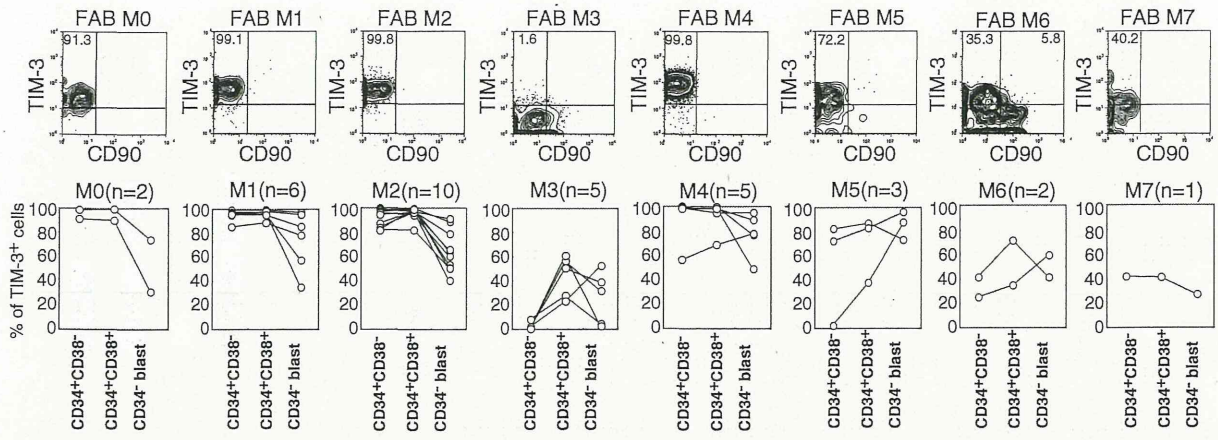

Figure 2. The Expression of TIM-3 in Stem and Progenitor Populations of AML of Each FAB Type
Expression of TIM-3 in each FAB type of AML. The representative expression pattern of TIM-3 in the $CD34^+CD38^-$ LSC fraction (top) and distribution of TIM-3 in $CD34^+CD38^-$ LSCs, $CD34^+CD38^+$ leukemic progenitors, 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

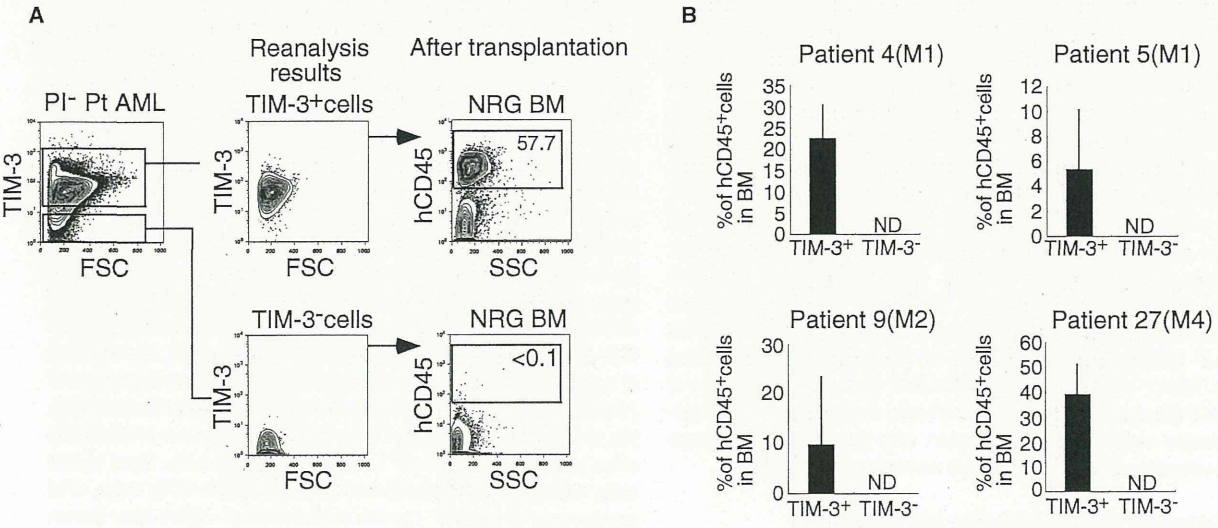

Figure 3. The TIM-3^+ AML Population Contains the Vast Majority of Functional LSC Activity
(A) A representative analysis of xenotransplantation of purified TIM-3^+ or TIM-3^- AML cells from patient 27 into NRG mice. Only TIM-3^+ cells reconstitute $hCD45^+$ AML cells after transplantation.
(B) Summarized 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.

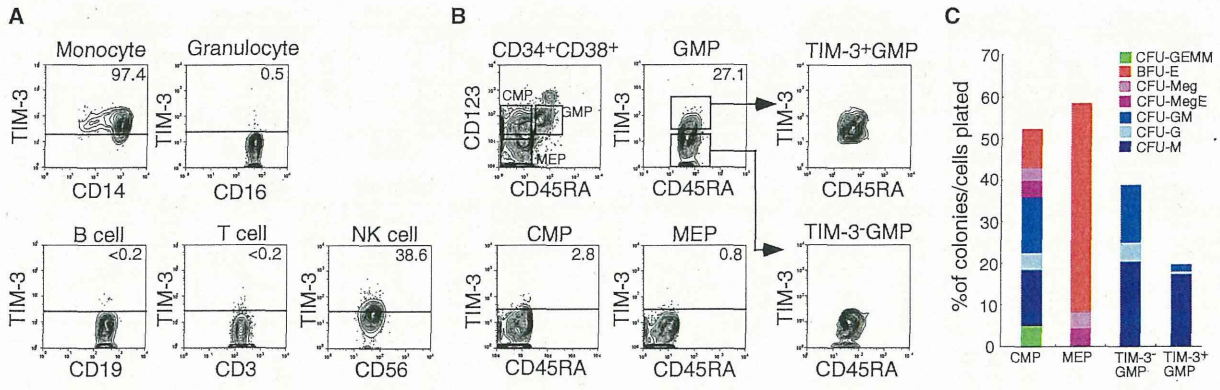

Figure 4. TIM-3 Is Expressed in Monocytes and Their Progenitors in Normal Hematopoiesis

(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 upregulation 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

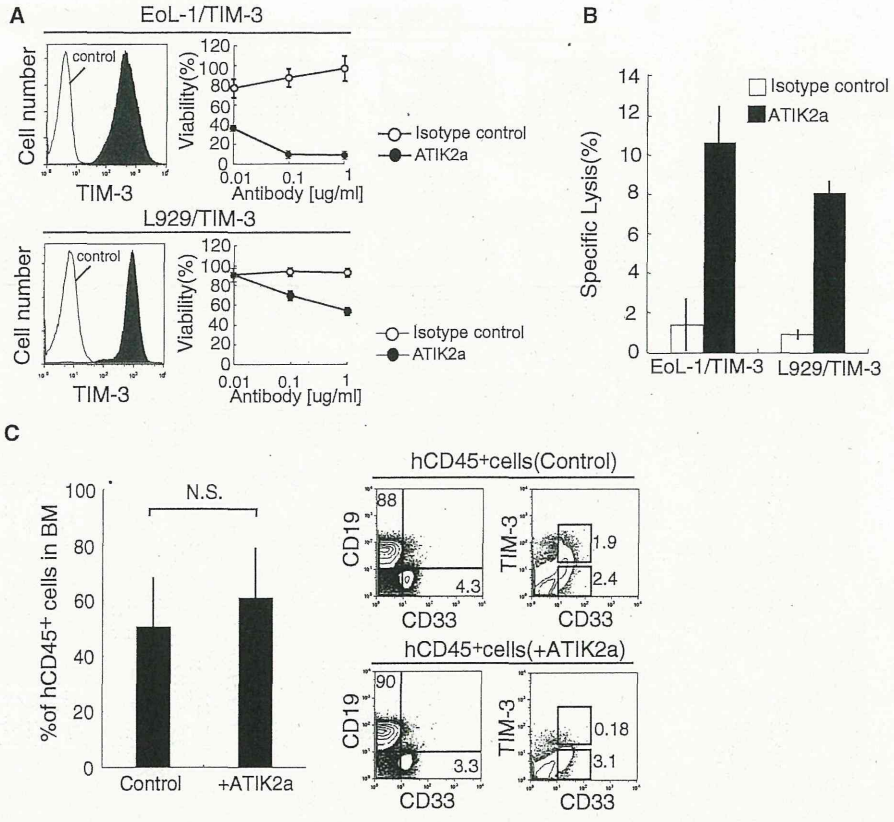

Figure 5. ATIK2a, a New Monoclonal Antibody against TIM-3, Has CDC and ADCC Activities and Does Not Harm Normal Hematopoietic Reconstitution

(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 hematopoiesis 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 $CD34^+TIM\text{-}3^+$ 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 $CD34^+CD38^-CD90^+TIM\text{-}3^-$ cells that was phenotypically normal HSCs, in addition to the major fraction of $CD34^+CD38^-CD90^-TIM\text{-}3^+$ 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

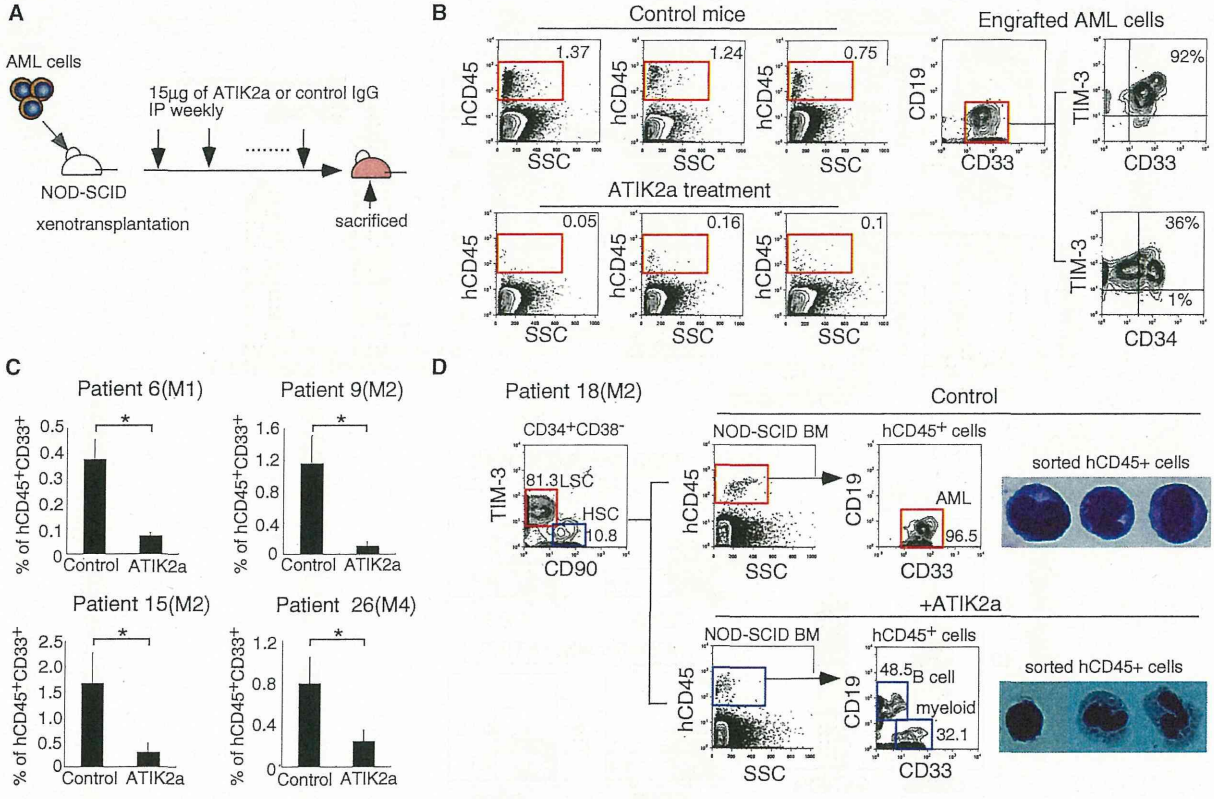

Figure 6. ATIK2a Antibodies Blocked AML Reconstitution in NOD-SCID Mice

(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 $TIM\text{-}3^+CD33^+$ 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 $TIM\text{-}3^+$ 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

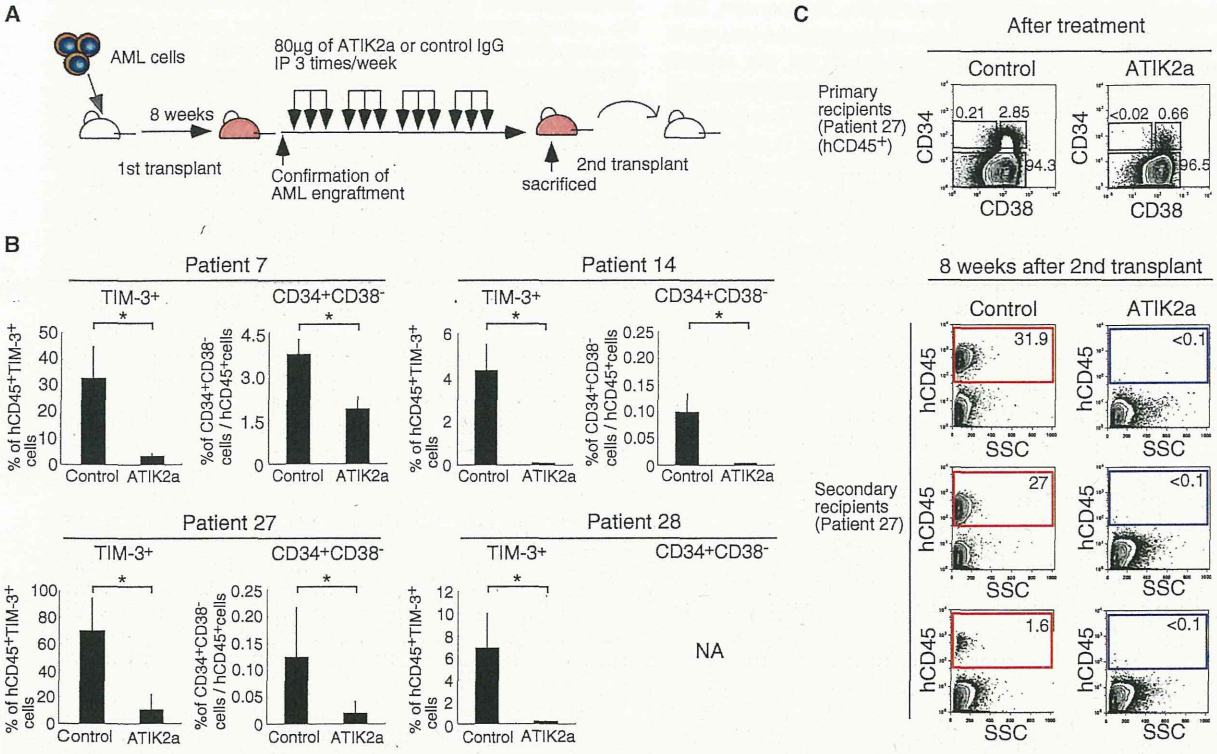

Figure 7. ATIK2a Antibodies Reduced the AML Burden at Least Targeting Functional LSCs

(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 methylcellulose 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% CO_2.

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.

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Self-Renewing Hematopoietic Stem Cell Is the Primary Target in Pathogenesis of Human Chronic Lymphocytic Leukemia

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SUMMARY

We report here that in chronic lymphocytic leukemia (CLL), the propensity to generate clonal B cells has been acquired already at the hematopoietic stem cell (HSC) stage. HSCs purified from patients with CLL displayed lymphoid-lineage gene priming and produced a high number of polyclonal B cell progenitors. Strikingly, their maturation into B cells was restricted always to mono- or oligo-clones with CLL-like phenotype in xenogeneic recipients. These B cell clones were independent of the original CLL clones because they had their own immunoglobulin VDJ genes. Furthermore, they used preferentially VH genes frequently used in human CLL, presumably reflecting the role of B cell receptor signaling in clonal selection. These data suggest that HSCs can be involved in leukemogenesis even in mature lymphoid tumors.

INTRODUCTION

Malignant transformation can occur through a multistep acquisition of critical somatic mutations. Therefore, the precursor of malignant stem cells should have a long life span to accumulate such mutations. In human hematopoiesis, genetic abnormalities for transformation should be accumulated in self-renewing hematopoietic stem cells (HSCs). HSCs can continuously produce a number of progenitors with the same genetic alteration, which are also potential targets for additional mutations (Rossi et al., 2008). Such HSCs or downstream progenitors finally become leukemia stem cells that possess self-renewal but lack normal differentiation activity (Huntly et al., 2004; So et al., 2003). This notion of leukemia development has been well accepted to explain acute myeloid leukemia (AML) development, and AML-initiating cells capable of reconstituting human leukemias in xenogeneic hosts have been purified (Bonnet and Dick, 1997) as a potential therapeutic target (Jin et al., 2006, 2009; Kikushige et al., 2010; Majeti et al., 2009; Saito et al., 2010). However, in lymphoid malignancies, leukemia or lymphoma cells usually have monoclonal immunoglobulin or T cell receptor gene rearrangements, suggesting that lymphoid malignant stem cells originate after cells have committed to the lymphoid lineage. Recent studies have shown that acute lymphoid leukemia (ALL)-initiating cells upon xenogeneic transplantation are composed of multiple genetically distinct subclones (Anderson et al., 2011; Notta et al., 2011). These data clearly show that lymphoid cells can easily accumulate genetic abnormalities, presumably because they can persist longer than myeloid cells, and are capable of clonal expansion simulating self-renewal (Luckey et al., 2006). Because of such property of lymphoid cells, the involvement of HSCs in lymphoid leukemogenesis has never been underscored.

Significance

HSCs capable of self-renewal should be the main target for accumulating mutational events to develop hematological malignancies. This paper shows that HSCs play such a role also in mature lymphoid malignancies. Most human CLL cases have a precursor phase, called monoclonal B lymphocytosis (MBL), that is asymptomatic monoclonal or oligoclonal proliferation of B cells. HSCs from patients with CLL but not normal HSCs developed monoclonal or oligoclonal B cells simulating MBL after xenogeneic transplantation. Acquisition of chromosomal abnormalities appeared to be secondary events to transform MBL into clinical CLL. Thus, even in CLL, accumulation of oncogenic events starts at the HSC stage. Our xenograft model might be very useful to understand the pathogenesis of human CLL.

Chronic lymphocytic leukemia (CLL), the most common leukemia in adults in western countries, is a mature B cell malignancy (Chiorazzi et al., 2005). It is characterized by accumulation of clonal B cells in the blood, the bone marrow, and the lymphoid tissues. The consistent clonal expansion of mature B cells frequently expressing CD5 is the major phenotype of patients with CLL. Unfortunately, the development of its xenograft models by transplanting primary CLL cells into immunodeficient hosts has failed because the engraftment was extremely inefficient (Dürig et al., 2007; Hummel et al., 1996). Thus, the search for CLL-initiating cells has never been successful.

Human CLL cells have functional B cell receptors (BCRs) on their surface as a result of productive rearrangement of immunoglobulin genes (Caligaris-Cappio and Ghia, 2008; Chiorazzi et al., 2005; Stevenson and Caligaris-Cappio, 2004). CLL has been divided into two subgroups based on the presence of somatic hypermutations within the variable regions of immunoglobulin heavy-chain (IGHV) genes, which normally occurs in the germinal center during naive to memory B cell transition. The group of CLLs with mutated BCRs has a more favorable prognosis than those with unmutated BCRs (Hamblin et al., 1999). However, recent studies suggest that both types of CLLs originate from self-reactive B cell precursors and that the status of somatic hypermutations does not indicate their origin (Hervé et al., 2005; Klein et al., 2001; Rosenwald et al., 2001). Interestingly, CLL cells preferentially use the IGHV genes, such as VH1, VH3, and VH4 regions (Chiorazzi and Ferrarini, 2003; Fais et al., 1998), and express a restricted BCR repertoire including antibodies with quasi-identical complementarity-determining region 3 (CDR3) (Ghiotto et al., 2004; Messmer et al., 2004; Tobin et al., 2003, 2004; Widhopf et al., 2004), suggesting specific antigen recognition by CLL cells (Chiorazzi and Ferrarini, 2003; Stevenson and Caligaris-Cappio, 2004).

To trace the origin of genetic aberration in human CLL, it is important to note the fact that CLL cells are not always monoclonal, but more than one CLL clone is found in up to ~14% of patients with CLL (Sanchez et al., 2003). Furthermore, a recent cohort study has shown that 44 out of 45 patients with CLL have a precursor state such as monoclonal B lymphocytosis (MBL) for 6 months to 7 years (Landgren et al., 2009). MBL represents asymptomatic proliferation of clonal B cells whose numbers in circulation are below 5000/μl (Marti et al., 2005). Of note, human MBL is frequently (20%–70% of total cases) composed of more than one B cell clone (Dagklis et al., 2009; Lanasa et al., 2010; Nieto et al., 2009). More than a half of such MBL clones express CD5 (Scarfò et al., 2010), and patients with these CLL-like MBL clones frequently develop into clinical CLL (Rawstron et al., 2008). Furthermore, like CLL cells, $CD5^+$ MBL clones use a biased set of VH genes, including VH1, 3, and 4 (Rawstron et al., 2008). The usage of such biased BCR types found in CLL and its precedent MBL clones strongly suggests that the antigenic drive contributes to clonal expansion and/or cell survival also during the transition from MBL to clinical CLL (Pleyer et al., 2009).

The question is: If progression from MBL to CLL reflects stepwise leukemogenesis, at what stage does the first oncogenic event occur. The existence of oligoclonal B cell clones in patients with CLL and with those MBL strongly suggests that the first oncogenic event could at least be traced up to the progenitor or HSCs that have not rearranged IGH genes. These data led us to search for CLL-initiating cells within the early hematopoietic stages utilizing an efficient xenotransplantation system.

RESULTS

Clonal Selection of CLL B Cells Occurs at the Mature B Cell Stage in Human CLL

To search for the cell population with CLL-initiating activity in human CLL, we first tried to locate the developmental stage at which CLL B cell clones appear. Patients' characteristics are shown in Table S1 available online.

Figure 1A shows the FACS analysis of the bone marrow of a patient with CLL. The bone marrow contained $CD34^+CD38^-$ HSCs (Bhatia et al., 1997), and the $CD34^+CD38^+$ progenitor fraction that contains myeloid and lymphoid progenitors (Manz et al., 2002). Interestingly, percentages of $CD10^+CD19^+$ proB cells in the bone marrow of patients with CLL were high in most patients: in 12 out of 13 patients with CLL, proB cell frequency was higher than the average of 7 normal controls, and the average proB cell frequency in patients with CLL was higher than that in normal controls by ~5-fold (Figure 1B). In contrast, frequencies of the $CD34^+CD38^-$ HSC population were equal (Figure 1B). Recent reports have shown that the $CD34^+CD38^-$ HSC population can further be divided into subpopulations including $CD90^+CD45RA^-$, $CD90^-CD45RA^-$, and $CD90^-CD45RA^+$ that mainly contain long-term HSCs (LT-HSCs), multipotent progenitors (Majeti et al., 2007), and early lymphoid/myeloid progenitors (Doulatov et al., 2010; Goardon et al., 2011), respectively. We performed the HSC subpopulation analysis in six CLL cases, and found that the distribution of these HSC subpopulations did not differ in normal and CLL bone marrow, and the majority (~60%) of $CD34^+CD38^-$ cells were the most primitive $CD90^+CD45RA^-$ population (Figure 1B). Thus, we tested whether the expansion at the proB stage reflects clonal proliferation of CLL precursors by analyzing the rearrangement status of the IGH gene.

As shown in Figure 1C, the purified $CD34^+CD38^-$ HSC population in patients with CLL (CLL-HSCs) presented the germline configuration, and $CD34^-CD19^+$ CLL cells had a clonal IGH rearrangement. Of note, proB cells in CLL bone marrow exhibited polyclonal rearrangement of IGH genes, suggesting that CLL clones are selected in vivo among such expanded polyclonal B cells. These data clearly show that $CD34^+CD38^-$ CLL-HSC populations do not rearrange the IGH gene, and therefore, are not contaminated with detectable CLL clones. However, CLL-HSCs are able to develop a higher number of polyclonal B cells as compared to normal HSCs, suggesting that developmental potential of CLL-HSCs is skewed toward B cell lineage probably reflecting their cell-intrinsic abnormality.

Purified HSCs from Patients with CLL Are Able to Generate Clonal B Cells with CLL-like Phenotype after Xenogeneic Transplantation

We then tried to identify the CLL-initiating cell population by transplanting subpopulations of CLL cells into immunodeficient mice. In these experiments, NOD/SCID/$IL2rg^{null}$ (NSG) (Ishikawa et al., 2005) newborns or NOD/RAG-$1^{-/-}$IL2rg^{null}