TIM-3 Is a Promising Target to Selectively Kill Acute Myeloid Leukemia Stem Cells

Yoshikane Kikushige,[1] Takahiro Shima,[1] Shin-ichiro Takayanagi,[2] Shingo Urata,[1] Toshihiro Miyamoto,[1] Hiromi Iwasaki,[1] Katsuto Takenaka,[1] Takanori Teshima,[1] Toshiyuki Tanaka,[3] Yoshimasa Inagaki,[2] and Koichi Akashi[1,*]
[1]Department of Medicine and Biosystemic Sciences, Kyushu University Graduate School of Medicine, Fukuoka 812-8582, Japan
[2]Innovative Drug Research Laboratories Kyowa Hakko Kirin Co., Ltd., Tokyo 194-8538, Japan
[3]School of Pharmacy, Hyogo University of Health Sciences, Kobe 650-8530, Japan
*Correspondence: akashi@med.kyushu-u.ac.jp
DOI 10.1016/j.stem.2010.11.014

SUMMARY

Acute myeloid leukemia (AML) originates from self-renewing leukemic stem cells (LSCs), an ultimate therapeutic target for AML. Here we identified T cell immunoglobulin mucin-3 (TIM-3) as a surface molecule expressed on LSCs in most types of AML except for acute promyelocytic leukemia, but not on normal hematopoietic stem cells (HSCs). TIM-3^{+} but not TIM-3^{−} AML cells reconstituted human AML in immunodeficient mice, suggesting that the TIM-3^{+} population contains most, if not all, of functional LSCs. We established an anti-human TIM-3 mouse IgG2a antibody having complement-dependent and antibody-dependent cellular cytotoxic activities. This antibody did not harm reconstitution of normal human HSCs, but blocked engraftment of AML after xenotransplantation. Furthermore, when it is administered into mice grafted with human AML, this treatment dramatically diminished their leukemic burden and eliminated LSCs capable of reconstituting human AML in secondary recipients. These data suggest that TIM-3 is one of the promising targets to eradicate AML LSCs.

INTRODUCTION

Acute myeloid leukemia (AML) is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs). LSCs self renew and generate leukemic progenitors that actively divide to produce a large number of immature clonogenic leukemic blasts (Bonnet and Dick, 1997; Hope et al., 2004; Lapidot et al., 1994). This hierarchical stem cell-progenitor-mature cell relationships in AML appears to simulate normal hematopoiesis that originates from hematopoietic stem cells (HSCs) with self-renewal activity. We have shown that like normal HSCs, AML LSCs are quiescent in vivo and appear to reside at the endosteal "osteoblastic" niche in the bone marrow based on our analysis in a xenograft model (Ishikawa et al., 2007). AML LSCs are resistant to chemotherapeutic reagents that usually target cycling malignant cells. In the majority (~90%) of AML patients, the conventional chemotherapies can diminish the leukemic clones to achieve remission. However,

~60% of such remission patients still relapse, and the recurrence of AML in these patients should originate from LSCs that survive the intensive chemotherapies. Therefore, the LSC should be the ultimate cellular target to cure human AML.

To eradicate the AML LSC without killing normal HSCs, it is critical to isolate a molecule that is expressed or functions specifically at the AML LSC stage (Krause and Van Etten, 2007). It has been shown that the AML LSCs mainly reside within the CD34^{+}CD38^{−} fraction of leukemic cells and can reconstitute human AML in immunodeficient mice (Lapidot et al., 1994), although recent studies have suggested that LSCs can exist also in CD34^{+}CD38^{+} (Taussig et al., 2008) or CD34^{−} blastic fractions at least in some types of AML (Martelli et al., 2010; Taussig et al., 2010). Normal HSCs with long-term reconstitution activity also have the CD34^{+}CD38^{−} phenotype (Bhatia et al., 1997; Ishikawa et al., 2005). However, the expression pattern of other surface molecules in the CD34^{+}CD38^{−} fraction of AML cells is different from that of normal controls. For example, CD34^{+}CD38^{−} AML cells possess many phenotypic characteristics analogous to normal granulocyte/macrophage progenitors (GMPs) (Yoshimoto et al., 2009). Previous studies have reported molecules preferentially expressed in AML cells. Such molecules include CLL-1 (van Rhenen et al., 2007), CD25, CD32 (Saito et al., 2010), CD33 (Florian et al., 2006; Hauswirth et al., 2007), CD44 (Florian et al., 2006; Jin et al., 2006), CD47 (Jaiswal et al., 2009; Majeti et al., 2009), CD96 (Hosen et al., 2007), and CD123 (Jin et al., 2009; Yalcintepe et al., 2006). However, in our hands, some of these molecules are expressed in LSCs at a level insufficient for clear distinction, are expressed also in normal HSCs at a considerable level, or are found only in a fraction of AML cases. It is therefore critical to isolate ideal targets for AML LSCs with sufficient specificity and sensitivity.

Here we report a new surface molecule that might be useful to eradicate AML LSCs leaving normal HSC intact. We performed differential transcriptional profiling of AML LSCs and HSCs and extracted the T cell immunoglobulin mucin-3 (TIM-3) as a promising AML LSC-specific target surface molecule. TIM-3 is originally found as a surface molecule expressed in CD4^{+} Th1 lymphocytes in mouse hematopoiesis and is an important regulator of Th1 cell immunity and tolerance induction (Monney et al., 2002; Sabatos et al., 2003; Sánchez-Fueyo et al., 2003). Murine TIM-3 is also expressed in CD11b^{+} macrophages and CD11c^{+} dendritic cells and recognizes apoptotic cells' phosphatidylserine through its IgV domain to mediate phagocytosis (Nakayama et al., 2009).

We found that human TIM-3 was expressed in the vast majority of CD34^{+}CD38^{−} LSCs and CD34^{+}CD38^{+} leukemic progenitors in AML of most FAB types, except for acute
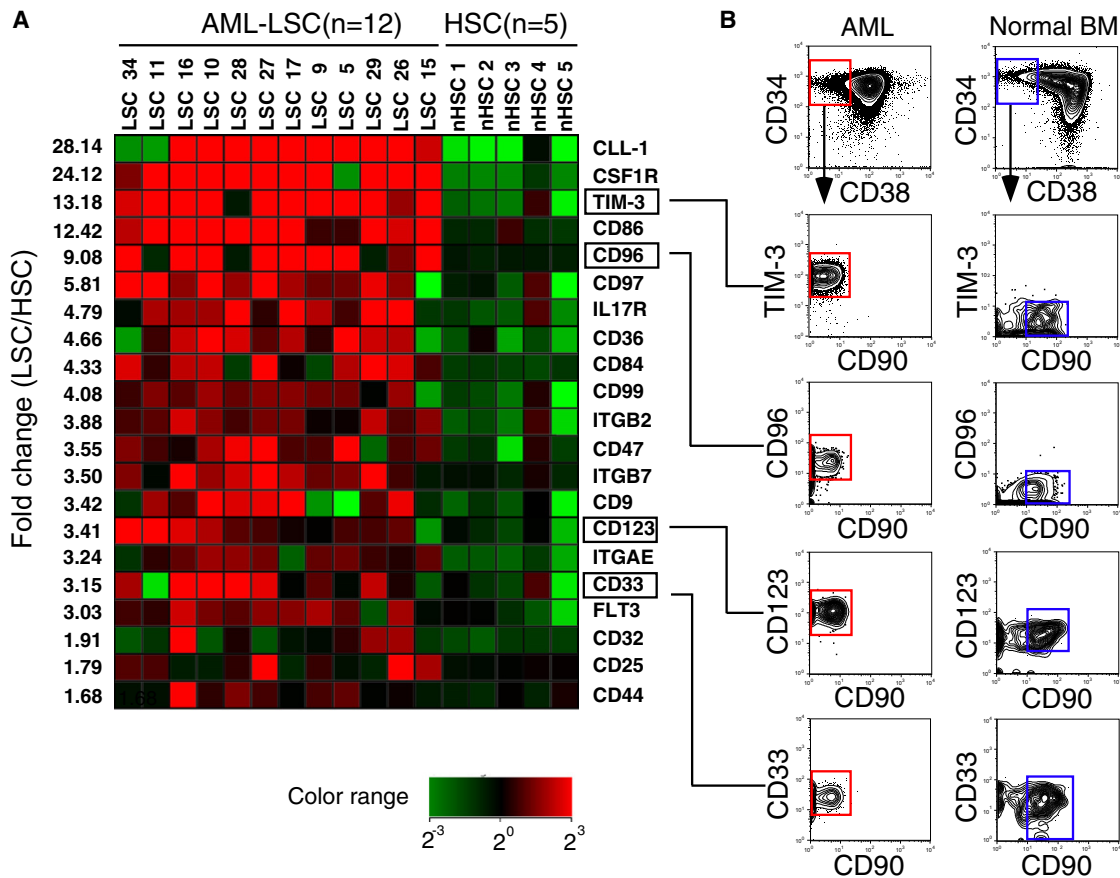
Figure 1. The Expression of LSC-Specific Surface Molecules in AML

The CD34+CD38− adult bone marrow HSCs and CD34+CD38− AML LSCs were purified and tested for their surface molecule expression.

(A) Results of cDNA microarray analysis of HSCs and AML LSCs. Representative genes coding surface molecules that are expressed highly in AML LSCs are shown. TIM-3 is expressed specifically in LSCs at high levels in the majority of AML patients. Patient numbers correspond to those in Table S1.

(B) The expression of representative surface proteins in HSCs and AML LSCs on FACS.

promyelocytic leukemia (M3). TIM-3 was not expressed in CD34+CD38− normal HSCs or the vast majority of CD34+ CD38+ normal progenitors. Administration of anti-human TIM-3 mouse antibodies with a complement-dependent cytotoxicity (CDC) and an antibody-dependent cellular cytotoxicity (ADCC) selectively inhibited engraftment and development of human AML in xenograft models. Our data strongly suggest that the use of TIM-3 to target AML LSCs is a promising approach for the improvement of leukemia therapy.

RESULTS

TIM-3 Is Expressed in the CD34+CD38− Fraction of AML Patients' Bone Marrow Cells

In most types of AML, LSCs are concentrated in the CD34+ CD38− fraction of AML cells (Ishikawa et al., 2007; Lapidot et al., 1994), whose phenotype is common to normal adult HSCs. Patients' characteristics are shown in Table S1 available online. To search for the AML LSC-specific molecules, 10,000 each of purified CD34+CD38− AML cells and CD34+CD38− normal HSCs were subjected to cDNA microarray analysis. We extracted 256 genes with >4-fold change between normal

HSCs and CD34+CD38− AML cells and then selected 197 differentially expressed genes with <0.01 of a cut-off p value (Table S2). Genes coding surface molecules that are expressed highly in CD34+CD38− AML cells were selected for this study. Figure 1A shows the mRNA levels of candidate LSC-specific surface molecules in purified CD34+CD38− AML cells and normal HSCs. The molecules expressed in CD34+CD38− AML cells at levels >8-fold higher as compared to normal HSCs included TIM-3 and previously identified LSC-specific molecules such as CLL-1 (van Rhenen et al., 2007), CSF1R (Aikawa et al., 2010), and CD96 (Figure 1A; Hosen et al., 2007). As shown in Figure 1B, TIM-3 protein was highly expressed in CD34+CD38− AML cells but not in normal HSCs. We focused on TIM-3 not only because it is expressed specifically in CD34+CD38− AML cells at high levels, but also because it is expressed in the majority of patients with most AML types.

We evaluated the TIM-3 protein expression on cell surface of AML cells by FACS analysis. As shown in Figure 2, the vast majority of the CD34+CD38− LSCs as well as CD34+CD38+ progenitor fractions in AML M0, M1, M2, and M4 types expressed TIM-3 at a high level in virtually all cases studied. In AML M5, M6, and M7, a considerable fraction of CD34+CD38− cells
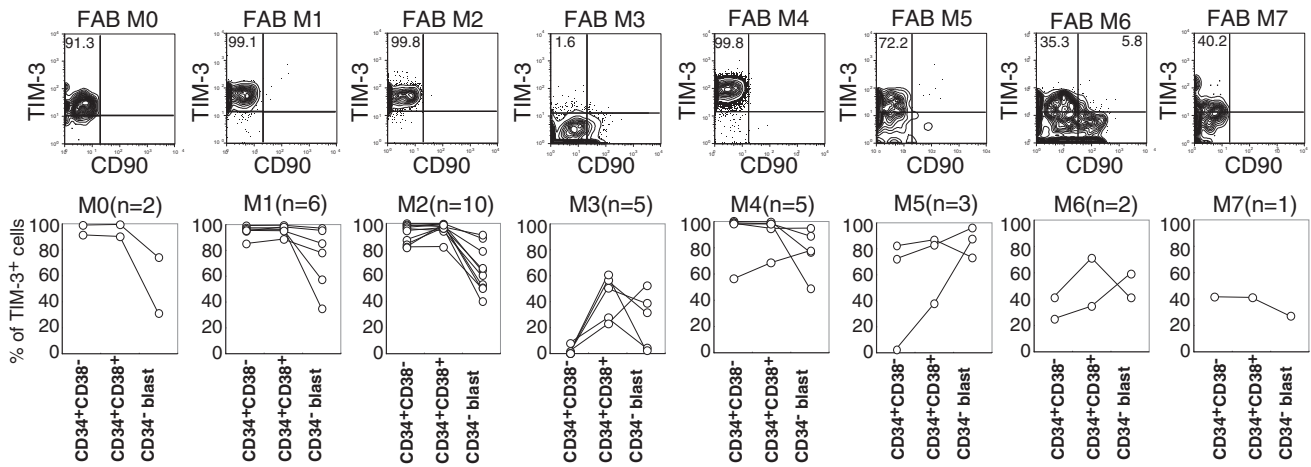
Figure 2. The Expression of TIM-3 in Stem and Progenitor Populations of AML of Each FAB Type
Expression of TIM-3 in each FAB type of AML. The representative expression pattern of TIM-3 in the CD34$^+$CD38$^-$ LSC fraction (top) and distribution of TIM-3 in CD34$^+$CD38$^-$ LSCs, CD34$^+$CD38$^+$ leukemic progenitors, and CD34$^-$ leukemic blasts (bottom) are shown.

expressed TIM-3. TIM-3 was, however, not expressed in the CD34$^+$CD38$^-$ population in all five M3 cases tested. In general, TIM-3 was expressed in both CD34$^+$CD38$^-$ LSCs and CD34$^+$CD38$^+$ leukemic progenitor fractions, but its expression tended to decline at the CD34$^-$ leukemic blast stage (Figure 2, bottom).

The TIM-3-Expressing of AML Fraction Contains the Vast Majority of Functional LSCs in a Xenograft Model
Recent studies have suggested that at least in some AML cases, LSCs that are capable of initiating human AML in xenograft models reside not only within the CD34$^+$CD38$^-$ fraction but

also outside of this population including CD34$^+$CD38$^+$ (Taussig et al., 2008) or CD34$^-$ (Martelli et al., 2010; Taussig et al., 2010) AML cells. To evaluate whether functional AML LSCs express TIM-3, 10^6 cells of human TIM-3$^+$ and TIM-3$^-$ AML populations were transplanted into sublethally irradiated immunodeficient mice. We used NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ (NRG) mice for the xenogeneic transplantation experiments, by which higher chimerism of human hematopoietic cells was observed in xenotransplantation assays (Pearson et al., 2008). Recipients transplanted with TIM-3$^+$ and TIM-3$^-$ AML cells were sacrificed 8–10 weeks after transplantation. As shown in Figure 3, human CD45$^+$CD33$^+$ AML cells were
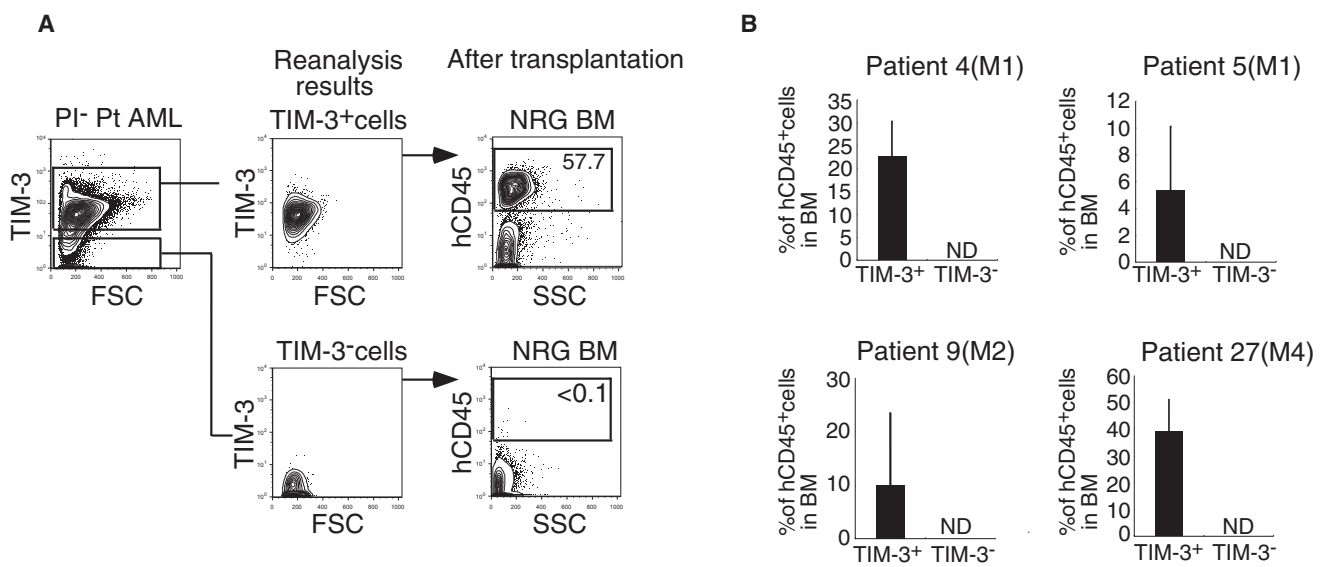

Figure 3. The TIM-3$^+$ AML Population Contains the Vast Majority of Functional LSC Activity
(A) A representative analysis of xenotransplantation of purified TIM-3$^+$ or TIM-3$^-$ AML cells from patient 27 into NRG mice. Only TIM-3$^+$ cells reconstitute hCD45$^+$ AML cells after transplantation.
(B) Summarized data of four independent experiments. Only TIM-3$^+$ (not TIM-3$^-$) AML cells reconstituted human AML cells in xenotransplantation experiments in all experiments, suggesting that most functional LSCs reside in the TIM-3$^+$ AML fraction.
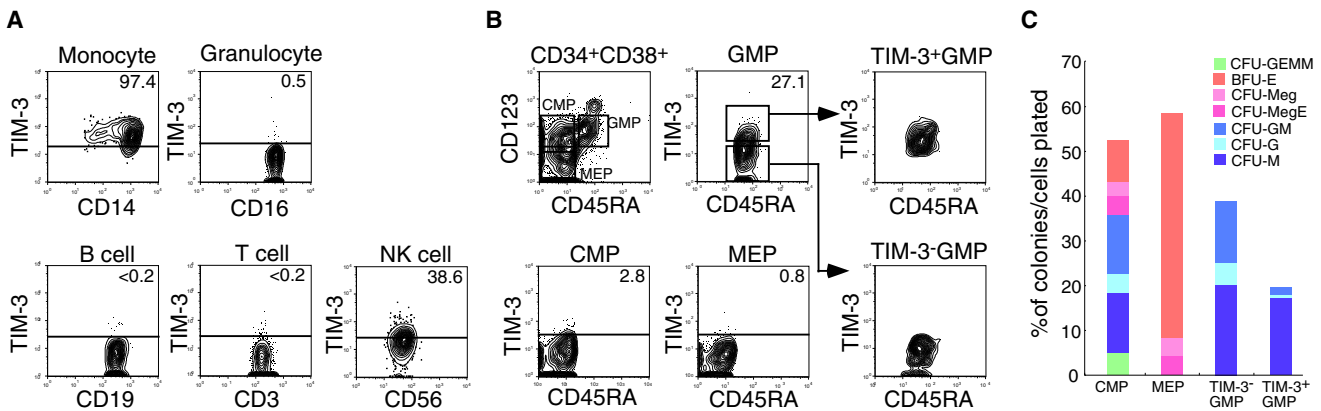
Figure 4. TIM-3 Is Expressed in Monocytes and Their Progenitors in Normal Hematopoiesis
(A) TIM-3 expression in normal mature blood cells.
(B) TIM-3 expression in normal hematopoietic progenitors. A fraction of GMPs but not other myeloid progenitors express TIM-3.
(C) Results of clonogenic assays of myelo-erythroid progenitors including single TIM-3+ GMPs out of five independent experiments. The vast majority of TIM-3+ GMPs gave rise to macrophage colonies (CFU-M).

reconstituted only in mice transplanted with TIM-3+ AML cells, whereas TIM-3− AML cells failed to reconstitution in all four AML cases tested (Figure 3B). Thus, all 23 mice injected with TIM-3+ AML cells reconstituted human AML, whereas 11 mice injected with TIM-3− AML never developed human AMLs after transplantation. These results strongly suggest that LSCs exclusively reside within the TIM-3+ fraction in human AML at least in these patients.

TIM-3 Is Not Expressed in Normal Adult HSCs, and Its Expression Begins after Cells Are Committed to the Monocyte Lineage

Murine TIM-3 is expressed in a fraction of Th1 cells, monocytes, dendritic cells, and mast cells (Anderson et al., 2007; Monney et al., 2002; Nakae et al., 2007). The expression of human TIM-3 protein in normal steady-state human hematopoiesis is shown in Figures 4A. In periphery, TIM-3 was expressed in monocytes and a fraction of NK cells, but not in granulocytes, T cells, or B cells (Figure 4A). In the bone marrow, TIM-3 was not expressed in normal HSCs (Figure 1) or the vast majority of the CD34+CD38+ progenitor population. Within the CD34+CD38+ fraction, TIM-3 was expressed only in a fraction of GMPs at a low level, but not in common myeloid progenitors (CMPs), megakaryocyte/erythrocyte progenitors (MEPs) (Figure 4B), or common lymphoid progenitors (CLPs) (not shown). In clonogenic colony-forming unit (CFU) assays, the vast majority of purified TIM-3+ GMPs gave rise to CFU-M, whereas colonies derived from TIM-3− GMP contained CFU-GM as well as CFU-G and CFU-M (Figure 4C). These data strongly suggest that TIM-3 up-regulation mainly occurs in concert with the monocyte lineage commitment at the GMP stage in normal hematopoiesis.

Anti-Human TIM-3 Antibodies Did Not Impair Development of Normal Hematopoiesis

To selectively eliminate TIM-3-expressing AML LSCs in vivo, we developed a monoclonal antibody against TIM-3 that has an efficient interaction with cellular Fc receptors on innate immune effector cells. It has become clear that the ADCC activity is one of

the most important factors to eliminate target cells in antibody therapies (Nimmerjahn and Ravetch, 2007). A TIM-3 monoclonal antibody (IgG2b) was obtained by immunizing Balb/c mice with L929 cells stably expressing human TIM-3 and soluble TIM-3 protein. The variable portion of the VH regions of the cloned hybridoma that recognize TIM-3 were then grafted onto IgG2a Fc regions, because IgG2a subclass is most efficient to induce ADCC activity in mice (Nimmerjahn and Ravetch, 2005; Uchida et al., 2004). The established clone, ATIK2a, possessed CDC activities in EoL-1 and L929 cells transfected with TIM-3 (Figure 5A), as well as Kasumi-3, an AML cell line that spontaneously expresses TIM-3 (not shown). Importantly, ATIK2a displayed strong ADCC activity against TIM-3-expressing EoL-1 and L929 cells in vitro (Figure 5B).

We first tested the effect of ATIK2a treatment on reconstitution of normal HSCs in a xenograft model. The major effectors in ADCC reaction are NK cells. Because NRG mice do not have NK cells because of γc mutation (Pearson et al., 2008), we used NOD-SCID mice for this experiment to potentiate ADCC activity of ATIK2a antibodies. NOD-SCID mice were sublethally irradiated and were transplanted with 10^5 CD34+ adult human bone marrow cells. 15 μg of ATIK2a was intraperitoneally injected to mice 12 hr after transplantation, which was followed by further injections of 15 μg of ATIK2a once a week until mice were sacrificed at 12 weeks after transplantation. Injection of ATIK2a did not affect reconstitution of normal hematopoiesis: The percentage of human cells were equal (∼1%), and human B and myeloid cells were normally reconstituted irrespective of ATIK2a treatment in three independent experiments (not shown). We also tested the effect of this ATIK2a treatment in NOD-SCID mice transplanted with 10^5 CD34+ cord blood cells. Cord blood cells have potent reconstitution activity in NOD-SCID mice, and percentage of hCD45+ human cells reached ∼50% after transplantation (Figure 5C). Again, the chimerism of human cells was equal, and CD19+ B cells and CD33+ myeloid cells were reconstituted irrespective of ATIK2a treatment (Figure 5C, left). In mice injected with ATIK2a, however, human TIM-3+ monocytes were removed (Figure 5C, right). These data suggest that
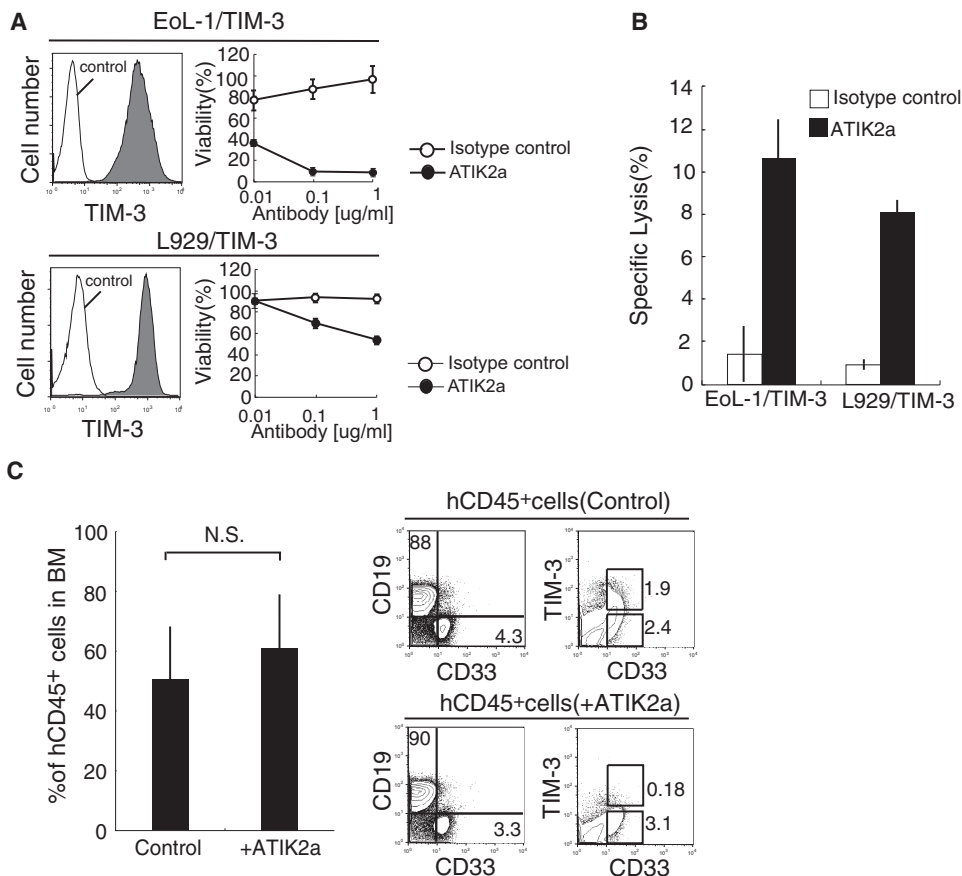
Figure 5. ATIK2a, a New Monoclonal Antibody against TIM-3, Has CDC and ADCC Activities and Does Not Harm Normal Hematopoietic Reconstitution

(A) CDC assays to evaluating the killing effect of ATIK2a antibodies on EoL-1 and L929 cell lines with enforced expression of human TIM-3.

(B) ADCC activities of ATIK2a on TIM-3-expressing EoL-1 and L929 cell lines.

(C) The effect of ATIK2a treatment on human hematopoietic reconstitution in NOD-SCID mice transplanted with 10^5 CD34$^+$ human cord blood cells. 15 μg of ATIK2a was intraperitoneally injected to mice 12 hr after transplantation, which was followed by further injections of 15 μg of ATIK2a once a week until mice were sacrificed at 12 weeks after transplantation. In this experiment, percentages of human cells in 10 each of mouse groups treated with control or ATIK2a antibodies were equivalent at 12 weeks after transplantation.

targeting TIM-3 does not affect development of normal hemato-poiesis but remove TIM-3-expressing monocytes.

Anti-Human TIM-3 Antibodies Effectively Blocked Development of AML LSCs but Not that of Normal HSCs

We then tried to test the effect of ATIK2a in AML LSCs. We transplanted 10^6 bone marrow cells of AML patients (patients 6, 9, 15, 18, and 26) into NOD-SCID mice. The bone marrow of patients 6, 9, 15, and 26 were completely occupied with AML clones, and normal HSCs were not seen on FACS. Samples of each patient were transplanted into six mice, and three mice each were treated with 15 μg of ATIK2a or control IgG 12 hr after transplantation and with the same dose of antibodies once a week (Figure 6A). Mice were sacrificed 16 weeks after xenotransplantation. As shown in Figure 6B, the chimerism of AML cells were low in the NOD-SCID xenotransplant system. Nonetheless, ATIK2a injection significantly blocked AML reconstitution in these mice. In all of these patients, mice injected with control IgG showed reconstitution of CD34$^+$TIM-3$^+$ cells that contained primitive AML stem or progenitors as well as CD33$^+$ AML blasts

(Figure 6B). In contrast, in mice treated with ATIK2a, the leukemic clone was barely detectable, and did not contain detectable numbers of CD34$^+$ cells (not shown), displaying significantly lower chimerisms as compared to control mice in all four independent experiments (Figure 6C).

The bone marrow of patient 18 possessed a small fraction of CD34$^+$CD38$^-$CD90$^+$TIM-3$^-$ cells that was phenotypically normal HSCs, in addition to the major fraction of CD34$^+$CD38$^-$CD90$^-$TIM-3$^+$ AML LSCs (Figure 6D). Interestingly, in mice transplanted with the bone marrow from this patient, ATIK2a injection induced reconstitution of normal myeloid and B cells, whereas control mice developed AML. These data strongly suggest that the ATIK2a treatment selectively inhibited development of human AML, presumably by targeting LSCs, instead allowing normal HSCs to reconstitute human hematopoiesis in vivo.

TIM-3 Targets Leukemic Stem Cells

In testing the inhibitory effect of ATIK2a on established human AML in a xenotransplant system, we used the NRG mice to increase engraftment efficiency of human AML cells. Eight
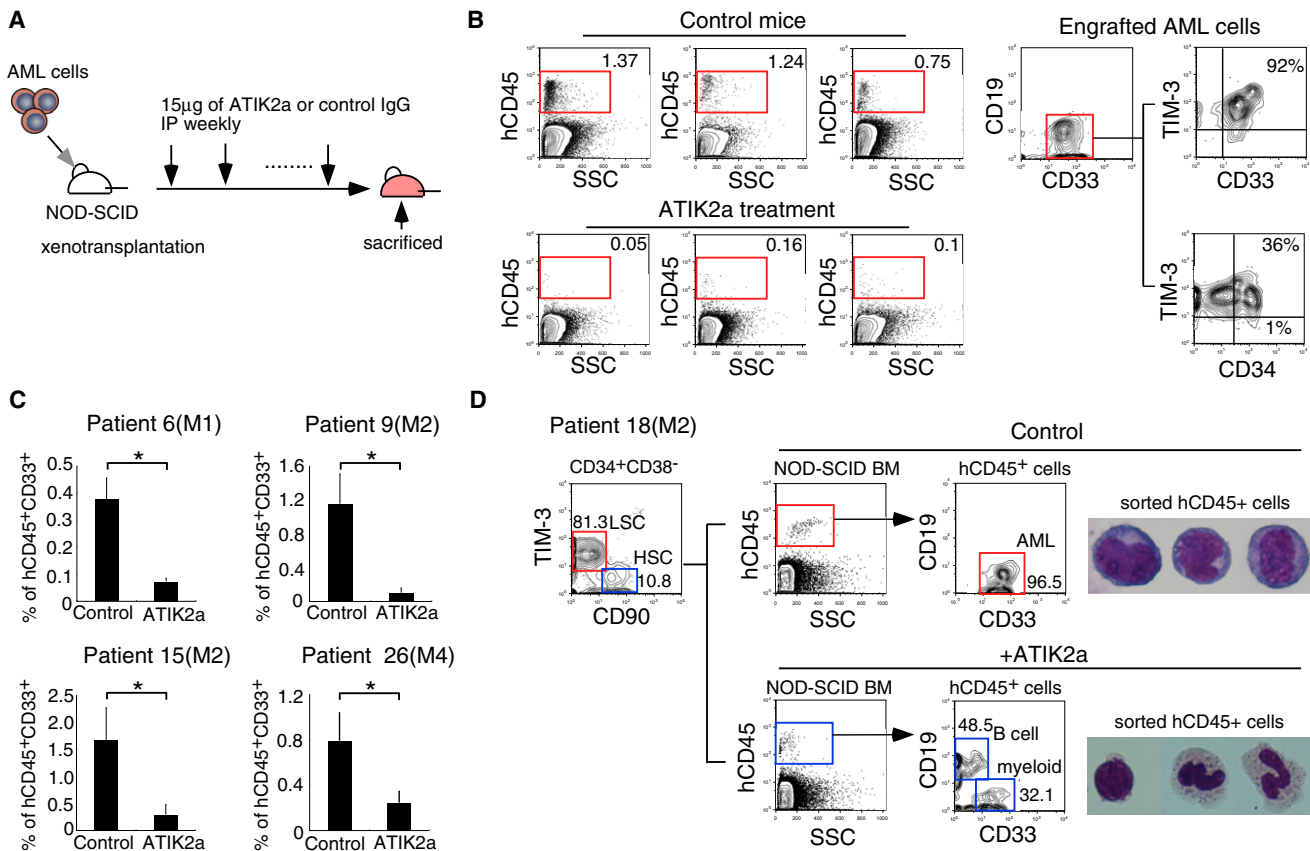
Figure 6. ATIK2a Antibodies Blocked AML Reconstitution in NOD-SCID Mice

(A) Schedule of ATIK2a administration in NOD-SCID experiment. ATIK2a treatment was started 12 hr after the transplantation.

(B) Analysis of mice transplanted with AML bone marrow cells at 16 weeks after transplantation. Three control mice (left top) showed reconstitution of human CD45+ cells, and the majority of these cells were TIM-3+CD33+ AML cells that contained CD34+ leukemic progenitor or stem cell populations (right). In contrast, mice treated with ATIK2a (left bottom) have only a small number of hCD45+ cells. Representative data of patient 9 are shown.

(C) Summary of four independent experiments to test the effect of ATIK2a on reconstitution of AML bone marrow cells from patients 6, 9, 15, and 26. In all experiments, ATIK2a treatment significantly inhibited the AML reconstitution. Three mice per group were analyzed.

(D) Selective inhibition of AML reconstitution by ATIK2a in mice reconstituted with the bone marrow of patient 18, which contained both normal HSCs and AML LSCs (left). Injection of the bone marrow cells resulted in AML development in control mice (right top), whereas mice treated with ATIK2a developed normal hematopoiesis (right bottom).

weeks after injection of 10^6 AML cells, engraftment of human AML cells were confirmed by blood sampling. In NRG mice, ATIK2a cannot fully exert its ADCC effects because of a lack of NK cells. Therefore, we injected a high dose (80 μg) of ATIK2a to maximize its CDC effects on AML cells in vivo. These mice were treated with ATIK2a or control IgG, 3 times a week for 4 weeks (Figure 7A). In all four cases tested (patients 7, 14, 27, and 28), ATIK2a treatment significantly reduced human CD45+ AML burden in vivo: ATIK2a strongly suppressed or eliminated the TIM-3+ AML fraction (Figure 7B, left) that contains all functional LSCs in our hand (Figure 3B), as well as the CD34+CD38− LSC fraction (Figure 7B, right, and Figure 7C), suggesting that reduction of leukemic burden by ATIK2a was achieved at least in part by killing LSCs.

In patients 7 and 27, in order to verify the anti-AML LSC effect of ATIK2a treatment, 10^6 human CD45+ AML cells from the primary NRG recipients were further retransplanted into secondary NRG recipients. In patients 14 and 28, however,

reduction of AML cells by ATIK2a in primary recipients was very severe, and we could not harvest sufficient numbers of AML cells to transplant into secondary recipients. We then evaluated the re-engraftment of AML cells in secondary recipients 8 weeks after transplantation. All seven mice transplanted with bone marrow cells from primary recipients treated with control IgG developed AML, whereas none of 10 mice transplanted with cells from ATIK2a-treated primary recipients developed AML. Representative data in patient 27 are shown in Figure 7C. These data again suggest that functional LSCs were effectively eliminated by ATIK2a treatment in primary recipients.

DISCUSSION

To selectively kill AML LSCs sparing normal HSCs, one of the most practical approaches is to target the AML LSC-specific surface or functionally indispensable molecules. To achieve specificity for LSCs, the target molecule should be expressed
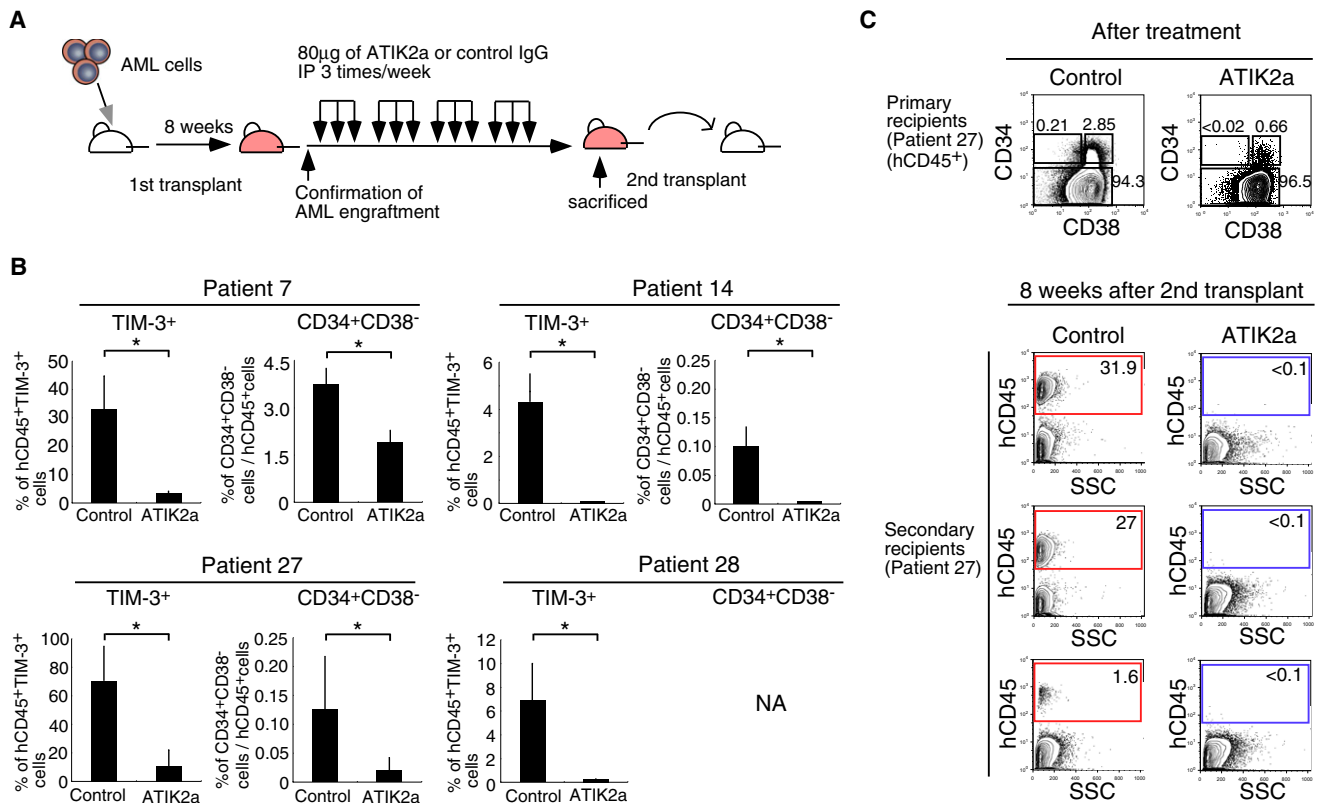
Figure 7. ATIK2a Antibodies Reduced the AML Burden at Least Targeting Functional LSCs

(A) Schedule of ATIK2a administration to test the effect on established human AML in NRG mouse experiments. ATIK2a treatment was started 8 weeks after transplantation.

(B) Summary of four independent experiments to assess the effect of ATIK2a on established human AML cells in vivo (patients 7, 14, 27, and 28). In all experiments, ATIK2a treatment significantly reduced hCD45$^+$ AML burden. Within the hCD45$^+$ population, the TIM-3$^+$ AML fraction that should contain AML LSCs (see Figure 3) was also reduced by this treatment. The percentages of CD34$^+$CD38$^-$ cells, in which LSCs were concentrated, were also reduced. Three to six mice in each group were analyzed.

(C) The phenotype of engrafted hCD45$^+$ cells in primary recipients (top). 10^6 hCD45$^+$ AML cells were then harvested from primary recipients treated with ATIK2a or control IgG, and then retransplanted into the secondary NRG recipients. ATIK2a efficiently blocked reconstitution of AML cells (bottom). Representative results of patient 27 are shown.

on LSCs at a high level but not on normal HSCs. In addition, when the molecule is expressed also in leukemic progenitors or blasts, it will help mass reduction of AML clones. It should not matter whether the molecule is expressed in normal mature blood cells or progenitor cells, because if normal HSCs are spared, they should be able to replenish all mature blood cells after treatment.

TIM-3 is expressed in the CD34$^+$CD38$^-$ AML LSC fraction as well as the majority of their downstream CD38$^+$ leukemic progenitors in most AML types except for M3. TIM-3$^+$ but not TIM-3$^-$ AML population engrafted and reconstituted human AML in NRG mice, suggesting that functional LSCs almost exclusively reside in TIM-3$^+$ cells. In contrast, normal HSCs do not express TIM-3. Thus, TIM-3 should be useful molecules to target AML LSCs without seriously affecting normal hematopoiesis. In steady-state human hematopoiesis, TIM-3 is not expressed in HSCs or myeloerythroid or lymphoid progenitor populations. TIM-3 expression begins at the GMP stage, in parallel with monocyte lineage commitment (Figure 4). Furthermore, in addition to TIM-3, the expression profiling data show that the CD34$^+$

CD38$^-$ LSC fraction expressed many monocyte lineage-related molecules such as CD86 and CSF1R at a high level (Figure 1). In this context, LSCs in most AML types, except for M3 that might be of granulocytic lineage leukemia, may activate some monocyte lineage-related programs.

ATIK2a, a TIM-3 antibody with ADCC and CDC activities, selectively blocked the human AML engraftment and/or development in NOD-SCID mice, whereas it did not disturb normal HSC engraftment. Furthermore, in NRG mice transplanted with human AML cells where percentage of engrafted human cells reached 5%–60% (Figure 7B), ATIK2a treatment reduced or eliminated CD34$^+$CD38$^-$ and TIM-3$^+$ LSC-containing fractions within the bone marrow of primary recipients, resulting in failure of re-engraftment of primary recipients' bone marrow cells into secondary recipients (Figure 7C). Collectively, it is likely that ATIK2a eradicated functional AML LSCs in vivo, sparing normal HSCs.

To use surface markers for targeting AML LSCs, specificity as well as sensitivity should be critical. TIM-3 has an advantage against other candidate markers in several aspects: Detectable levels of TIM-3 protein is not expressed in normal HSCs or other

progenitors except for only a fraction of GMPs. Furthermore, TIM-3 is expressed in LSCs at a high level, and its expression was found in the vast majority of CD34⁺CD38⁻ cells of M0, M1, M2, and M4 AMLs in all cases tested. As shown in Figure 1, the mRNA expression level of CD25, CD32, CD44, and CD47 in LSCs was only 2- to 3-fold higher as compared to normal HSCs, and in some AML cases, LSCs did not express these molecules. CD33 and CD123 proteins were detectable in normal HSCs (Figure 1B) as well as most myeloid progenitors including CMPs and GMPs (Taussig et al., 2005). In fact, prolonged cytopenias have been observed in AML patients treated with gemtuzumab, a recombinant humanized CD33 monoclonal antibody conjugated with the cytotoxic antibiotic calicheamicin, and this side effect could be due to CD33 expression in normal HSCs (Taussig et al., 2005). CLL-1, CSF1R, TIM-3, and CD96 are the group of molecules that are specifically expressed in LSCs. Among all, the sensitivity of TIM-3 is likely to be the highest at least for AML M0, M1, M2, and M4 (Figures 1B and 2). Thus, TIM-3 might be one of the most useful therapeutic targets at least for these AML types.

It may also be important to understand function of these molecules in maintenance or reconstitution capability of LSCs. For example, it was shown that CD44 monoclonal antibodies reduced the leukemic burden and blocked secondary engraftment in a NOD-SCID model (Jin et al., 2006). This effect on LSCs was mediated in part by the disruption of LSC-niche interactions (Jin et al., 2006). CD47 antibodies can block LSC reconstitution and inhibited the growth of engrafted human AML in a NOD-SCID model (Majeti et al., 2009). However, the interpretation of this result is difficult because the anti-LSC effect of CD47 antibody treatment in this xenograft model could be due to induction of xenogeneic rejection by blocking the ligation of human CD47 expressed on LSCs with mouse SIRPA: NOD-type SIRPA expressed on host macrophage is agonistic for human CD47 to block phagocytotic signals, resulting in the induction of tolerance for human cells in this model (Takenaka et al., 2007). The effect of TIM-3 antibodies in our study might be due to killing activity for their target cells that should include LSCs. It is, however, still important to understand the role of TIM-3 signaling in LSC functions by, for example, testing the effect of activation or suppression of TIM-3 signaling on LSC fate decision.

In summary, TIM-3 is a promising surface molecule to target AML LSCs of most FAB types. Our in vivo experiments strongly suggest that targeting this molecule by monoclonal antibody treatment is a practical approach to eradicate human AML.

EXPERIMENTAL PROCEDURES

Clinical Samples
The bone marrow samples of 34 adult AML cases diagnosed according to French-American-British (FAB) and WHO criteria were enrolled. Human adult bone marrow and peripheral blood cells were obtained from healthy donors. Cord blood cells were obtained from full-term deliveries. Informed consent was obtained from all patients and controls in accordance with the Helsinki Declaration of 1975 that was revised in 1983. The Institutional Review Board of Kyushu University Hospital approved all research on human subjects.

Antibodies, Cell Staining, and Sorting
For the analyses and sorting of human HSCs and progenitors, cells were stained and sorted by FACS Aria (BD Biosciences) as we have previously reported (Kikushige et al., 2008; Yoshimoto et al., 2009). In brief, for the analyses

and sorting of HSCs and myeloid progenitors, cells were stained with a Cy5-PE- or PC5-conjugated lineage cocktail, including anti-CD3 (HIT3a), CD4 (RPA-T4), CD8 (RPA-T8), CD10 (HI10a), CD19 (HIB19), CD20 (2H7), CD11b (ICFR44), CD14 (RMO52), CD56 (NKH-1), and GPA (GA-R2); FITC-conjugated anti-CD34 (8G12), anti-CD90 (5E10), or anti-CD45RA (HI100); PE-conjugated anti-TIM-3 (344823), CD33 (HIM3-4), CD96 (NK92.39), or anti-CD123 (6H6); APC-conjugated anti-CD34 (8G12) or anti-CD38 (HIT2); and Pacific Blue conjugated anti-CD45RA (HI100), and biotinylated anti-CD38 (HIT2), or anti-CD123 (9F5). For analysis and sorting of human cells in the immunodeficient mice, FITC-conjugated anti-CD33 (HIM3-4), PE-conjugated anti-CD19 (HIB19), PE-Cy7-conjugated anti-CD38 (HIT2), and APC-conjugated anti-CD45 (J.33) monoclonal antibodies were used in addition to the antibodies described above. Streptavidin-conjugated APC-Cy7 or PE-Cy7 was used for visualization of the biotinylated antibodies (BD PharMingen, San Jose, CA). Nonviable cells were excluded by propidium iodide (PI) staining. Appropriate isotype-matched, irrelevant control monoclonal antibodies were used to determine the level of background staining. The cells were sorted and analyzed by FACS Aria (BD Biosciences, San Jose, CA). The sorted cells were subjected to an additional round of sorting withthe same gate to eliminate contaminating cells and doublets. For single-cell assays, an automatic cell-deposition unit system (BD Biosciences, San Jose, CA) was used.

In Vitro Assays to Determine the Differentiation Potential of Myeloid Progenitors
Clonogenic colony-forming unit (CFU) assays were performed with a methyl-cellulose culture system that was set up to detect all possible outcomes of myeloid differentiation as reported previously (Kikushige et al., 2008; Manz et al., 2002). Colony numbers were enumerated on day 14 of culture. All of the cultures were incubated at 37°C in a humidified chamber under 5% CO₂.

Microarray Analysis
Twelve AML samples and five normal adult HSCs samples were investigated with Sentrix Bead Chip Assay For Gene Expression, Human-6 V2 (Illumina). In brief, total RNA was extracted with TRIzol (Invitrogen) from FACS-sorted AML CD34⁺CD38⁻ cells and normal CD34⁺CD38⁻Lin⁻ HSCs, and biotinylated complementary RNA was synthesized with two round amplification steps via MessageAmpII aRNA Amplification Kit and Illumina TotalPrep RNA Amplification Kit (Applied Biosystems). 1.5 μg of cRNA from each sample was hybridized to the Bead Chip. After staining and washing, Bead Chip was scanned with an Illumina Bead Array reader. Microarray data were analyzed with Gene Spring GX11.01 software (Agilent Technologies). According to the guided workflow for Illumina single color experiment, normalization algorithm of 75-percentile shift was used, and the preprocessing baseline was adjusted to median of all samples.

Production of Recombinant Anti-Human TIM-3 Mouse Monoclonal Antibody
Human TIM-3 cDNA were cloned from normal pancreas cDNA (Clontech). Female Balb/C mouse (7-week-old, Purchased from Charles River) was immunized with L929 cells stably expressing TIM-3 four times and soluble human TIM-3 protein once. Four days after the final injection, spleen cells were fused with SP2/O cells by the PEG method and selected in the HAT-medium. Hybridomas were screened by FACS and clone-sorted. cDNAs encoding the variable regions amplified by SMART RACE cDNA Kit (Clontech) and specific primers (Doenecke et al., 1997) were ligated to mouse IgG2a or Igκ constant region.

Evaluation of ADCC and CDC Activities of ATIK2a Antibodies
ADCC and CDC were determined as previously described with slight modification (Shields et al., 2001; Tawara et al., 2008). For ADCC, target cells and IL-2-cultured peripheral blood mononuclear cells prepared from healthy volunteers were incubated with antibodies (1 μg/mL, Effecter/Target ratio = 25). Cytotoxicity was analyzed by CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) as follows: specific lysis [%] = $(A_E - A_{Allo})/(A_{Max} - A_{TS}) \times 100$, where A_E is absorbance of experiment, A_{Allo} is allogenic reaction (no antibody control), A_{Max} is maximum, A_{TS} is target spontaneous release. For CDC, viability of target cells incubated with rabbit sera was assayed by CellTiterGlo (Promega, no antibody control = 100%). UPC 10 (Sigma) replaced in PBS was used as an isotype control.

Transplantation of AML Cells into Immunodeficient Mice

NOD-SCID and NRG mice (stock#7799) were purchased from The Jackson Laboratory. The mice were housed in a specific-pathogen-free facility in micro-isolator cages at the Kyushu University. Animal experiments were performed in accordance with institutional guidelines approved by the Kyushu University animal care committee. NOD-SCID and NRG mice were irradiated at a sublethal dose (2.4 Gy and 4.8 Gy, respectively). In transplantation of AML cells, NOD-SCID mice additionally received a single intraperitoneal injection of 200 μg purified CD122 antibodies that were generated from TM-β1 hybridoma (Tanaka et al., 1993) before transplantation, based on the expectation that it induces transient reduction of NK cells and helps human cell engraftment. We did not inject CD122 antibodies in transplantion of normal bone marrow or cord blood cells. AML cells or CD34$^+$ cells from adult bone marrow and cord blood cells were transplanted via a tail vein.

Statistical Analysis

Data were presented as the mean ± standard deviation. The significance of the differences between groups was determined via Student's t test.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number 24395.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at doi:10.1016/j.stem.2010.11.014.

REFERENCES

Aikawa, Y., Katsumoto, T., Zhang, P., Shima, H., Shino, M., Terui, K., Ito, E., Ohno, H., Stanley, E.R., Singh, H., et al. (2010). PU.1-mediated upregulation of CSF1R is crucial for leukemia stem cell potential induced by MOZ-TIF2. Nat. Med. 16, 580–585, 1p, 585.

Anderson, A.C., Anderson, D.E., Bregoli, L., Hastings, W.D., Kassam, N., Lei, C., Chandwaskar, R., Karman, J., Su, E.W., Hirashima, M., et al. (2007). Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. Science 318, 1141–1143.

Bhatia, M., Wang, J.C., Kapp, U., Bonnet, D., and Dick, J.E. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc. Natl. Acad. Sci. USA 94, 5320–5325.

Bonnet, D., and Dick, J.E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat. Med. 3, 730–737.

Doenecke, A., Winnacker, E.L., and Hallek, M. (1997). Rapid amplification of cDNA ends (RACE) improves the PCR-based isolation of immunoglobulin variable region genes from murine and human lymphoma cells and cell lines. Leukemia 11, 1787–1792.

Florian, S., Sonneck, K., Hauswirth, A.W., Krauth, M.T., Schernthaner, G.H., Sperr, W.R., and Valent, P. (2006). Detection of molecular targets on the surface of CD34+/CD38-/- stem cells in various myeloid malignancies. Leuk. Lymphoma 47, 207–222.

Hauswirth, A.W., Florian, S., Printz, D., Sotlar, K., Krauth, M.T., Fritsch, G., Schernthaner, G.H., Wacheck, V., Selzer, E., Sperr, W.R., and Valent, P. (2007). Expression of the target receptor CD33 in CD34+/CD38-/CD123+ AML stem cells. Eur. J. Clin. Invest. 37, 73–82.

Hope, K.J., Jin, L., and Dick, J.E. (2004). Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. Nat. Immunol. 5, 738–743.

Hosen, N., Park, C.Y., Tatsumi, N., Oji, Y., Sugiyama, H., Gramatzki, M., Krensky, A.M., and Weissman, I.L. (2007). CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. Proc. Natl. Acad. Sci. USA 104, 11008–11013.

Ishikawa, F., Yasukawa, M., Lyons, B., Yoshida, S., Miyamoto, T., Yoshimoto, G., Watanabe, T., Akashi, K., Shultz, L.D., and Harada, M. (2005). Development of functional human blood and immune systems in NOD/SCID/IL2 receptor gamma chain(null) mice. Blood 106, 1565–1573.

Ishikawa, F., Yoshida, S., Saito, Y., Hijikata, A., Kitamura, H., Tanaka, S., Nakamura, R., Tanaka, T., Tomiyama, H., Saito, N., et al. (2007). Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. Nat. Biotechnol. 25, 1315–1321.

Jaiswal, S., Jamieson, C.H., Pang, W.W., Park, C.Y., Chao, M.P., Majeti, R., Traver, D., van Rooijen, N., and Weissman, I.L. (2009). CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell 138, 271–285.

Jin, L., Hope, K.J., Zhai, Q., Smadja-Joffe, F., and Dick, J.E. (2006). Targeting of CD44 eradicates human acute myeloid leukemic stem cells. Nat. Med. 12, 1167–1174.

Jin, L., Lee, E.M., Ramshaw, H.S., Busfield, S.J., Peoppl, A.G., Wilkinson, L., Guthridge, M.A., Thomas, D., Barry, E.F., Boyd, A., et al. (2009). Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. Cell Stem Cell 5, 31–42.

Kikushige, Y., Yoshimoto, G., Miyamoto, T., Iino, T., Mori, Y., Iwasaki, H., Niiro, H., Takenaka, K., Nagafuji, K., Harada, M., et al. (2008). Human Flt3 is expressed at the hematopoietic stem cell and the granulocyte/macrophage progenitor stages to maintain cell survival. J. Immunol. 180, 7358–7367.

Krause, D.S., and Van Etten, R.A. (2007). Right on target: Eradicating leukemic stem cells. Trends Mol. Med. 13, 470–481.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 367, 645–648.

Majeti, R., Chao, M.P., Alizadeh, A.A., Pang, W.W., Jaiswal, S., Gibbs, K.D., Jr., van Rooijen, N., and Weissman, I.L. (2009). CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell 138, 286–299.

Manz, M.G., Miyamoto, T., Akashi, K., and Weissman, I.L. (2002). Prospective isolation of human clonogenic common myeloid progenitors. Proc. Natl. Acad. Sci. USA 99, 11872–11877.

Martelli, M.P., Pettirossi, V., Thiede, C., Bonifacio, E., Mezzasoma, F., Cecchini, D., Pacini, R., Tabarrini, A., Ciurnelli, R., Gionfriddo, I., et al. (2010). CD34+ cells from AML with mutated NPM1 harbor cytoplasmic mutated nucleophosmin and generate leukemia in immunocompromised mice. Blood Press. Published online July 15, 2010. 10.1182/blood-2009-08-238899.

Monney, L., Sabatos, C.A., Gaglia, J.L., Ryu, A., Waldner, H., Chernova, T., Manning, S., Greenfield, E.A., Coyle, A.J., Sobel, R.A., et al. (2002). Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature 415, 536–541.

Nakae, S., Iikura, M., Suto, H., Akiba, H., Umetsu, D.T., Dekruyff, R.H., Saito, H., and Galli, S.J. (2007). TIM-1 and TIM-3 enhancement of Th2 cytokine production by mast cells. Blood 110, 2565–2568.

Nakayama, M., Akiba, H., Takeda, K., Kojima, Y., Hashiguchi, M., Azuma, M., Yagita, H., and Okumura, K. (2009). Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. Blood 113, 3821–3830.

Nimmerjahn, F., and Ravetch, J.V. (2005). Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science *310*, 1510–1512.

Nimmerjahn, F., and Ravetch, J.V. (2007). Antibodies, Fc receptors and cancer. Curr. Opin. Immunol. *19*, 239–245.

Pearson, T., Shultz, L.D., Miller, D., King, M., Laning, J., Fodor, W., Cuthbert, A., Burzenski, L., Gott, B., Lyons, B., et al. (2008). Non-obese diabetic-recombination activating gene-1 (NOD-Rag1 null) interleukin (IL)-2 receptor common gamma chain (IL2r gamma null) null mice: A radioresistant model for human lymphohaematopoietic engraftment. Clin. Exp. Immunol. *154*, 270–284.

Sabatos, C.A., Chakravarti, S., Cha, E., Schubart, A., Sánchez-Fueyo, A., Zheng, X.X., Coyle, A.J., Strom, T.B., Freeman, G.J., and Kuchroo, V.K. (2003). Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nat. Immunol. *4*, 1102–1110.

Saito, Y., Kitamura, H., Hijikata, A., Tomizawa-Murasawa, M., Tanaka, S., Takagi, S., Uchida, N., Suzuki, N., Sone, A., Najima, Y., et al. (2010). Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. Sci. Transl. Med. *2*, ra9.

Sánchez-Fueyo, A., Tian, J., Picarella, D., Domenig, C., Zheng, X.X., Sabatos, C.A., Manlongat, N., Bender, O., Kamradt, T., Kuchroo, V.K., et al. (2003). Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. Nat. Immunol. *4*, 1093–1101.

Shields, R.L., Namenuk, A.K., Hong, K., Meng, Y.G., Rae, J., Briggs, J., Xie, D., Lai, J., Stadlen, A., Li, B., et al. (2001). High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. J. Biol. Chem. *276*, 6591–6604.

Takenaka, K., Prasolava, T.K., Wang, J.C., Mortin-Toth, S.M., Khalouei, S., Gan, O.I., Dick, J.E., and Danska, J.S. (2007). Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells. Nat. Immunol. *8*, 1313–1323.

Tanaka, T., Kitamura, F., Nagasaka, Y., Kuida, K., Suwa, H., and Miyasaka, M. (1993). Selective long-term elimination of natural killer cells in vivo by an anti-interleukin 2 receptor beta chain monoclonal antibody in mice. J. Exp. Med. *178*, 1103–1107.

Taussig, D.C., Pearce, D.J., Simpson, C., Rohatiner, A.Z., Lister, T.A., Kelly, G., Luongo, J.L., Danet-Desnoyers, G.A., and Bonnet, D. (2005). Hematopoietic stem cells express multiple myeloid markers: Implications for the origin and targeted therapy of acute myeloid leukemia. Blood *106*, 4086–4092.

Taussig, D.C., Miraki-Moud, F., Anjos-Afonso, F., Pearce, D.J., Allen, K., Ridler, C., Lillington, D., Oakervee, H., Cavenagh, J., Agrawal, S.G., et al. (2008). Anti-CD38 antibody-mediated clearance of human repopulating cells masks the heterogeneity of leukemia-initiating cells. Blood *112*, 568–575.

Taussig, D.C., Vargaftig, J., Miraki-Moud, F., Griessinger, E., Sharrock, K., Luke, T., Lillington, D., Oakervee, H., Cavenagh, J., Agrawal, S.G., et al. (2010). Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. Blood *115*, 1976–1984.

Tawara, T., Hasegawa, K., Sugiura, Y., Harada, K., Miura, T., Hayashi, S., Tahara, T., Ishikawa, M., Yoshida, H., Kubo, K., et al. (2008). Complement activation plays a key role in antibody-induced infusion toxicity in monkeys and rats. J. Immunol. *180*, 2294–2298.

Uchida, J., Hamaguchi, Y., Oliver, J.A., Ravetch, J.V., Poe, J.C., Haas, K.M., and Tedder, T.F. (2004). The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. J. Exp. Med. *199*, 1659–1669.

van Rhenen, A., van Dongen, G.A., Kelder, A., Rombouts, E.J., Feller, N., Moshaver, B., Stigter-van Walsum, M., Zweegman, S., Ossenkoppele, G.J., and Jan Schuurhuis, G. (2007). The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. Blood *110*, 2659–2666.

Yalcintepe, L., Frankel, A.E., and Hogge, D.E. (2006). Expression of interleukin-3 receptor subunits on defined subpopulations of acute myeloid leukemia blasts predicts the cytotoxicity of diphtheria toxin interleukin-3 fusion protein against malignant progenitors that engraft in immunodeficient mice. Blood *108*, 3530–3537.

Yoshimoto, G., Miyamoto, T., Jabbarzadeh-Tabrizi, S., Iino, T., Rocnik, J.L., Kikushige, Y., Mori, Y., Shima, T., Iwasaki, H., Takenaka, K., et al. (2009). FLT3-ITD up-regulates MCL-1 to promote survival of stem cells in acute myeloid leukemia via FLT3-ITD-specific STAT5 activation. Blood *114*, 5034–5043.