Prospective separation of normal and leukemic stem cells based on differential expression of TIM3, a human acute myeloid leukemia stem cell marker

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Hematopoietic tissues in acute myeloid leukemia (AML) patients contain both leukemia stem cells (LSC) and residual normal hematopoietic stem cells (HSC). The ability to prospectively separate residual HSC from LSC would enable important scientific and clinical investigation including the possibility of purged autologous hematopoietic cell transplants. We report here the identification of TIM3 as an AML stem cell surface marker more highly expressed on multiple specimens of AML LSC than on normal bone marrow HSC. *TIM3* expression was detected in all cytogenetic subgroups of AML, but was significantly higher in AML-associated with core binding factor translocations or mutations in *CEBPA*. By assessing engraftment in NOD/SCID/IL2Rγ-null mice, we determined that HSC function resides predominantly in the TIM3-negative fraction of normal bone marrow, whereas LSC function from multiple AML specimens resides predominantly in the TIM3-positive compartment. Significantly, differential TIM3 expression enabled the prospective separation of HSC from LSC in the majority of AML specimens with detectable residual HSC function.

Acute myeloid leukemia (AML) is an aggressive malignancy of the bone marrow with a 5-y overall survival between 30% and 40%, and much poorer outcomes for patients over age 65 (1, 2). Cytogenetic abnormalities are prognostic in AML (3, 4), although the majority of cases have a normal karyotype (NKAML). Among NKAML patients, numerous prognostic molecular mutations have been identified (5), most commonly internal tandem duplications in the *FLT3* tyrosine kinase (*FLT3*-ITD), which confer a poor prognosis, and cytoplasmic mislocalizing mutations in *NPM1* (NPM1c), which confer a better prognosis (6).

According to the cancer stem cell model, tumors are organized as cellular hierarchies initiated and maintained by a small pool of self-renewing cancer stem cells (CSC) (7, 8). The first in vivo demonstration of this model was achieved in human AML, where leukemia-initiating potential was identified exclusively in the $CD34^+CD38^-$ leukemic subpopulation (9). Notably, recent experiments suggest that leukemia stem cell (LSC) activity may exist in additional subpopulations in some cases (10, 11). Normal hematopoietic stem cells (HSC) also reside within the $Lin^-CD34^+CD38^-$ compartment and are contained in the subpopulation expressing CD90 (12, 13).

Hematopoietic tissues in AML patients contain both LSC and residual HSC, as indicated by the recovery of normal hematopoietic cells in most patients treated with induction chemotherapy (1). Experimental evidence of the presence of both of these stem cell populations is demonstrated by the observation that normal lymphoid-myeloid hematopoiesis can occasionally be detected upon transplantation of AML samples into immunodeficient mice (10).

The ability to prospectively separate residual HSC from AML LSC would facilitate important clinical and scientific investigation. Clinically, prospective separation raises the possibility of purged autologous hematopoietic cell transplants as part of AML therapy. Scientifically, we have demonstrated that preleukemic mutations occur in self-renewing HSC (14–16). Thus, it is possible that residual functionally normal HSC in leukemia patients are preleukemic, and the ability to prospectively isolate these cells provides a unique opportunity to investigate early events in leukemogenesis.

Recently, a number of investigators have reported the identification of cell surface proteins preferentially expressed on AML LSC compared with normal HSC, including CD123 (17), CD44 (18), CLL-1 (19), CD25 (20), CD32 (20), CD96 (21), and CD47 (22, 23). Monoclonal antibodies targeting several of these antigens have shown promise in preclinical models and are in active clinical development (18, 23–25). LSC surface markers may be used to monitor minimal residual disease by flow cytometry. Indeed, increased expression of CLL-1 within the $Lin^-CD34^+CD38^-$ compartment can predict relapse in AML patients in remission (19). Thus far, prospective separation of HSC from LSC has been reported by using LSC marker expression (23, 26) and in $CD34^-$ AML (27), but not from a diverse collection of patient samples.

Here, we report the identification of a unique AML stem cell surface marker, T-cell Ig mucin-3 (TIM3). We determined that most HSC function resides in the TIM3-negative fraction of normal bone marrow (NBM), whereas most LSC function from multiple AML specimens was observed in the TIM3-positive compartment. Significantly, differential expression of TIM3 enabled the prospective separation of HSC from LSC in multiple primary human AML samples.

Results

TIM3 Is More Highly Expressed on AML LSC Than on NBM HSC. Using our previously published gene expression data, we identified increased *TIM3* expression in primary human AML LSC compared with NBM HSC (28). Cell surface expression of TIM3 protein in AML was investigated by flow cytometry. TIM3 was more highly expressed on multiple specimens of $Lin^-CD34^+CD38^-CD90^-$ AML LSC compared with $Lin^-CD34^+CD38^-CD90^+$ NBM HSC; however, TIM3 expression was not significantly different between bulk AML cells and the LSC-enriched fraction (Fig. 1 *A* and *B*). Moreover, a greater percentage of $Lin^-CD34^+CD38^-$ cells

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Conflict of interest statement: I.L.W. is a Director of Stem Cells, Inc., and is a cofounder of Cellerant, Inc. and Stem Cells, Inc. I.L.W. and R.M. have filed International Patent Application No. PCT/US2009/000224 entitled "Markers of Acute Myeloid Leukemia Stem Cells."

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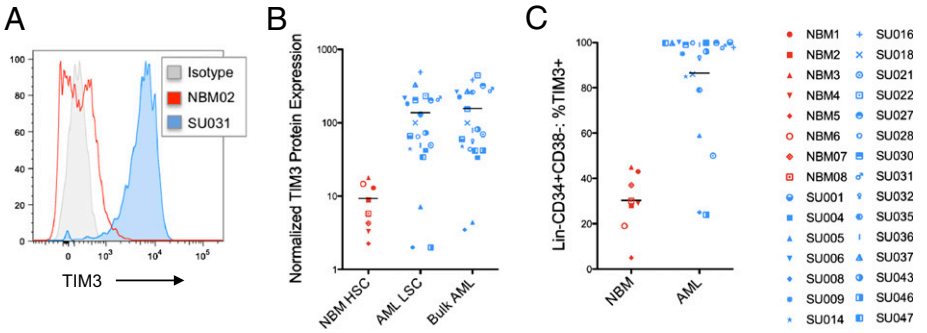
expressed TIM3 in multiple specimens of AML than in NBM (Fig. 1*C*). TIM3 was expressed on 20 of 22 patient specimens examined, which consisted of diverse clinical and molecular subtypes (Fig. 1*D*).

Because TIM3 expression was similar on bulk AML cells and the LSC-enriched fraction, bulk AML gene expression data from a previously described cohort of 526 adult AML patients (29) was used to examine *TIM3* expression across cytogenetic and molecularly defined subgroups of AML. Across cytogenetic subgroups, *TIM3* was more highly expressed in AML associated with core binding factor translocations, *t*(8;21)(q22;q22) and *idt*(16), but was also detected in samples from other subtypes (Fig. S1*A*). In NKAML, no significant associations were identified between *TIM3* expression and either *FLT3*-ITD or NPM1c mutations; however, higher *TIM3* expression was detected in the presence of mutations in one or both copies of *CEBPA* (Fig. S1*B*).

Most Functional NBM HSC Do Not Express TIM3. To investigate the self-renewal and differentiation potential of $TIM3^+$ and $TIM3^-$ cells in the HSC-enriched $Lin^-CD34^+CD38^-$ fraction of NBM, $TIM3^+$ and $TIM3^-$ cells from four independent NBM specimens were purified by fluorescence-activated cell sorting (FACS) and transplanted into newborn NOD/SCID/IL2Rγ^{null} (NSG) mice (Fig. 2*A*). In vivo HSC activity was indicated by the presence of both human $CD45^+CD19^+$ lymphoid cells and human $CD45^+CD33^+$ myeloid cells in the bone marrow 12 wk after transplant (Fig. 2*B*). When transplanted at equal cell doses, HSC engraftment occurred more frequently in $TIM3^-$ (8/12 mice) than $TIM3^+$ subpopulations (2/12 mice) ($P = 0.04$, Fisher's exact test) (Fig. 2*C*). In summary, although $TIM3^+$ cells from normal bone marrow have engraftment potential, HSC reside predominantly within the $TIM3^-$ compartment.

Most AML LSC Express TIM3. Lin^-CD34^+ cells from seven AML cases were tested for LSC function, as defined by the ability to transplant long-term leukemic engraftment in NSG mice. In two of these cases, $TIM3^-$ cells were not detected, and leukemic engraftment was observed from the single $TIM3^+$ fraction (Fig. 2*E*). In the remaining five cases, $TIM3^+$ and $TIM3^-$ cells were tested for LSC function. When transplanted at equal cell doses, leukemic engraftment was observed more frequently from $TIM3^+$ (10/11 mice) than $TIM3^-$ (4/10 mice) cells ($P = 0.02$, Fisher's exact test) (Table S1). Whereas only the $TIM3^+$ subpopulation transplanted leukemic engraftment from AML cases SU018, SU047, and SU030, both $TIM3^+$ and $TIM3^-$ cells transplanted leukemic engraftment from SU028 and the $Lin^-CD34^+CD38^+$ fraction of SU046 by transplantation of >80,000 cells. The leukemic engraftment potential of $TIM3^+$ and $TIM3^-$ fractions of $Lin^-CD34^+CD38^+$



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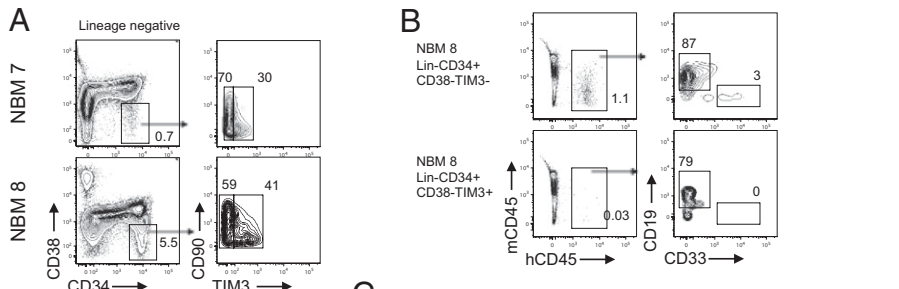
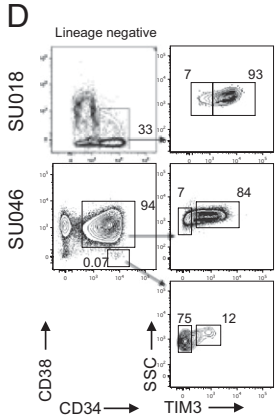
Sample	Age	Gender	Primary/ Secondary	De Novo/ Relapsed	Cytogenetics	Flt-3 ITD	%CD34+	WHO Classification	FAB
SU001	59	Female	Primary	Relapsed	Normal	Negative	99	AML not-otherwise specified	M2
SU004	47	Female	Primary	Relapsed	Normal	Positive	38	AML not-otherwise specified	M5
SU005	56	Male	Secondary	De Novo	t(8;9),-21	Negative	52	AML not-otherwise specified with prior hx of CEL	N/A
SU006	51	Female	Primary	De Novo	Failed to grow	Negative	94	AML not-otherwise specified	M1
SU008	64	Male	Primary	De Novo	Normal	Negative	73	AML with multilineage dysplasia without antecedent MDS	M2
SU009	43	Male	Primary	De Novo	Normal	Negative	73	AML with multilineage dysplasia without antecedent MDS	M2
SU014	59	Male	Primary	De Novo	Normal	Positive	18	AML not-otherwise specified	N/A
SU016	59	Male	Primary	De Novo	Normal	Positive	10	AML with multilineage dysplasia without antecedent MDS	NS
SU018	21	Male	Primary	Relapsed	Normal	Positive	55	AML not-otherwise specified	M5
SU021	60	Male	Secondary	Relapsed	Normal	Negative	74	AML with multilineage dysplasia following MDS	N/A
SU022	62	Male	Secondary	De Novo	-Y; -9	Unknown	ND	AML with multilineage dysplasia following MDS	M2
SU027	61	Male	Primary	Relapsed	Failed to grow	Unknown	10	AML not-otherwise specified	M5
SU028	53	Female	Primary	Relapsed	Complex	Positive	50	AML not-otherwise specified	M5
SU030	53	Female	Primary	De Novo	Normal	Positive	40	AML with multilineage dysplasia without antecedent MDS	M4
SU031	31	Female	Primary	De Novo	inv(16)	Negative	97	AML with inv(16)(p13;q22) or t(16;16)(p13;q22)	M4eo
SU032	47	Male	Primary	De Novo	Normal	Negative	68	AML not-otherwise specified	M5
SU035	46	Male	Primary	De Novo	Failed to grow	Negative	68	AML not-otherwise specified	M5
SU036	71	Female	Primary	De Novo	t(8;21)	Negative	ND	AML with t(8;21)(q22;q22)	N/A
SU037	21	Female	Primary	De Novo	t(8;21)	Negative	ND	AML with t(8;21)(q22;q22)	M2
SU043	39	Female	Primary	De Novo	Normal	Positive	1	AML not-otherwise specified	M4
SU046	53	Female	Primary	De Novo	t(6;11)	Negative	94	AML with 11q23 (MLL)	M5a
SU047	60	Male	Primary	De Novo	Normal	Positive	4	AML with multilineage dysplasia without antecedent MDS	M5a

Fig. 1. TIM3 is more highly expressed on AML LSC than on NBM HSC. (*A*) Representative flow cytometry histograms indicating TIM3 expression on NBM HSC ($Lin^-CD34^+CD38^-CD90^+$) and AML LSC ($Lin^-CD34^+CD38^-CD90^-$). (*B*) TIM3 protein expression was assessed by flow cytometry for multiple specimens of NBM HSC, primary AML LSC, and bulk AML. Mean fluorescence intensity was normalized for cell size and against lineage-positive cells for comparison between measurements conducted on different days. Normalized mean expression (and range) for each population were as follows: NBM HSC 8.8 (2.3–18.9), AML LSC 130.0 (3.0–488.5), bulk AML 148.1 (3.5–443.7). Using a two-sided Student's *t* test, differences in mean expression between NBM HSC and AML LSC ($P = 0.01$) and between NBM HSC and bulk AML ($P = 0.01$) were statistically significant, whereas the difference in mean expression between AML LSC and bulk AML ($P = 0.67$) was not statistically significant. (*C*) The percentage of cells positive for TIM3 expression by flow cytometry within the $Lin^-CD34^+CD38^-$ compartment of AML and normal bone marrow samples was determined by comparison with isotype control. Mean (and median) percentage of cells positive for TIM3 expression were as follows: NBM 30% (30%), AML 86% (98%). The difference in mean percentage of cells positive for TIM3 expression between NBM and AML ($P < 0.0001$) was statistically significant using a two-tailed Student's *t* test. (*D*) Clinical features of AML patients whose samples were studied here.



C

NBM Sample	TIM3- Cell Dose	TIM3- CD19+/CD33+ Engraftment	TIM3- CD19+ Engraftment	TIM3+ Cell Dose	TIM3+ CD19+/CD33+ Engraftment	TIM3+ CD19+ Engraftment
5	600	2/3	1/3	600	0/3	1/3
6	1,230	1/2	0/2	1,230	0/3	0/3
	5,500	2/2	0/2			
7	125	1/2	0/2	125	0/2	0/2
8	2,000	4/5	0/5	2,000	2/4	1/4
Summary		10/14	1/14		2/12	2/12



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AML Sample	Lin- Population	TIM3- Cell Dose x 10^3	TIM3- Leukemic Engraftment	TIM3- Lymphoid and Myeloid Engraftment	TIM3+ Cell Dose x 10^3	TIM3+ Leukemic Engraftment	TIM3+ Lymphoid and Myeloid Engraftment
SU001	CD34+				344	3/3	0/3
SU004	CD34+CD38-				240	3/3	0/3
SU018	CD34+	12	0/2	0/2	12	2/2	0/2
					350	2/2	0/2
SU028	CD34+	22	2/2	0/2	22	2/2	0/2
					350	2/2	0/2
SU030	CD34+	0.4	0/4	2/4	20	2/2	0/2
					200	2/2	0/2
SU046	CD34+CD38+	80	2/2	0/2	80	2/2	0/2
	CD34+CD38-	1	0/2	1/2	1	2/2	0/2
SU047	CD34+CD38-	2	0/2	2/2	2	2/3	0/3
					125	1/1	0/2
Summary			4/14	5/14		25/26	0/26

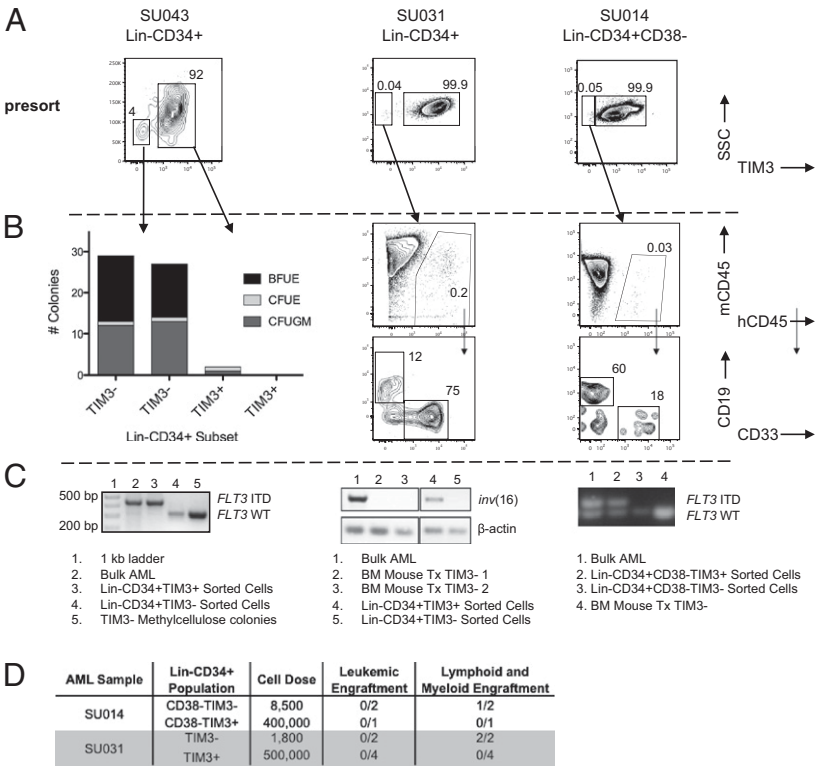
Fig. 2. Functional NBM HSC and LSC differ in TIM3 expression. (*A*) TIM3$^+$ and TIM3$^-$ fractions of the Lin$^-$CD34$^+$CD38$^-$ compartment from normal human bone marrow were identified by flow cytometry. (*B*) These cells were purified by two rounds of FACS and transplanted into NSG pups. Twelve weeks later, bone marrow cells were harvested and analyzed by flow cytometry for the presence of human CD45$^+$ leukocyte engraftment (*Left*) whose lineage was further defined by expression of CD19 on lymphoid cells and CD33 on myeloid cells (*Right*). (*C*) Summary of long-term engraftment in NSG mice from normal bone marrow populations. (*D*) TIM3$^+$ and TIM3$^-$ subpopulations were observed in Lin$^-$CD34$^+$ (SU018) or both Lin$^-$CD34$^+$CD38$^+$ and Lin$^-$CD34$^+$CD38$^-$ (SU046) AML cells by flow cytometry. (*E*) Summary of bone marrow engraftment 12 wk after transplantation in NSG mice from multiple AML cases.

SU046 cells was investigated in further detail. Limiting dilution analysis for leukemic engraftment revealed similar LSC frequencies within TIM3$^+$ and TIM3$^-$ subpopulations of SU046 (Table S2). To formally demonstrate LSC function, secondary transplantation experiments were conducted for AML cases SU018 and SU028. All cell populations capable of engrafting primary recipients also initiated secondary xenografts (Table S3). In summary, LSC function was restricted to the TIM3$^+$ fraction of the Lin$^-$CD34$^+$compartment in five of seven AML cases.

Prospective Separation of Normal HSC From LSC in a Series of AML Patients. We identified eight primary AML specimens with a high percentage of leukemic cells (mean and median blast counts of 80% and 86%, respectively) that exhibited evidence of residual normal HSC function as assessed by long-term engraftment of lymphoid-myeloid hematopoiesis in NSG mice (n = 6) or HPC function as assessed by morphologically normal erythro-myeloid colony formation in complete methylcellulose (n = 2). Because TIM3 expression differed between functional NBM HSC and AML LSC, we investigated the ability of TIM3 expression to discriminate residual HSC from LSC in the same patient sample.

Within the HSC- and LSC-enriched Lin$^-$CD34$^+$ population, rare TIM3$^-$ and majority TIM3$^+$ populations were detected and FACS-separated in 6 of these 8 AML cases (Figs. 3*A* and 4*A*). All HSC engraftment in vivo and most HPC colony formation in vitro was restricted to each TIM3$^-$ subpopulation (Figs. 3 *B* and *D* and 4*B*). In AML cases SU043, SU031, and SU014, neither leukemic in vitro colony formation nor in vivo leukemic engraftment was observed, consistent with the previously reported inability of some primary human AML specimens to engraft in immunodeficient mice (30) (Fig. 3 *B* and *D*). To further investigate the relationship between these functionally normal hematopoietic cells and their patient-matched leukemias, FACS-purified TIM3$^-$ cells and their progeny from in vivo or in vitro assays were assessed for molecular mutations known to be present in each leukemia sample [*FLT3*-ITD or inv(16)]. In each case, the molecular mutation was detected in the TIM3$^+$ cells but not in the rare TIM3$^-$ cells or their progeny (Fig. 3*C*). In summary, these data are consistent with prospective separation of functionally normal HSC and HPC from leukemic cells in three AML patient samples based on differential expression of TIM3.

In the additional three cases in which Lin$^-$CD34$^+$TIM3$^-$ populations were detected, engraftment studies in vivo identified functional HSC in the TIM3$^-$ fraction and LSC activity in the TIM3$^+$ fraction (Fig. 4 *A* and *B*). In one of these cases, AML SU030, PCR analysis confirmed that FACS-purified TIM3$^-$ cells or their progeny did not contain the *FLT3*-ITD mutation, unlike FACS-sorted TIM3$^+$ cells (Fig. 4*C, Left*). In the second of these cases, AML SU047, human CD45$^+$ cells from one mouse engrafted with TIM3$^+$ cells were homozygous for the *FLT3*-ITD mutation, whereas human CD45$^+$ cells from one mouse engrafted with TIM3$^-$ cells were heterozygous for the *FLT3*-ITD and *FLT3*-WT alleles (Fig. 4*C, Center*). These data can be explained either by mixed engraftment of *FLT3*-WT normal HSC and *FLT3*-ITD-homozygous LSC or by engraftment of heterozygous *FLT3*-ITD



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AML Sample	Lin-CD34+ Population	Cell Dose	Leukemic Engraftment	Lymphoid and Myeloid Engraftment
SU014	CD38-TIM3-	8,500	0/2	1/2
	CD38-TIM3+	400,000	0/1	0/1
SU031	TIM3-	1,800	0/2	2/2
	TIM3+	500,000	0/4	0/4

Fig. 3. Prospective separation of residual normal HSC from leukemic cells in three AML cases. (*A*) TIM3 expression was determined on the Lin$^-$CD34$^+$ or Lin$^-$CD34$^+$CD38$^-$ fraction of each AML sample by flow cytometry. (*B*) TIM3$^-$ and TIM3$^+$ cells were double-sorted to >99% purity by FACS. For AML SU043, these sorted cells were plated in duplicate into complete methylcellulose media and, 14 d later, myeloid colony formation was determined by microscopy. No leukemic blast colonies were observed. For AML SU014 and SU031, sorted cells were transplanted into NSG pups and, 12 wk later, human engraftment in mouse bone marrow was analyzed by flow cytometry as in Fig. 2*B*. (*C*) Sorted cell populations, methylcellulose colonies from TIM3$^-$ cells, and/or bone marrow cells from mice engrafted with TIM3$^-$ cells were assessed by PCR for the presence of the *FLT3*-ITD mutation for AML SU043 and SU014. For AML SU031, sorted cells or human CD45$^+$ cells purified from engrafted mice were assessed by RT-PCR for the presence of the *CBFB-MYH11* fusion transcript produced by inv(16) and human β-actin. (*D*) Summary of long-term engraftment in NSG mice of subpopulations from multiple AML cases.

preleukemic HSC in the TIM3$^-$ compartment. In the third case, AML SU046, FISH analysis demonstrated MLL rearragement in 38/40 sorted Lin$^-$CD34$^+$CD38$^-$TIM3$^+$ cells, 13/40 sorted Lin$^-$CD34$^+$CD38$^-$TIM3$^-$ cells, and 1/40 human CD45$^+$ cells from one mouse engrafted with TIM3$^-$ cells (Fig. 4*C*, *Right*). MLL rearrangement-positive cells within the Lin$^-$CD34$^+$CD38$^-$TIM3$^-$ compartment may be either leukemic or preleukemic. Therefore, differential TIM3 expression discriminated functional normal HSC from functional LSC in these 3 AML patient samples, permitting the prospective separation of these critical stem cell populations. In total, we identified Lin$^-$CD34$^+$TIM3$^-$ subpopulations mostly lacking patient matched leukemic molecular markers with HSC ($n = 5$) or HPC ($n = 1$) function in eight AML cases.

Discussion

We report here the identification of TIM3 as a unique AML stem cell surface marker that is highly expressed on multiple specimens of AML LSC, but not on NBM HSC. *TIM3* is a negative regulator of Th1–T-cell immunity (31, 32). TIM3 is also expressed on innate immune cells including dendritic cells, monocytes, and macrophages, where it can synergize with Toll-like receptor signaling via crosslinking with Galectin-9 (33) and mediate phagocytosis of apoptotic cells by binding phosphatidylserine (34). *TIM3* expression was detected in all cytogenetic subgroups of AML, but was significantly higher in AML-associated with core binding factor translocations or mutations in *CEBPA*. By assessing engraftment in NSG mice, we determined that most NBM HSC do not express TIM3, whereas LSC from multiple AML specimens express high levels of TIM3. Finally, TIM3 expression enabled the prospective separation of HSC from leukemic cells in the majority of AML samples with residual normal HSC function.

It is not known why *TIM3* is highly expressed in AML. Elevated *TIM3* expression may be a direct consequence of the molecular mutations present in AML. Indeed, *TIM3* expression was detected in all cytogenetic subgroups of AML, but was significantly higher in AML-associated with core binding factor translocations or mutations in *CEBPA* (Fig. S1). Mutations in these transcription factors that regulate myeloid differentiation (35) may directly regulate *TIM3* transcription or may arrest leukemic cells in a stage of differentiation normally associated with high *TIM3* expression.

TIM3 is a candidate for AML LSC-targeted therapeutic monoclonal antibodies, as recently reported (36). The absence of TIM3 expression on functional NBM HSC and residual HSC in AML samples, in contrast to high expression on multiple samples of AML LSC, provides the rationale for developing such antibodies. Recently, we demonstrated that a blocking monoclonal antibody directed against CD47, a protein overexpressed on LSC, eliminated AML LSC by stimulating phagocytosis through Fc receptor-independent mechanisms (23). In this context, anti-TIM3 antibodies able to bind and activate Fc receptors might synergize with anti-CD47 antibodies to induce more effective phagocytic elimination of AML LSC (37).

The ability to prospectively separate HSC from AML LSC is an unmet and important goal. Previous reports of prospective separation of HSC and LSC show promise but have not been successfully demonstrated on a diverse cohort of AML specimens. Aberrant expression of mature hematolymphoid cell markers

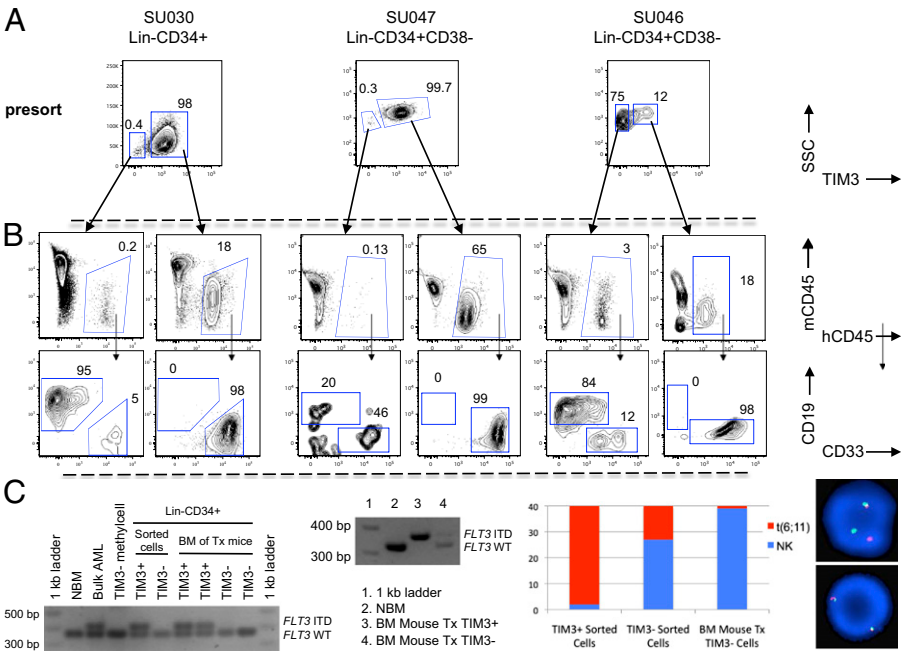

Fig. 4. Prospective separation of functional HSC and functional LSC from three AML cases. (*A*) TIM3 expression was determined on the Lin^-CD34^+ or $Lin^-CD34^+CD38^-$ fractions of each AML sample by flow cytometry. (*B*) $TIM3^+$ and $TIM3^-$ cells were double-sorted to >99% purity by FACS. These sorted cells were transplanted into newborn NSG mice. Twelve weeks after transplantation, bone marrow cells were harvested and analyzed by flow cytometry for the presence of human $CD45^+$ leukocyte engraftment (*Left*) whose lineage was further defined by expression of CD19 on lymphoid cells and CD33 on myeloid cells (*Right*). (*C*) Sorted cell populations and/or bone marrow cells from engrafted mice were assessed by PCR for the presence of the *FLT3*-ITD mutation for AML SU030 and SU047. For AML SU046, FISH analysis was used to assess the presence of the *MLL* translocation produced by *t*(6;11) in FACS-sorted cell populations and human $CD45^+$ cells purified from engrafted mice. Representative normal and abnormal *MLL* FISH results using a break-apart probe are shown.

including CD7, CD19, and CD56 within the $Lin^-CD34^+CD38^-$ fraction of AML specimens can distinguish malignant marker-positive cells from nonmalignant marker-negative cells (26). However, cell populations with malignant and normal stem cell function have not yet been separated by this scheme. In NPM-mutated AML, which is associated with low CD34 expression, CD34 and CD38 expression can prospectively separate functional NPM–wild-type HSC and functional NPM-mutated LSC in ≈50% of AML cases considered (27). This strategy is specific to CD34-negative AML, which represents a minority of all AML cases. CD47 has been shown to separate functional HSC from LSC in a single AML case (23). In summary, TIM3 is a significant addition to current markers used for HSC and LSC discrimination.

Ultimately, prospective separation may have several clinical applications. First, autologous hematopoietic cell transplantation (AHCT) has been used in the treatment of AML, including investigation of protocols using ex vivo chemotherapy to eradicate residual leukemic cells in the autograft (38–40). AHCT has fallen out of mainstream clinical practice because of equivalence to conventional chemotherapy (39, 41). However, purification of LSC-depleted, functionally normal HSC may improve AHCT outcomes and potentially broaden the use of AHCT to patients who do not achieve complete remission. Second, the ability to discriminate HSC and LSC by flow cytometry may enable evaluation of LSC-targeted therapeutics and prediction of relapse based on minimal residual disease monitoring at the level of the LSC (42). This possibility is supported by the demonstration that increased expression of the LSC marker CLL-1 in the $CD34^+CD38^-$ bone marrow fraction from two AML patients in remission correlated with relapse (19).

Finally, prospective separation of residual functionally normal HSC from LSC based on TIM3 expression provides an opportunity to map the accumulation of mutations leading to AML. Because leukemogenesis involves the multistep accumulation of rare mutations (43), preleukemic mutations likely accumulate in self-renewing HSC (16). The ability to prospectively isolate functionally normal HSC from AML patients could enable the direct identification of preleukemic mutations.

Materials and Methods

Human Samples. Normal human bone marrow mononuclear cells were purchased from AllCells. Human AML samples were obtained from patients at the Stanford Medical Center with informed consent, according to Institutional Review Board (IRB)-approved protocols (Stanford IRB no. 76935 and 6453).

Animal Care. All mouse experiments were conducted according to an Institutional Animal Care and Use Committee-approved protocol and in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Flow Cytometry Analysis and Cell Sorting. A panel of antibodies was used for analysis and sorting of AML LSC ($Lin^-CD34^+CD38^-$) and HSC ($Lin^-CD34^+CD38^-CD90^+$) as described (23, 28). For AML samples, the lineage consisted of CD3, CD19, and CD20. TIM3 antibody clone 344823 (R&D Systems) was used. Human CD34-positive cells were enriched from NBM by magnetic selection (StemCell Technologies).

Methylcellulose Colony Assay. Erythro-myeloid colony formation was assayed by culturing hematopoietic cells in complete methylcellulose (Methocult GF$^+$ H4435; Stem Cell Technologies) as described (13). Colony formation was assayed after 14 d in culture by microscopy. Colony types scored were as follows: CFU-GEMM, colony forming unit–granulocyte, erythrocyte, monocyte, megakaryocyte; BFU-E, blast forming unit–erythrocyte; CFU-E, colony forming unit–erythrocyte; and CFU-GM–colony forming unit–granulocyte, monocyte.

NSG Xenotransplantation Assay. FACS-purified cell populations were transplanted into newborn NSG mice conditioned with 100 rads of irradiation as described (13, 23, 44). After 12 wk, mice were killed and peripheral blood and bone marrow were analyzed for human myeloid engraftment ($hCD45^+CD33^+$) and human lymphoid engraftment ($hCD45^+CD19^+$) as described (13, 23, 44).

PCR Analysis for inv(16) and *FLT3*-ITD. Total RNA was isolated by Rneasy Micro Kit (Qiagen). cDNA was reverse-transcribed with SuperScript III First-Strand Synthesis Kit (Invitrogen). inv(16) was detected by PCR with the following primers: CBFB-C (F) 5′-GGGCTGTCTGGAGTTTGATG-3′ and MYH11-B2 (R) 5′-TCCTCTTCTCCTCATTCTGCTC-3′ as described (45). Genomic DNA was isolated by Gentra Puregene Cell Kit (Qiagen). *FLT3*-ITD was detected by PCR with the following primers: *FLT3* 11F (F) 5′-gcaatttaggtatgaaagccagc-3′ and *FLT3* 12R (R) 5′-CTTTCAGCATTTTGACGGCAACC-3′ as described (23).

FISH Analysis for MLL Translocation. Slide preparations were analyzed by FISH by using the MLL breakapart probe (Abbott Molecular) specific for the MLL gene locus at chromosomal band 11q23. Briefly, dried, dehydrated specimens, pretreated per the manufacturer's instructions, were denatured (75 °C, 5 min) and hybridized (37 °C, 24 h) by using a VysisHYBrite instrument. Slides were washed with 2× SSC/0.3% Nonidet P-40 at 73 °C for 2 min, counterstained with DAPI, and analyzed with an Olympus BX51 microscope equipped with an 100× oil immersion objective, appropriate fluorescent filters, and CytoVision imaging software (Genetix).

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