kit$^{low\text{-}neg}$, CD19$^-$, and Sca1$^-$ (Figures 4A and 4B). The cells did not express CD21, CD23, sIgM, λ5, Bp1, or Igβ (Figures S4A and S4B), or CD4 or NK1.1 (data not shown). Transcriptional profiling by RT-PCR

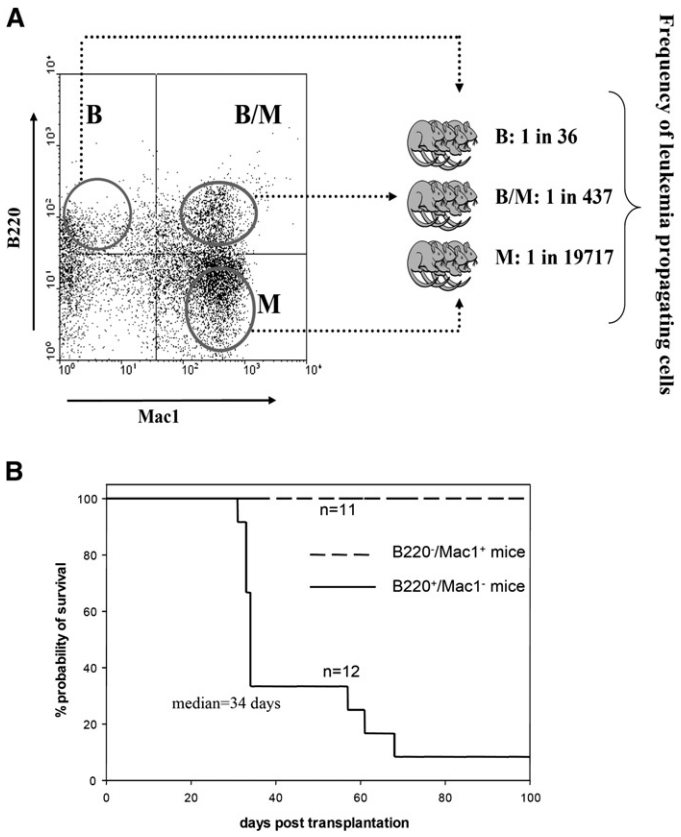

Figure 3. Frequency of leukemia-propagating cells

A: Determination of the frequency of leukemia-propagating cells. Cohorts of secondary mice were injected with highly purified B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, and B220$^-$/Mac1$^+$ populations from primary leukemic mice in a limiting dilution assay. The frequency of leukemia-propagating cells in each population is indicated. Details of cell numbers, number of mice per arm, and the median latency of disease are described in Table S2.

B: Survival of mice transplanted with leukemic BM depleted of B220$^+$ cells (B220$^-$/Mac1$^+$cells, n = 11; 50 cells [n = 4], 500 cells [n = 4], and 5000 cells [n = 3] isolated from the primary leukemic population). In comparison the survival of mice (n = 12) transplanted with B220$^+$/Mac1$^-$ cells with the same range of cell numbers (50 [n = 4], 500 [n = 4]), and 5000 cells [n = 4]) is shown.

showed lineage-promiscuous expression of the lymphoid factors *EBF*, *VpreB*, and *Rag2* and the MM *MPO* (Figure 4C; Figure S4C). Importantly, there was no transcription of *Pax5*, which is compatible with the incomplete rearrangement status at the IgH locus (data not shown) and the CD19 negativity of the cells (Figure 4B). In the B220$^+$/Mac1$^+$ and in the B220$^-$/Mac1$^+$ cells, transcripts for the M-CSF receptor became detectable, whereas transcription for *EBF* was downregulated. There was no detectable transcription of *Gata3*, *Zfpn1a3* (*Aiolos*) (Figure 4C), or *mb1* and only low expression of *λ5* transcripts (Figure S4C). In addition, we could not detect rearrangements of the TCRγ receptor, specifically Vγ 1.1-Jγ4, Vγ 2/4-Jγ1, or Vγ 5/7-Jγ1 by PCR (data not shown). The surface marker expression as well as the transcription factor expression profile collectively defined the B220$^+$/MM$^-$ population as a lymphoid progenitor (Figure S5).

In an effort to determine whether an earlier B220-negative cell could be transformed by the *CALM/AF10* fusion gene, we next analyzed whether transduction of *CALM/AF10* into highly purified B220-negative cells with IgH germline configuration resulted in the induction of AML of the same phenotype as described for the transduction of unfractionated bone marrow: two of two animals tested developed acute myeloid leukemia after transplantation of 1–2 × 10^5 B220-negative cells proven to have IgH germline configuration by PCR before injection, indicating that early B220-negative progenitors with IgH germline configuration can acquire leukemogenic potential after *CALM/AF10* transduction. However, whereas one of the AML cases showed B220$^+$ cells with IgH DJ rearrangement as described before, the other case did not display a B220$^+$ subpopulation. In addition, the leukemic bulk had a germline IgH configuration, a phenotype not observed in the transplantation assays using 5-FU-mobilized unfractioned bone marrow for viral infection (Figure S3B and Table 1). In contrast to early B220$^-$ progenitors, murine cell populations comprising pro-B cells (B220+/CD43+/CD25−; input number 2 × 10^6 cells per mouse; transduction efficiency, 10%) and pre-B cells (B220+/CD43+/CD25+ cells; input number 2 × 10^5 cells per mouse; transduction efficiency, 2%) did not acquire in vivo repopulating activity with no short- or long-term engraftment in lethally irradiated mice 4 or 8 weeks posttransplantation, respectively (data not shown).

To examine whether our findings in the murine CALM/AF10 leukemia model parallel characteristics of human *CALM/AF10*-positive AML, patient samples were analyzed for *IG* DH-JH rearrangement status and the presence of a CD45RA (the human homolog to B220) population. In addition, we further characterized the CD45RA$^+$ population by performing CFU-blast colony assays in vitro and FISH analysis for the presence of the *CALM/AF10* fusion gene. Table S4 summarizes the characteristics of patients diagnosed with AML or in one case with acute undifferentiated leukemia (AUL); two of the nine patients were children, six of nine patients had additional genetic alterations to the *CALM/AF10* t(10;11) translocation. Treatment consisted mainly of chemotherapy with high-dose Ara-C and anthracyclines, and outcome was dismal in all the patients with longer follow-up. Multiplex PCR and clonality analysis by GeneScanning techniques showed that seven out of nine AML samples demonstrated clonal *IG* DH-JH rearrangements (Table 2 and Figure 5A). We could also detect immature TCRδ (Dδ2,Vδ2) and TCRβ (TCRβ DJ) as well as TCRγ (Vγ10) rearrangements in six out of six patients tested (Table 2).

In all three patients tested, a substantial proportion of CD45RA$^+$ cells could be detected, ranging from 50.6% to 99.8% of bone marrow cells. In two of the three patients, the CD45RA-positive population coexpressed CD34. In all the patients tested, the CD34$^+$/CD45RA$^+$ cells harbored the *CALM/AF10* fusion gene as detected by FISH analysis (Figure 5Bb) (Table 3). Furthermore, CD34$^+$/CD45RA$^+$/CD10$^+$ cells, corresponding to the stage of early human lymphoid progenitors (Haddad et al., 2004), were also positive for the fusion gene (data not shown). The involvement of the CD45RA population in the malignant transformation process was further supported by the observation that the CD45RA population generated myeloid CFU-blasts (Table 3) in vitro, which showed the identical *IG* DH-JH rearrangement status at a single-colony level as detected in the myeloid bulk population (Figure 5C). To test whether early CD34$^+$/CD45RA$^-$ cells could be positive for the CALM/AF10 fusion gene, we highly purified CD34$^+$/CD45RA$^-$/lineage marker (lin)-negative (lin: CD38$^-$/CD19$^-$/CD10$^-$) cells of one patient with *C/A*-positive AML. Notably, the fusion gene could be detected in the majority of cells (92%) in this primitive

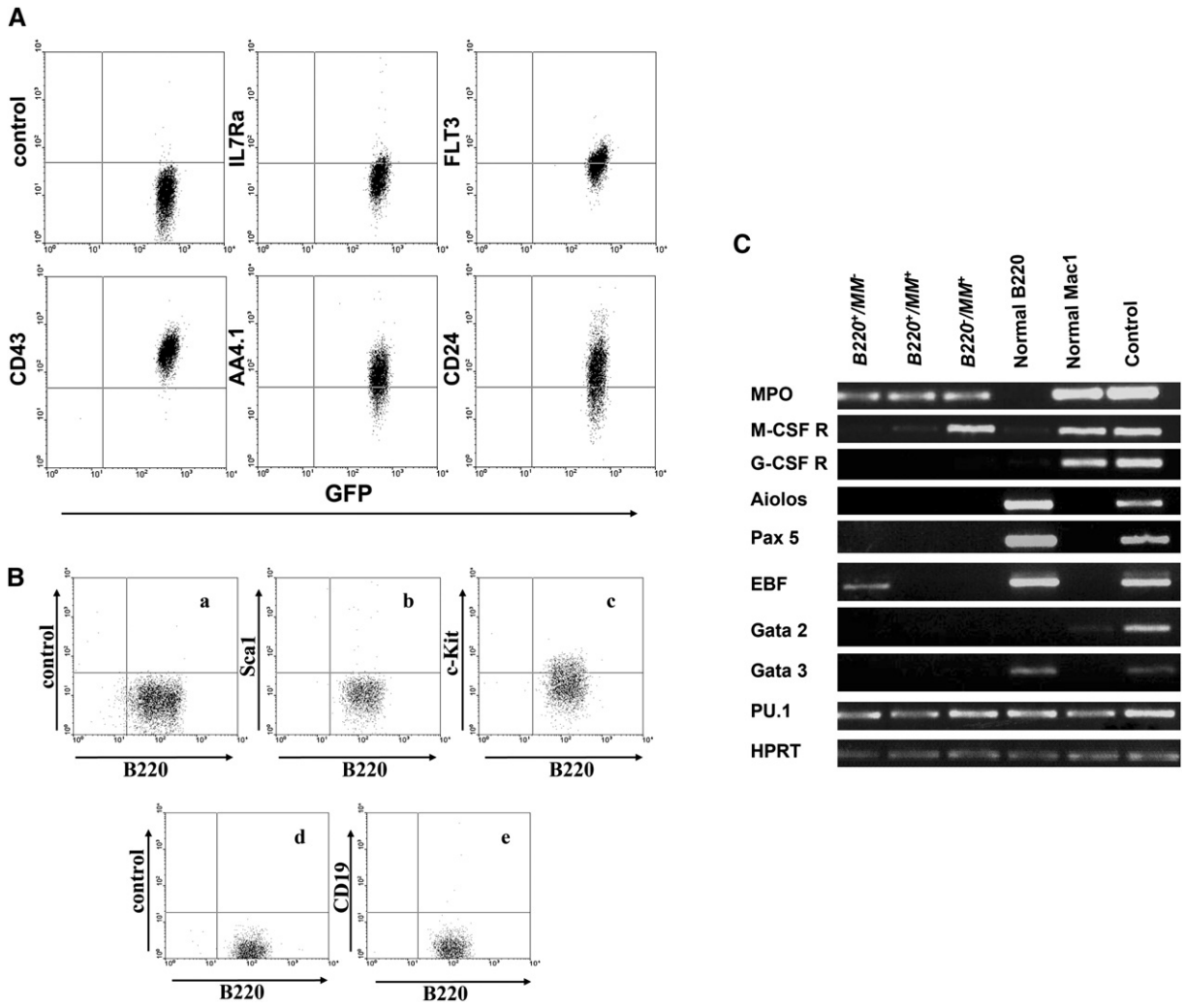

Figure 4. Leukemic B220$^+$/Mac1$^-$ cells express lymphoid markers

A: Expression of B cell-associated markers on leukemic B220$^+$/Mac1$^-$ cells: flow cytometric analysis after staining with various markers as indicated.
B: Expression of early and late markers on leukemic B220$^+$/Mac1$^-$ cells propagated in vitro for Sca1 (**Bb**) and c-Kit (**Bc**) or CD19 (**Be**); **Ba** and **Bd** are controls as indicated.
C: RT-PCR analysis of lineage associated genes. B220$^+$/MM$^-$, B220$^+$/MM$^+$, and B220$^-$/MM$^+$ cells clonally generated from a single B220$^+$/Mac1$^-$ cell were analyzed for the expression of genes associated with different hematopoietic lineages as indicated. B220$^+$ sorted cells from the spleen, Mac1$^+$ sorted cells from the bone marrow, and unsorted BM cells (all populations isolated from a nontransplanted control mouse) were used as controls for lymphoid and myeloid transcripts, respectively. MPO, myeloperoxidase; EBF, early B cell factor; HPRT, hypoxanthine phosphoribosyl transferase; *B220$^+$/MM$^-$*, B220$^+$/Mac1$^-$ cells; *B220$^+$/MM$^+$*, B220$^+$/Mac1$^+$ cells; and *B220$^-$/MM$^+$*, B220$^-$/Mac1$^+$ cells.

cell compartment (Figure 5Ba, patient number 9), indicating the involvement of early CD45RA-negative cells in the transformation process in this patient. In summary, these findings indicate that patients with *CALM/AF10*-positive AML are characterized by *IG* DH-JH rearrangements of their leukemic blasts and a CD45RA-positive population, which is positive for the leukemia-specific fusion gene and is able to generate myeloid blast colonies with the clonal *IG* DH-JH rearrangement ex vivo, reminiscent of several characteristics of murine CALM/AF10-induced AML in transplanted mice.

Discussion

Leukemias are considered to originate from a rare subset of transformed cells, which are able to initiate and maintain the disease. In particular, studies in patients with acute myeloid leukemia have shown that the transformation process involves early hematopoietic progenitor cells characterized by expression of CD34 and lack of CD38 and the different lineage markers (Blair et al., 1998; Dick, 2005). The concept that hematopoietic stem cells (HSCs) are a key target of leukemic transformation was further supported by murine models, which demonstrated that HSCs could acquire leukemic stem cell properties by forced expression of appropriate oncogenes, recurrently found in patients with AML (Huntly and Gilliland, 2005; Reya et al., 2001). Furthermore, inactivation of certain transcription factors such as JunB caused leukemia only when the targeted deletion took place in the HSC compartment and not in the more differentiated cell pool (Passegué et al., 2004). However, more recent data have provided in vivo evidence that also more

Table 2. IgH DJ and TCR rearrangements in CALM/AF10 AML patients

Patient no.	Type	IgH DJ rearrangements		TCR rearrangements		
		IgH DJ	IgH DJ sequences	TCRγ	TCRδ	TCRβ DJ
1	AML M0	monoclonal DH3	D39/ATTTTGACTGGTTATTATAAC*GT*CTGGTACTT CGATCTCTGGGGCCT/JH2	monoclonal Vγ10	Dδ2 biallelic	polyclonal
2	AML M2	monoclonal biallelic DH2 DH6	D2-15/TGGTAGCTGCTACTC*TT*TACGGTATGGAC GTCTGGGGCCA/JH6B D6-13/GGGTATAGCAG C*GGCTACAAGGGT*ACTACTGGGGCCA/JH4B	monoclonal Vγ10	Vδ2	monoclonal D1
3	AML M1	polyclonal DH1–7	—	polyclonal	Dδ2/Dδ3 biallelic	polyclonal
4	AUL	monoclonal DH1	DH1-26/TATAGTGGGAGCTACT*GTG*CTACTGG GGCCA/JH4B	monoclonal Vγ1-8 and Vγ10-11	Dδ2	monoclonal TCRβ DJ and TCRβ JA
5	AML M1	monoclonal DH7	ND	polyclonal	Dδ2 biallelic	polyclonal
6	AML M1	monoclonal DH7	ND	monoclonal Vγ10-11	Dδ2 biallelic	monoclonal TCRβ DJ
7	AML M1	monoclonal DH6	DH6-6/TATAGCAGCTCGT*TGT*ACTACTTTGACT ACTGGGGCCA/JH4B	ND	ND	ND
8	AML	monoclonal DH7	DH7-27/TAACCACTGGGGAC*CTCCCGGG* CTTTGACTACGGGGCCA/JH4B	ND	ND	ND
9	AML M5a	polyclonal DH1-7	—	ND	ND	ND

ND, not determined.

differentiated cells can acquire properties of leukemic stem cells: in a murine bone marrow transplantation model, retrovirally driven expression of the *MLL-GAS7* fusion gene induced acute leukemias with myeloid, lymphoid, or mixed phenotype. Immunophenotyping, transcriptional profiling, and the ability of the LSC to induce three distinct leukemia subtypes indicated

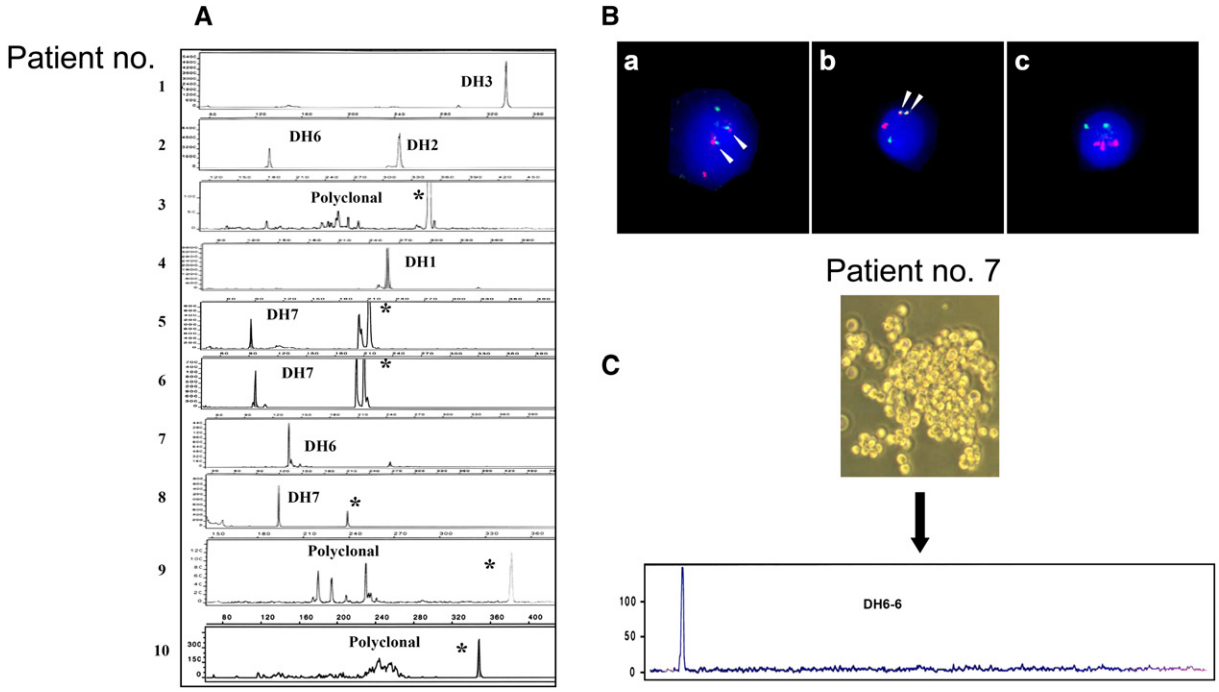

Figure 5. CALM/AF10-positive human AML samples show IgH DH-JH rearrangements and express CD45RA(B220)

A: GeneScanning analysis of IgH DH-JH rearrangements in nine cases of human CALM/AF10-positive AML using bone marrow of the leukemic patients with >80% myeloid blasts and polyclonal nonleukemic control cells. GeneScanning demonstrated a clonal IgH DH-JH rearrangement in seven out of nine cases. Unspecific background peaks resulting from annealing to nonrearranged alleles in the reactions are indicated with an asterisk.

B: Detection of the CALM/AF10 rearrangement in flow-sorted patient cells. **Ba:** A representative CD34+/CD45RA$^-$/lin$^-$ cell with the CALM/AF10 fusion, showing a normal AF10 (red) and a normal CALM (green) locus as well as two red/green signals, indicating the two reciprocal fusion genes (arrowheads) (92% of the cells were positive for the fusion gene; one representative CALM/AF10-negative cell of the same compartment is shown in **Bc**) (patient number 9). **Bb:** A representative CD34$^+$/CD45RA$^+$ cell carrying the CALM/AF10 fusions, showing the same signal pattern as in **Ba** (fusion signals are indicated by arrowheads) (patient number 7). **Bc:** A CD34$^+$/CD45RA$^-$/lin$^-$ cell without CALM/AF10 fusion showing two normal AF10 (red) and two normal CALM (green) signals (patient number 9).

C: Myeloid blast colony formation in methylcellulose of CD45RA$^+$ cells of a representative human AML patient (patient 7, magnification 100×). Lower panel shows the clonal IgH DJ rearrangement peak from a singly isolated myeloid blast colony analyzed by GeneScan. The rearrangement was identical to the rearrangements detected in the leukemic bulk of the patient (Figure 5A).

Table 3. Proportion of CD45RA-positive cells and CFU-blast frequency of C/A-positive AML patients

Patient no.	$CD34^+$/$CD45RA^+$	Overall $CD45RA^+$	CD34+/$CD45RA^-$/lin$^-$[b]	Positivity for CALM/AF10[c]		CFU-blast frequency[a]	IgH DJ of CFU-blast colonies
				$CD34^+/CD45RA^+$	$CD34^+/CD45RA^-$/lin$^-$		
3	94.52%	98.59%	0.13%	+	ND	92 ± 14	NA
7	96.21%	99.8%	0.01%	+	ND	12 ± 3	monoclonal DH6
9	1.68%	50.6%	33.07%	ND	+	ND	ND

ND, not determined; NA, not applicable.
[a]Per 10^6 $CD45RA^+$ bone marrow cells isolated from C/A AML patients (0.2–2 × 10^5 cells plated per dish).
[b]Lineage markers comprised CD38, CD19, and CD10.
[c]As determined by CALM- and AF10-specific FISH probes.

that the fusion gene targeted a multipotential progenitor in this murine model (So et al., 2003). In addition, retroviral expression of the *MLL-ENL* or *MOZ-TIF2* fusion gene in murine myeloid committed progenitor cells clearly demonstrated that committed progenitor cells can function as leukemic stem cells in these models and that transformation of HSC is not mandatory for the development and maintenance of acute leukemia (Cozzio et al., 2003; Huntly et al., 2004). The murine data presented here now provide evidence that a progenitor cell with lymphoid characteristics can propagate acute myeloid leukemia. Detailed characterization of the leukemic stem cell showed that the cells expressed B220, CD43, AA4.1, and CD24 but lacked expression of MMs as well as Sca1. Importantly, transcriptional profiling demonstrated a lineage-promiscuous expression of lymphoid factors such as *EBF*, *VpreB*, and *Rag2* (Busslinger et al., 2000) with progressive downregulation following differentiation into the $B220^+/Mac1^-$ and the $B220^-/Mac1^+$ cells together with the MM *MPO*. Of note, we did not detect transcription of *Pax5*, which is compatible with our finding that the $B220^+$ cells had an incomplete rearrangement status at the *IG* DH-JH locus and did not express CD19. Even though this progenitor showed characteristics of the early B lineage, the classification of this CALM/AF10 leukemia-propagating cell according to the different B cell classification systems remains difficult because of its leukemogenic characteristics and the potential impact of the fusion gene on its cell surface phenotype and transcriptional network. The phenotype we observed differed from the $B220^+/CD19^+$ cell stage, which is associated with *Pax5* positivity and the entire commitment to the B cell lineage (Hardy, 2003), but also from the profile of the earliest lymphoid progenitor, the common lymphoid progenitor (CLP), which lacks expression of B220 (Kondo et al., 1997).

In our model, a single transformed Pax5-negative progenitor with lymphoid characteristics was able to generate myeloid cells with IgH immunoglobulin rearrangements. Interestingly, $Pax5^{-/-}$ B cell progenitors show lineage-promiscuous transcription of B-lymphoid and myeloid genes, display a remarkable multilineage potential in vitro, and are able to be directed into the myeloid lineage (Heavey et al., 2003; Schaniel et al., 2002). In addition, the latent myeloid differentiation capacity of the CLP, which does not express *Pax5* yet, has become obvious due to the observation that it can be redirected to the myeloid lineage by retrovirally induced *IL-2Rβ* chain or *GM-CSF* receptor expression (Kondo et al., 2000). Thus, one intriguing possibility is that *CALM/AF10* instructed myeloid development of an early Pax5-negative lymphoid progenitor in our model. This commitment into the myeloid pathway was irreversible in the *C/A* model, as highly purified $B220^+/Mac1^-$ cells were not able to differentiate along the B lineage pathway in vitro on stromal OP9 feeders in lymphoid growth medium (data not shown).

The scenario that expression of *C/A* directs the lineage choice of *Pax5*-negative lymphoid progenitors is only one potential explanation for the myeloid phenotype of the leukemia. We cannot exclude that the fusion gene instructed myeloid lineage decision at the later stage of *Pax5*-positive cells, as it was recently shown that the myeloid transcription factors C/EBPα and C/EBPβ could convert *Pax5*-positive B cells into macrophages by downregulation of *Pax5* expression and upregulation of myeloid genes (Xie et al., 2004). However, we could not observe any significant downregulation of *Pax-5* expression following CALM/AF10 expression for 48 hr in the in vitro cultivated murine pro-B cell line Ba/F3 or primary murine fetal pre-B cell lines by real-time quantitative PCR and did not observe repopulating ability of murine pro-B or pre-B cells in lethally irradiated mice after transduction with CALM/AF10 (data not shown).

Another important scenario would be that the CALM/AF10 fusion gene targets a rare and early B220-negative progenitor with IgH germline configuration and activates lymphoid developmental programs in these cells, resulting in a $B220^+/MM^-$ IgH rearranged cell population with high leukemia-propagating potential as described in the murine model. Indeed, highly purified B220-negative progenitor cells with IgH germline configuration acquired leukemogenic properties in vivo in two independent experiments after infection with the CALM/AF10 retrovirus, indicating that an early progenitor cell can be transformed by the fusion gene. Interestingly, one of the mice developed an AML that lacked a $B220^+$ population and displayed an IgH germline configuration, which was never observed when unfractionated bone marrow was infected with the *CALM/AF10* retrovirus and transplanted into recipients. One explanation for this might be that C/A can target IgH rearranged $B220^+$ lymphoid progenitors, giving rise to AML with the consistent phenotype of a $B220^+$ transformed progenitor population, as well as early $B220^-$ progenitors with IgH germline configuration, resulting in AML with or without the $B220^+$ cell population.

Importantly, the experiments performed in this study do not rule out the other possibility, that the *CALM/AF10* fusion gene had an instructive effect on myeloid progenitors, initiating a lymphoid-specific program in these cells. In this scenario a myeloid cell would acquire lymphoid characteristics as a result of transformation by an appropriate oncogene. Another possibility is that *C/A* directly transformed a rare cell subset of B/macrophage progenitors, which was identified in fetal liver but also in adult bone marrow (Lacaud et al., 1998; Montecino-Rodriguez et al., 2001). However, this cell was described to be

CD19/*Pax5* positive and negative for CD45R/B220, a phenotype different than the phenotype of the LSC described here.

Of note, these different scenarios would result in the development of AML with a $B220^+$/MM-negative cell population with a very high frequency of leukemia repopulating cells, which would be accessible to an antibody-based B220 depletion. Indeed antibody-mediated depletion of the $B220^+/MM^-$ cells from leukemic transplants efficiently prevented leukemic development in mice, underlining the key role of this population for disease propagation. One implication of our findings is that in a subset of AML the leukemia-propagating cell might show a distinct cell surface and transcriptional phenotype, setting it apart from the leukemic myeloid bulk population, but also from the normal stem cell pool. In particular, the expression of lymphoid antigens would allow to discriminate this cell from the normal hematopoietic stem cells, which would facilitate the development of treatment strategies using the lymphoid surface antigens to target the leukemia-propagating cell but to spare the normal stem cell (Passegué et al., 2003). As illustrated in the murine CALM/AF10 leukemia model, such a strategy would have therapeutic potential. Depletion of the $B220^+$ transformed progenitor cells would fail to eradicate the malignant clone, if a rare leukemic B220-negative progenitor cell population existed, which gives rise to the $B220^+/MM^-$ population described. But even in this scenario, diminishing the B220-positive leukemia-propagating cells would promise clinical benefit, as mice transplanted with B220-depleted bone marrow failed to develop leukemia up to an observation time of 100 days, compared to rapid development of AML in mice transplanted with $B220^+$ transformed progenitors.

Recently, substantial progress has been made to characterize very early stages in human B cell development (Haddad et al., 2004; Hou et al., 2005; LeBien, 2000; Ryan et al., 1997). These reports have shown that similar to the murine system these early human progenitors express the B220 human homolog CD45RA, as well as CD34 and CD10, but lack CD19 and *Pax5* expression. In an effort to determine potential similarities between the murine CALM/AF10 AML and human disease, we analyzed nine patients with *CALM/AF10*-positive AML. Interestingly, the cases tested showed a CD45RA population with coexpression of CD34 in two of three patients. Furthermore, in seven out of nine patients tested, clonal IgH immunoglobulin rearrangements could be detected in the myeloid bulk. The leukemic nature of the CD45RA population in these cases was indicated by the presence of the fusion gene, its clonal IgH rearrangement, and its ability to form CFU-blast colonies ex vivo. Interestingly, in the $CD34^+/CD45RA^-/lin^-$ progenitor compartment of one patient we detected a mosaic of cells carrying the fusion gene or showing normal signal pattern by FISH, indicating that this cell compartment might be involved in the malignant transformation process.

In addition to IgH rearrangements, six of six patients were also positive for clonal rearrangements of the T cell receptor (TCR). These observations are in line with reports associating the *CALM/AF10* fusion gene with T-ALL, in particular with T-ALLs expressing the γ/δ TCR (Asnafi et al., 2003). In the murine model the constitutive expression of CALM/AF10 failed to induce T cell leukemia, and the observed myeloid leukemias did not show the TCRγ rearrangements that were analyzed (Vγ 1.1-Jγ4, Vγ 2/4-Jγ1, or Vγ 5/7-Jγ1). This could be attributable to various factors predisposing the murine model to leukemias of the myeloid lineage, such as the cell population initially used for retroviral infection; the in vitro culture conditions with IL3, IL6, and SCF stimulation before transplantation; or the level of expression of the fusion gene in the murine system compared to human disease. However, real-time PCR quantification of *CALM/AF10* transcripts showed low *CALM/AF10* expression in both the murine and human myeloid bulk (data not shown). Insertional mutagenesis was identified as an important factor in murine leukemogenesis (Akagi et al., 2004; Suzuki et al., 2002) and might explain the predisposition to AML development in the CALM/AF10 model: in an analysis of the proviral integration sites in leukemic mice, 10 of 18 detected sites were described before as common integration sites or transposon-tagged cancer genes according to the Retroviral Tagged Cancer Gene Database (RTCGD; http://RTCGD.ncifcrf.gov/) (Akagi et al., 2004). However, there were no recurrent insertion sites or a correlation of the integrations with the consistent phenotype of the leukemias in this model. As described for human *CALM/AF10*-positive T-ALLs (Dik et al., 2005; Soulier et al., 2005), the murine AML cases also displayed aberrant expression of *HoxA7* and *Meis1* (unpublished data); furthermore, we also observed deregulation of HOX genes in patients with *CALM/AF10*-positive AML (unpublished data). This indicates that deregulation of this transcription factor family is a common characteristic of CALM/AF10 leukemias independent of the phenotype.

In summary, this murine leukemia model demonstrates that a progenitor cell with lymphoid characteristics and a phenotype that differs from that of normal hematopoietic stem cells and the leukemic myeloid bulk, is able to propagate AML. These data provide a rationale for the development of therapeutic strategies targeting the leukemia-propagating stem cell without harming the normal stem cell pool and raise the hope that such innovative concepts may improve treatment outcome in a subgroup of patients with AML in the future.

Experimental procedures

Retroviral transduction of primary bone marrow cells and BM transplantation

Breeding and maintenance of the mice were conducted as described previously (Rawat et al., 2004). Production of high-titer helper-free retrovirus was carried out following standard procedures by using the ecotropic packaging cell line GP^+E86 (Schessl et al., 2005). Lethally irradiated (0.80 Gy) mice were transplanted with either highly purified $EGFP^+$ cells alone (4×10^5 cells per mouse) (FACSVantage, Becton Dickinson) or with a mixture of transduced and nontransduced cells (on average 1.76×10^5 transduced cells with 1.9×10^5 nontransduced cells per mouse). Lethally irradiated secondary and tertiary recipients were injected with 1×10^6 BM cells from a primary and secondary diseased mouse, respectively, with an equal number of nontransduced cells from a syngenic disease-free mouse bone marrow for radioprotection. Both animal and human studies were approved by the Ethics Committee of Ludwig Maximilians University and abided by the tenets of the revised World Medical Association Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm).

In order to determine the frequency of leukemia-propagating cells, $B220^+/Mac1^-$, $B220^+/Mac1^+$, and $B220^-/Mac1^+$ cells were isolated and highly purified from the BM of a leukemic primary mouse. The sort purity of these cells was analyzed with the FACSCalibur and determined to be over 98%. In each cohort 10-fold serial dilutions of these cells were injected intravenously (50 cells to 5×10^5 cells per mouse cohort) into lethally irradiated secondary recipient mice. Carrier cells (1×10^6) (nontransduced BM from a syngenic disease-free mouse) were added to each sample for radioprotection. Diseased mice were sacrificed and assessed for leukemia development. Mice that did not develop disease within 20 weeks posttransplantation were sacrificed and

tested for engraftment. The frequency of leukemia-propagating cells was calculated using Poisson statistics using the L-Calc Limiting dilution analysis software (Version 1.1 StemSoft Inc., Vancouver, Canada) (Buske et al., 2002).

Patient samples

BM samples from adult patients diagnosed with AML were analyzed. The diagnosis of AML was performed according to the French-American-British criteria and the World Health Organization classification (Bennett et al., 1985; Harris et al., 1995). Cytomorphology, cytochemistry, cytogenetics, and molecular genetics were applied in all cases as described below. The clinical features of patients are provided in Table S4. The study abided by the rules of the local internal review board and the tenets of the revised Helsinki protocol (http://www.wma.net/e/policy/b3.htm).

Sorting of cells from human AML patient samples

Frozen mononuclear peripheral blood or bone marrow cells from patients with AML were rapidly thawed and were washed twice in IMDM with 10% FBS, resuspended in PBS, and stained with various antibodies. For lineage depletion experiments, immunostaining was done with FITC-labeled anti-human CD34 (Beckman Coulter, Marseille, France) and PE-labeled anti-human CD38, PE-labeled anti-human CD19, PE-labeled anti-human CD10, and PE-labeled anti-human CD45RA (all from BD Pharmingen, Heidelberg, Germany). Cells were incubated with appropriate antibodies for 20 min on ice and washed with cold PBS prior to sorting. The trial was approved by the responsible local ethics committees, and all patients gave written informed consent according to the Helsinki Declaration.

FISH

Dual Colour Dual Fusion (DCDF) probes were developed with bacterial artificial chromosome (BAC) clones RP11-418C1 for the 5′-AF10 region (Texas Red; red in Figure 5B), RP11-249M6 for the 3′-AF10 region (red), RP11-12D16 (FITC, green) for the 5′-CALM region, and RP11-90K17 (green) for the 3′-CALM region (kindly provided by Dr. Mariano Rocchi, Bari). BAC DNA preparation, labeling, and FISH were performed as described (Crescenzi et al., 2004). Normal nuclei display a two-red and two-green (2R2G) hybridization pattern, whereas nuclei carrying the translocation display a one-red, one-green, twored/green fusion (1R1G2F) pattern. Other hybridization patterns, e.g., 1R1G1F (found in 0.5%–2% of nuclei) or 1R1G3F (found in 0.5%–1% of nuclei), were interpreted as technical artifacts.

The cells were directly sorted onto aminoalkylsilane-coated slides (SilanePrep Slides, Sigma, Germany) with or without preloading of 15 μl 3% bovine serum albumin solution in PBS and fixed in 1:3 acetic acid-methanol fixative.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, five supplemental figures, and five supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/5/363/DC1/.

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Accession numbers

The microarray data in Figure 2C can be accessed from NCBI's Gene Expression Omnibus (GEO) with accession number GSE5030.