rate (CR + PR + MR) was 23%. A univariate analysis to identify possible relationships between clinical parameters and overall response failed to show any statistically significant factors. The conditioning regimen did not significantly affect the response rate, although the statistical power was not enough due to the small number of patients in each group. Response was observed in two of the seven patients who received the most intensive regimen including fludarabine, BU and gemcitabine, while it was seen in one of the six patients who received the least intensive regimen with fludarabine and low-dose TBI. None of the patients with mixed chimerism showed a response, but this difference was not statistically significant. DLI (donor lymphocyte infusion) was performed in four patients who had progressive disease after RICT, and the number of infused CD3-positive cells was between 2.7×10^7 and 1.8×10^8 cells/kg. One patient showed tumor shrinkage after DLI, but the response was transient.

Figure 1a shows overall survival after RICT. Median survival was only 139 days and the major cause of death was tumor progression. Other causes of death included infection in one and chronic GVHD in two. In a univariate analysis, ECOG-PS below 2 and infused CD34-positive cell dose greater than 4.0×10^6 cells/kg were associated with significantly longer survival after RICT (Table 2; Figures 1b and c). A multivariate analysis revealed that these two factors were almost independently significant (Table 2). With regard to post transplantation factors, while the development of grade II–IV acute GVHD did not significantly affect survival ($P = 0.76$), the eight patients who developed chronic GVHD tended to survive longer than those who survived longer than 100 days after RICT but did not develop chronic GVHD ($P = 0.092$; Figure 2). This analysis was unlikely to be biased by the fact that patients who survived longer had more chance to develop chronic GVHD, as most of the patients developed chronic GVHD as a progressive type from acute GVHD.

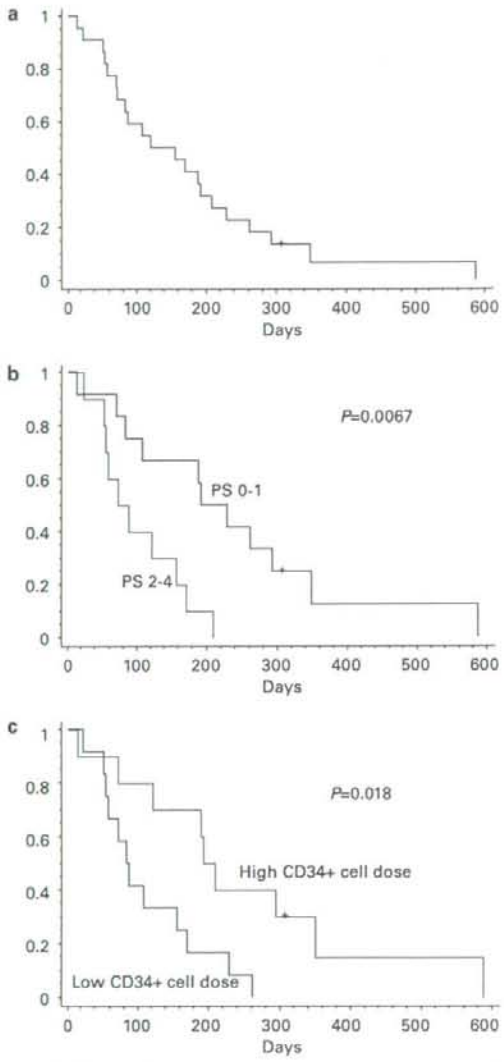

Figure 1 Patient survival, overall (**a**) and grouped according to risk factors (**b** and **c**).

Discussion

To summarize these findings, 23% of the 22 patients in this series showed a response to RICT. However, the duration of the response was generally short and most of the patients eventually died with progressive disease. The median survival after RICT was only 139 days and only one survived longer than 1 year after transplantation. Good ECOG-PS and higher number of CD34-positive cells in the graft were independently associated with longer survival.

The relationship between the number of infused CD34-positive cells and transplant outcome has been studied in PBSC transplantation for hematological malignancies.[17] The infusion of a higher number of CD34-positive cells has been associated with faster recovery of neutrophils and plts, but chronic GVHD was more frequently observed in patients who received a very high dose of CD34-positive cells (that is, $>8.0 \times 10^6$ cells/kg). In this study, two patients failed to achieve engraftment, and both had received less than 4.0×10^6 cells/kg of CD34-positive cells. However, a statistically significant survival advantage was confirmed even after these two patients were excluded from the analysis. If we consider that the major cause of death in this study was progressive disease, the infusion of a higher number of CD34-positive cells might have protected patients from disease progression by a graft-versus-host reaction, although we failed to show a significant difference between the number of infused CD34-positive cells and tumor response or the incidence of chronic GVHD, probably due to the small number of patients. Patients who developed chronic GVHD showed better survival than those who did not, with a borderline significance, suggesting that they had some immunological protection against the progression of pancreatic cancer.

Table 2 Univariate and multivariate analyses for overall survival

Factor	*Median survival (days)*	P-*value*
A. Univariate		
Age (years)		
<55	115	0.63
⩾55	180	
Sex		
Male	87	0.69
Female	170	
ECOG-PS		
0–1	211	0.0067
2–4	80	
Stage		
Locally advanced	192	0.21
Metastatic	121	
Serum CEA		
Negative	122	0.70
Positive	192	
Serum CA19-9		
Negative	157	0.84
Positive	132	
Regimen		
Flu + BU based	191	0.25
Flu + CY	156	
Flu + TBI	71	
CD34+ cell dose		
$\leqslant 4.0 \times 10^6$/kg	85	0.018
$> 4.0 \times 10^6$/kg	201	
GVHD prophylaxis		
CsA alone	132	0.55
CsA + MTX	191	
CsA + MMF	96	
B. Multivariate	*Relative risk (95% CI)*	P-*value*
ECOG-PS		
0–1	1.00	0.032
2–4	3.39 (1.11–10.3)	
CD34+ cell dose		
$\leqslant 4.0 \times 10^6$/kg	1.00	0.068
$> 4.0 \times 10^6$/kg	0.37 (0.13–1.07)	

Abbreviations: CI = confidence interval; ECOG-PS = Eastern Cooperative Oncology Group performance status; Flu = fludarabine; MMF = mycophenolate mofetil.

This study was limited by the heterogeneity of transplantation procedures among centers. However, considering the difficulty of performing a large-scale prospective study on RICT against pancreatic cancer, this small survey may currently represent the best evidence of the efficacy of this novel treatment strategy against advanced pancreatic cancer and may suggest a future direction for improving the treatment outcome. We showed that pancreatic cancer can be a possible target for allogeneic immunotherapy. However, the immunological effect was not strong or durable enough to prevent tumor progression. A possible strategy for enhancing a graft-versus-tumor effect against pancreatic cancer without enhancing GVHD is a combination with specific immunotherapy using antigens including CA19-9,

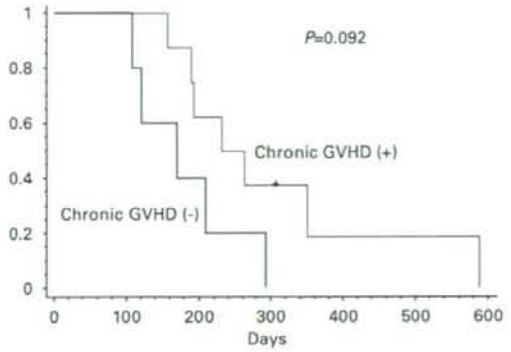

Figure 2 Overall survival of patients who survived at least 100 days after transplantation grouped according to the presence or absence of chronic GVHD.

CA242, CEA, Her-2, mutated K-ras and MUC-1.[12] Among these, CEA is attractive, since it is expressed in 85–90% of pancreatic cancer, and a specific immunotherapy against CEA could also be applied to other gastrointestinal cancers. An increase in the serum anti-CEA antibody level associated with a tumor response was observed in the University of Tokyo Study.[15] In addition, Kim *et al.*[18] showed that a peptide CEA652, TYACFVSNL, binds to HLA-A24 and induces CEA-specific cytotoxic T cells. Therefore, vaccination with such a peptide may be promising as a post transplantation immunotherapy against pancreatic cancer. Another approach is to add molecular targeting agents such as erlotinib after RICT. This may induce tumor cell death, leading to the enhanced presentation of tumor antigens to donor T cells. In addition, RICT can be combined with surgical resection, since the prognosis of pancreatic cancer is very poor even after complete resection.[19,20] Maximum graft-versus-tumor effect can be expected when the tumor load is at its lowest level.

In conclusion, a tumor response was observed in approximately one-fourth of the patients who underwent RICT against advanced pancreatic cancer. Although the response was not durable, our findings, such as the relationship between longer survival and the infusion of a higher number of CD34-positive cells or the development of chronic GVHD, should support a future study to enhance the specific immunological effect against pancreatic cancer.

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Human Flt3 Is Expressed at the Hematopoietic Stem Cell and the Granulocyte/Macrophage Progenitor Stages to Maintain Cell Survival[1]

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FLT3/FLK2, **a member of the receptor tyrosine kinase family, plays a critical role in maintenance of hematopoietic homeostasis, and the constitutively active form of the FLT3 mutation is one of the most common genetic abnormalities in acute myelogenous leukemia. In murine hematopoiesis, Flt3 is not expressed in self-renewing hematopoietic stem cells, but its expression is restricted to the multipotent and the lymphoid progenitor stages at which cells are incapable of self-renewal. We extensively analyzed the expression of Flt3 in human (h) hematopoiesis. Strikingly, in both the bone marrow and the cord blood, the human hematopoietic stem cell population capable of long-term reconstitution in xenogeneic hosts uniformly expressed Flt3. Furthermore, human Flt3 is expressed not only in early lymphoid progenitors, but also in progenitors continuously along the granulocyte/macrophage pathway, including the common myeloid progenitor and the granulocyte/macrophage progenitor. We further found that human Flt3 signaling prevents stem and progenitors from spontaneous apoptotic cell death at least through up-regulating Mcl-1, an indispensable survival factor for hematopoiesis. Thus, the distribution of Flt3 expression is considerably different in human and mouse hematopoiesis, and human FLT3 signaling might play an important role in cell survival, especially at stem and progenitor cells that are critical cellular targets for acute myelogenous leukemia transformation.** ***The Journal of Immunology*****, 2008, 180: 7358–7367.**

Hematopoiesis is one of the most intensely studied stem cell systems where hematopoietic stem cells (HSCs)[3] self-renew, generate a variety of lineage-restricted progenitors, and continuously supply all types of mature blood cells. The technical advances of the multicolor FACS and the use of mAbs have enabled the prospective isolation of hematopoietic stem and progenitor cells according to the surface marker expression. In mice, multipotent hematopoietic activity resides in a small fraction of bone marrow (BM) cells lacking the expression of lineage-associated surface marker (Lin) but expressing high levels of Sca-1 and c-Kit (1, 2). Within the c-Kit$^+$Lin$^-$Sca-1$^+$ (KLS) fraction, the most primitive self-renewing HSCs with long-term reconstituting activity (LT-HSCs) do not express murine (m) CD34, but they do express mCD38 and a low level of mCD90 (Thy1), whereas mCD34$^+$, mCD38$^-$, or mThy1$^-$ KLS cells are short-term HSCs (ST-HSCs) or multipotent progenitors that do not self-renew (3–5). Downstream of the mCD34$^+$ ST-HSC stage, common lymphoid progenitors (CLPs) (6) and common myeloid progenitors (CMPs) (7) that can differentiate into all lymphoid cells and myelo-erythroid cells, respectively, have been purified. CMPs differentiate into granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs), both of which are also prospectively isolatable by FACS (7).

Interestingly, the expression pattern of these surface markers in early stem and progenitor populations are considerably different in human (h) hematopoiesis. In humans, LT-HSCs express hCD34 (8). The hLT-HSC resides in the hCD34$^+$hCD38$^-$ (9, 10) or the hCD34$^+$hCD90$^+$ (11–13) fractions in both human BM and cord blood (CB). It is still unclear what percent of hCD34$^+$hCD38$^-$ or hCD34$^+$hCD90$^+$ cells are LT-HSCs in human hematopoiesis. The human counterpart for mCMPs, mGMPs, mMEPs, or mCLPs is also isolatable in the BM and the CB within the hCD34$^+$hCD38$^+$ progenitor fraction (14, 15). It has thus been suggested that, despite the difference in the expression patterns of key Ags in human and mouse hematopoiesis, lineage commitment processes from HSCs to mature blood cells might be generally preserved in both species. For example, the existence of prospectively isolatable CMPs and CLPs suggests that lineage commitment from HSCs involves myeloid vs lymphoid bifurcation in both mouse and human.

Recently, two independent groups have reported that in murine hematopoiesis, Flt3/Flk2, a tyrosine kinase receptor, is expressed in ST-HSCs but not in LT-HSCs. One group showed that mCD34$^-$

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[3] Abbreviations used in this paper: HSC, hematopoietic stem cell; AML, acute myelogenous leukemia; BM, bone marrow; KLS, c-Kit$^+$Lin$^-$Sca-1$^+$; LT-HSC, HSC with long-term reconstituting activity; ST-HSC, short-term HCS; m, murine; h, human; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GM, granulocyte/macrophage; GMP, GM progenitor; MEP, megakaryocyte/erythrocyte progenitor; CB, cord blood; MegE, megakaryocyte/erythrocyte; FL, Flt3 ligand; PI, propidium iodide; SCF, stem cell factor; Tpo, thrombopoietin; Epo, erythropoietin; CFU-GEMM, CFU-granulocyte/erythroid/macrophage/megakaryocyte; RTK, receptor tyrosine kinase; ITD, internal tandem duplication.

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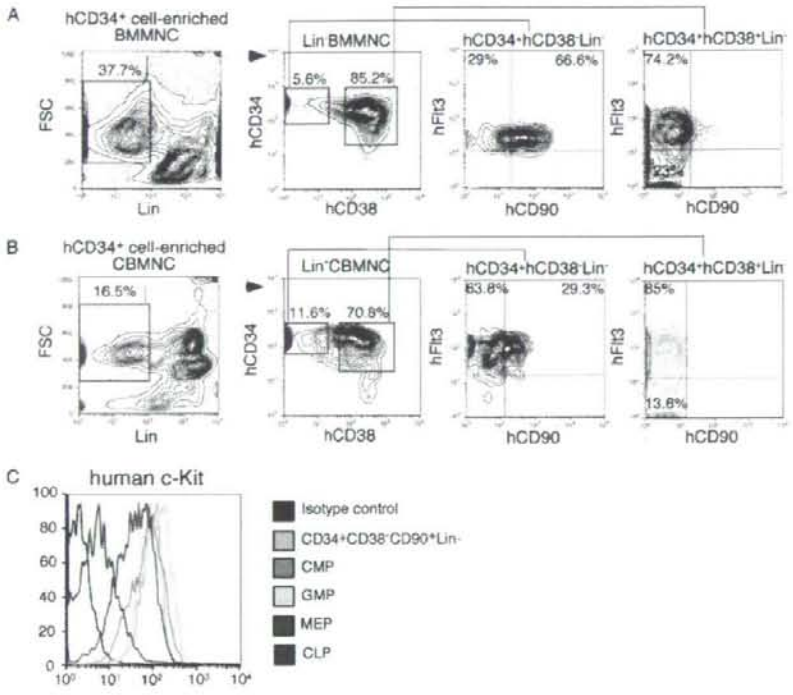

FIGURE 1. A flow cytometric analyses of human early hematopoietic populations in the BM and the CB. In hCD34$^+$hCD38$^-$ immature BM (*A*) and CB (*B*) cells, hFlt3 was expressed at a low level in both hCD90 (Thy1) positive and negative fractions. In contrast, the hCD34$^+$hCD38$^+$ BM and CB progenitor populations did not express hCD90, and hFlt3 was expressed in only a fraction of these populations. *C*, hHSCs and myeloid progenitors expressed c-Kit at high levels, and CLPs at a low level. Representative data of independent five experiments are shown here.

KLS cells (LT-HSCs) are mFlt3$^-$ (16), and the other showed that only the mFlt3$^-$ fraction of mCD90^{low} KLS cells possesses LT-HSC activity (17). Each group further studied the detailed differentiation activity of mFlt3$^+$ KLS cells, but drew different conclusions. Adolfsson et al. (18) reported that the mFlt3$^+$mCD34$^+$ KLS population maintains the granulocyte/macrophage (GM) and the T/B lymphoid, but not the megakaryocyte/erythrocyte (MegE) potential, if any. This result suggests that, in addition to the lymphoid vs myeloid developmental pathway represented by CLPs and CMPs, respectively, there is a critical stage common to GM, T, and B lymphoid cells. The other group, however, showed that mFlt3$^+$mCD90$^-$ KLS cells are multipotent, thus claiming that the stage common to GM/lymphoid lineages proposed by Adolfsson et al. (18) does not constitute a major pathway for hematopoietic development (19). In contrast, downstream of the mST-HSC stage, there is a general agreement that mFlt3 is expressed in progenitors with lymphoid potential, such as the majority of CLPs and a minor fraction of CMPs, that retain a weak B cell potential (20), whereas it is down-regulated in late myeloid stages, such as GMPs and MEPs (20, 21). The Flt3 ligand (FL) is required for development of CLPs from mFlt3$^+$ KLS cells, whereas mFlt3 is dispensable for HSC maintenance and myeloid development (22). These results suggest that in mouse hematopoiesis, Flt3 signaling plays an important role in lymphoid, but not in HSC or myeloid, development.

The precise expression and the role of hFlt3 in human hematopoiesis, however, remain unclear. Around 40–80% of hCD34$^+$ BM and CB cells express hFlt3 (23, 24). Although a fraction of both the hFlt3$^+$ and the hFlt3$^-$ populations gave rise to multilineage "mixed" colonies containing all myelo-erythroid components, the hFlt3$^+$hCD34$^+$ and hFlt3$^-$hCD34$^+$ populations predominantly formed GM and erythroid colonies, respectively (23–25). It has also been shown that cells with NOD/SCID reconstitution activity reside in the hCD34$^+$hFlt3$^+$ fraction (24). These data collectively suggest that LT-HSCs and GMPs may reside mainly in the hFlt3$^+$hCD34$^+$ fraction, whereas MEPs may be contained in the hFlt3$^-$hCD34$^+$ fraction. Therefore, the expression pattern of Flt3 could be quite different in mouse and human hematopoiesis. Flt3 expression has also been implicated in development of human acute myelogeneous leukemia (AML). Flt3 is expressed in leukemic blasts in most cases with AML (26, 27). Furthermore, *FLT3* is one of the most frequently mutated genes in AML (28, 29), and the FLT3 mutants transduce the constitutively active FLT3 signaling, that could be the cause of poor prognosis in AML with *FLT3* mutations (30–32).

In this study, we extensively analyzed the expression and function of hFlt3 in steady-state human BM and CB hematopoiesis. Interestingly, hFlt3 was expressed in the entire human BM and CB HSC population, and purified hFlt3$^+$ HSCs could reconstitute multilineage cells for a long-term in our xenogeneic transplantation system (33). Therefore, unlike mouse hematopoiesis, the negative expression of Flt3 does not mark LT-HSCs in human. Furthermore, in striking contrast to mouse hematopoiesis where mFlt3 is expressed in CLPs but not GMPs (20, 21), hFlt3 was expressed in GMPs as well as in CLPs at a high level. The hFlt3 signaling did not affect the lineage fate decision of hHSCs, but supported cell survival of hFlt3$^+$ stem and progenitor cells, at least through the up-regulation of Mcl-1, a survival promoting Bcl-2 homologue (34). These data collectively suggest that Flt3 signaling plays a critical role in maintenance of self-renewing LT-HSCs, and of GM and lymphoid progenitors in human hematopoiesis.

Materials and Methods

BM and CB samples

Fresh human steady-state BM and CB samples were collected from healthy adults and newborns after normal deliveries. Informed consent was obtained from all subjects. The Institutional Review Board of each institution participating in this project approved all research on human subjects.

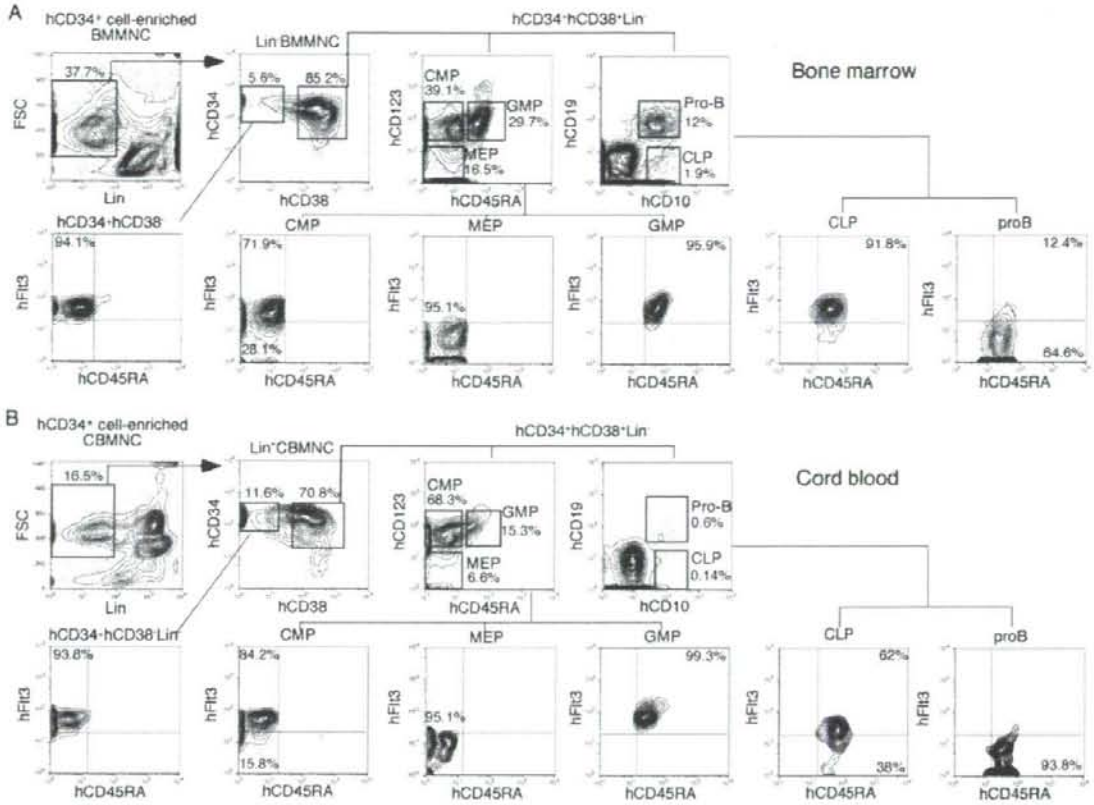

FIGURE 2. The expression patterns of hFlt3 are similar in early human BM (*A*) and CB (*B*) hematopoiesis. In the myeloid pathway in both the BM and CB, hFlt3 was up-regulated into the GM pathway, but was down-regulated in the MegE pathway; GMPs expressed hFlt3 at a high level, whereas MEPs did not express hFlt3. CMPs contained both $hFlt3^+$ and $hFlt3^-$ fractions. In the lymphoid pathway, CLPs expressed hFlt3 at a high level in BM (*A*) and a low level in CB (*B*), whereas $hCD10^+hCD19^+$ proB cells did not express hFlt3 in either the BM or the CB. Representative data of independent five experiments are shown here.

Cell preparation, flow cytometric analysis, and cell sorting

The BM and CB mononuclear cells were prepared by gradient centrifugation and the $CD34^+$ cells were enriched from mononuclear cells by using the Indirect CD34 MicroBead kit (Miltenyi Biotec) as described previously (14). For the analyses and sorting of myeloid progenitors, cells were stained with a Cy5-PE- or PC5-conjugated lineage mixture, including anti-hCD3 (HIT3a), hCD4 (RPA-T4), hCD7, hCD8 (RPA-T8), hCD10 (HI10a), hCD19 (HIB19), hCD20 (2H7), hCD11b (ICFR44), hCD14 (RMO52), hCD56 (NKH-1), and hGPA (GA-R2), FITC-conjugated anti-hCD34 (8G12), or anti-hCD45RA (HI100), PE-conjugated anti-hFlt3 (CD135) (4G8), or anti-hCD123 (6H6), allophycocyanin-conjugated anti-hCD34 (8G12), or anti-hCD38 (HIT2), Pacific Blue-conjugated anti-hCD45RA (HI100), and biotinylated anti-hCD38 (HIT2), or anti-hCD123 (9F5). The lymphoid progenitors were stained with the same lineage mixture except for the omission of anti-hCD10 and hCD19 followed by FITC-conjugated anti-hCD10 (SS2/36), PE-Cy7-conjugated anti-hCD19 (SJ25C1), and anti-hFlt3, hCD34, hCD38, and hCD45RA as described above. For additional analyses, PE-Cy7-conjugated anti-hCD34 (8G12), FITC-conjugated anti-hCD90 (5E10), PE-conjugated anti- hCD117 (YB5.B8), biotinylated anti-hFlt3 (BV10A4H2), and PE-conjugated anti-hCD127 (R34.34) mAbs were used. Streptavidin-conjugated allophycocyanin-Cy7 or PE-Cy7 was used for visualization of the biotinylated Abs (BD Pharmingen). The dead cells were excluded by propidium iodide (PI) staining. Appropriate isotype-matched, irrelevant control mAbs were used to determine the level of background staining. The cells were sorted and analyzed by FACS Aria (BD Biosciences). The sorted cells were subjected to an additional round of sorting using the same gate to eliminate contaminating cells and doublets. For single-cell assays, the re-sort was performed by using an automatic cell-deposition unit system (BD Biosciences).

In vitro assays to determine the differentiation potential of progenitors

Clonogenic CFU assays were performed using a methylcellulose culture system that was set up to detect all possible outcomes of myeloid differentiation as reported previously (14, 35). For myeloid colony formation, cells were cultured in IMDM-based methylcellulose medium (Methocult H4100; StemCell Technologies) supplemented with 20% FCS, 1% BSA, 2 mM L-glutamine, 50 μM 2-ME, and antibiotics in the presence of human cytokines such as IL-3 (20 ng/ml), stem cell factor (SCF) (20 ng/ml), FL (20 ng/ml), IL-11 (10 ng/ml), thrombopoietin (Tpo) (50 ng/ml), erythropoietin (Epo) (4 U/ml), and GM-CSF (50 ng/ml). All cytokines were obtained from R&D Systems. Colony numbers were enumerated on day 14 of culture. For the short-term liquid cell culture, cells were cultured in IMDM with 10% FCS in the presence of the cytokines described above. All of the cultures were incubated at 37°C in a humidified chamber under 5% CO_2.

Apoptosis assay and cytokine stimulation assays in the serum-free medium

To exclude the unexpected effects of FCS and to evaluate the effects of cytokine stimulation precisely, the cells were prepared in the FCS-free condition. The anti-apoptotic effect of FL and SCF was evaluated after 24 h

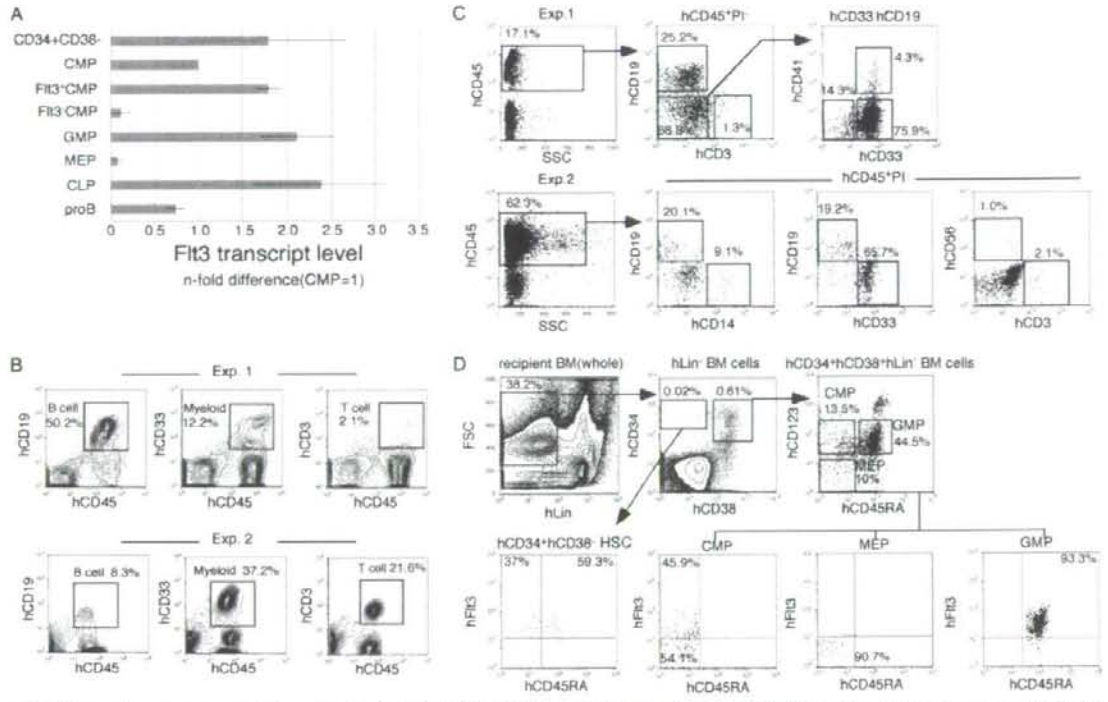

FIGURE 3. Long-term reconstitution potential of hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$Lin$^-$ cells in NOD/SCID/IL2rγ^{null} newborn mice. *A*, Analyses of the relative expression levels of hFlt3 transcript by real-time PCR. Each bar shows the *n*-fold difference of the level of hFlt3 mRNA in comparison to that of the whole CMP. The mean value and SD of BM samples from three independent normal donors are shown. Note that the levels of hFlt3 transcripts are well correlated with those of surface hFlt3 expression determined by FACS (Fig. 2*A*). *B*, The long-term and multilineage reconstitution of human cells in mice injected with 1×10^3 hFlt3$^+$hCD34$^+$hCD38$^-$Lin$^-$ CB cells 4 (*upper panels*) or 6 (*lower panels*) mo after transplantation. Representative two results out of five experiments are shown. *C*, Multilineage reconstitution 6 (*upper panels*) and 15 wk (*lower panels*) after i.v. injection of 5×10^3 hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$Lin$^-$ BM HSCs into NOD/SCID/IL2rγ^{null} newborns. Donor-derived viable human cells were evaluated as hCD45$^+$PI$^-$ cells. hCD33$^+$ granulocytes, hCD14$^+$ monocytes, hCD41$^+$ megakaryocytes, hCD19$^+$ B cells, hCD3$^+$ T cells, and hCD56$^+$ NK cells were detected in the BM of recipient mice. *D*, Stem and progenitor analyses of BM from mice reconstituted with hFlt3$^+$ HSCs. The BM contained hFlt3$^+$hCD34$^+$hCD38$^-$ HSCs, and all types of myeloid progenitors within the hCD34$^+$hCD38$^+$ population, including hCD45RA$^-$hCD123^{low} CMPs, hCD45RA$^+$hCD123^{low} GMPs, and hCD45RA$^-$hCD123$^-$ MEPs. The expression patterns of hFlt3 in each population were identical with those of freshly isolated stem and progenitor cells. A representative experiment by using BM samples from three independent normal donors is shown.

serum-free liquid culture, using Annexin V and PI staining (BD Pharmingen). The sorted cells were cultured in the serum-free medium (STEMPRO-34 SFM; Invitrogen) with or without FL (20 ng/ml) and/or SCF (20 ng/ml) for 24 h. The living cells were defined as Annexin V$^-$/PI$^-$ among the live-gated cells (as shown in Fig. 5*B*). For the cytokine stimulation assays, cells were sorted in the IMDM and then the cytokines were added.

In vivo assays to determine the differentiation potential and reconstitution capacity

The NOD.Cg-Prkdc^{scid}IL-2rg^{tm1Wjl}/Sz (NOD/SCID/IL2rγ^{null}) mice were developed at The Jackson Laboratory. The NOD/SCID/IL2rγ^{null} strain was established by backcrossing a complete null mutation at γc locus (36) onto the NOD.Cg-Prkdc^{scid} strain. The establishment of this mouse line has been reported elsewhere (37). For the reconstitution assays, the sorted cells were transplanted into irradiated (100cGy) NOD/SCID/IL2rγ^{null} newborns via a facial vein within 48 h of birth. To confirm the long-term reconstitution by hHSCs, the chimerism of circulating human blood cells were analyzed until at least 24 wk after transplantation, as previously reported (33). In addition to the Abs described above, the following mAbs were used: allophycocyanin-conjugated anti-hCD45 (J33), PE-Cy7-conjugated anti-hCD123 (6H6), FITC-conjugated anti-hCD33 (HIM3–4) or hCD14 (M5E2), and PE-conjugated anti-hCD41 (VIPL3), hCD56 (B159), anti-hGlycophorin A (GPA) (GA-R2), or anti-hCD3 (HIT3a).

Quantitative real-time PCR

To examine the gene expression profile of each population, RNA was isolated from 2,000-sorted cells using Isogen reagent (Nippon gene) according to the manufacturer's instructions. The total RNA was reverse transcribed to cDNA using a TaKaRa RNA PCR kit (Takara Shuzo). The mRNA levels were quantified in triplicate using a real-time PCR (7500 Real-Time PCR system; Applied Biosystems). hβ2-microglobulin mRNA was separately amplified in the same plate to be used for internal control. The primer and probes were designed by Primer Express software (Applied Biosystems).

Results

The hCD34$^+$hCD38$^-$ HSC fraction express hFlt3 at a low level in both BM and CB

The hCD34$^+$Lin$^-$ population was divided into hCD38$^+$ and hCD38$^-$ populations (Fig. 1, *A* and *B*). It has been shown that HSCs with long-term reconstitution activity reside in the hCD38$^-$ fraction within the hCD34$^+$ BM and CB populations (9, 10). As shown in Fig. 1*A*, in the BM, hCD38$^-$ cells constituted only ~5% of the Lin$^-$hCD34$^+$ population. This population uniformly expressed hFlt3 at a low level. More than

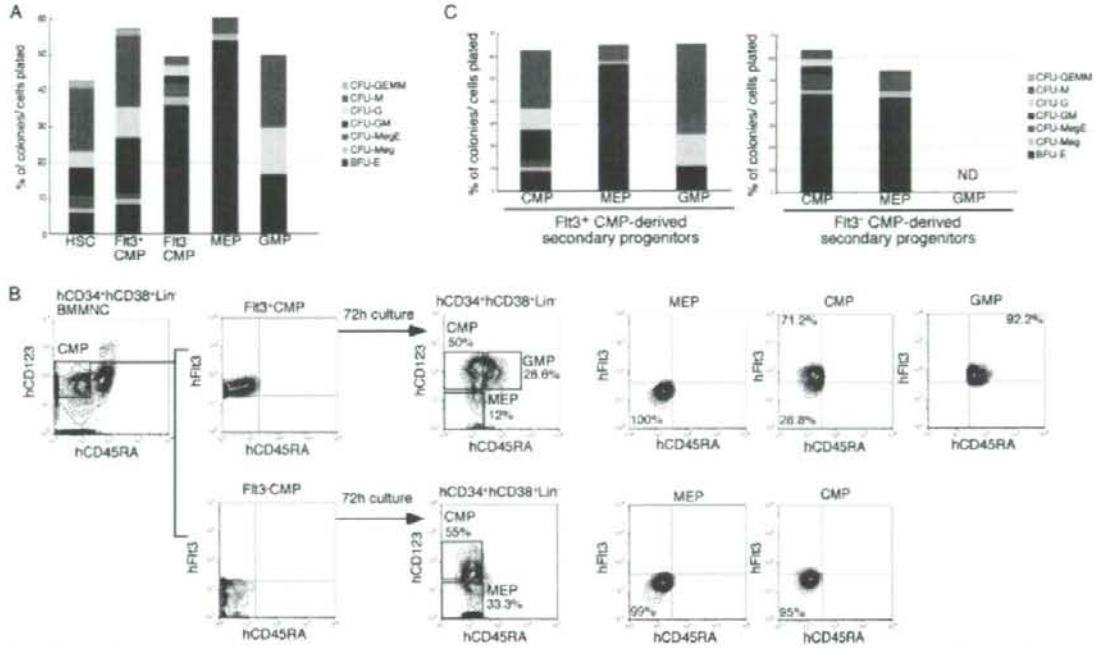

FIGURE 4. The lineage potential and the relationship of myeloid progenitor populations. *A*, Clonogenic colony formation of purified populations on methylcellulose in the presence of cytokine mixture. The hCD34$^+$hCD38$^-$ HSCs and hFlt3$^+$CMPs gave rise to various myeloid colonies including CFU-GEMM, whereas GMPs and MEPs formed exclusively GM and MegE lineage-related colonies, respectively. In contrast, hFlt3$^-$CMPs predominantly gave rise to MegE lineage-related colonies but failed to form CFU-GEMM. The mean value of eight independent experiments is shown. CFU-M: CFU-macrophage, CFU-G: CFU-granulocyte, CFU-GM: CFU-granulocyte/macrophage, CFU-MegE: CFU-megakaryocyte/erythroid, CFU-Meg: CFU-megakaryocyte, and BFU-E: burst-forming units-erythroid. *B*, The lineal relationship between hFlt3$^+$CMPs and hFlt3$^-$CMPs. After 72 h of culturing, hFlt3$^+$CMPs gave rise to hFlt3$^-$CMPs, GMPs, and MEPs. In contrast, hFlt3$^-$CMPs differentiated into only MEPs, thus suggesting hFlt3$^-$CMP to be a transitional intermediate population from hFlt3$^+$CMPs to hFlt3$^-$MEPs. *C*, The colony formation activity of phenotypically defined secondary CMPs, GMPs, and MEPs purified from the primary culture of hFlt3$^+$CMPs or hFlt3$^-$CMPs. Each population displayed the colony formation activity consistent with their phenotypic definition. The mean value of four independent experiments is shown.

60% of the hCD34$^+$hCD38$^-$ BM cells also expressed hCD90, another critical marker for hHSCs (11–13), whereas the hCD34$^+$hCD38$^+$Lin$^-$ fraction was constituted of hCD90$^-$ lineage-committed progenitors.

In the CB, only ~30% of hCD34$^+$hCD38$^-$ cells expressed hCD90 (Fig. 1*B*). In the NOD/SCID/IL2rγ^{null} newborn system, the hCD34$^+$hCD38$^-$hCD90$^+$ population was highly enriched for HSCs capable of long-term reconstitution as compared with the hCD34$^+$hCD38$^-$hCD90$^-$ CB fraction (F. Ishikawa, unpublished data). The vast majority of hCD34$^+$hCD38$^-$ cells expressed hFlt3 at a low level as previously reported (38). Furthermore, the hCD34$^+$hCD38$^-$hCD90$^+$ CB population expressed hFlt3.

These data clearly show that hFlt3 is expressed in all cells with the hHSC phenotype in both the BM and the CB, and suggest that Flt3 expression does not discriminate ST-HSCs from LT-HSCs in human as it does in mouse (16, 17). In contrast, the BM and the CB hCD34$^+$hCD38$^+$ progenitor fraction expressed negative to high levels of hFlt3. We thus further subfractionated the hCD34$^+$hCD38$^+$ population to evaluate the hFlt3 expression in a variety of lineage-restricted progenitors.

The expression of hFlt3 within the hCD34$^+$hCD38$^+$ progenitor fraction

In mouse hematopoiesis, the expression of mFlt3 is associated with early lymphoid progenitor activities; it is expressed in the majority of CLPs, and in the minority of CMPs with weak B cell potential (20), but not in MEPs or GMPs (20) (21). Fig. 2 shows the expression of hFlt3 in the myeloid and lymphoid progenitor populations. According to the phenotypic definition of human myeloid and lymphoid progenitors (14, 15, 39, 40), hCD34$^+$hCD38$^+$ cells were subfractionated into myeloid and lymphoid progenitors, including the hCD45RA$^-$hCD123 (IL-3Rα)^{low} CMP, the hCD45RA$^-$hCD123$^-$ MEP, the hCD45RA$^+$hCD123^{low} GMP, the hCD10$^+$hCD19$^-$ CLP, and the hCD10$^+$hCD19$^+$proB populations. Interestingly, in both the human BM and CB, ~70–80% of CMPs expressed hFlt3, whose level was progressively up-regulated at the GMP stage. In contrast, hFlt3 expression was completely shut down in MEPs. In the lymphoid lineage, the hCD34$^+$hCD38$^+$hCD10$^+$ CLP (15) strongly expressed hFlt3, whereas hFlt3 was down-regulated in the proB cells. The expression level of hFlt3 in GMPs and CLPs appears to be higher than that in hCD34$^+$hCD38$^-$hCD90$^+$ HSCs (Fig. 2). We also tested the level of hFlt3 transcripts in purified hBM HSCs and progenitor populations (Fig. 3*A*). The pattern of hFlt3 mRNA expression was generally consistent with that in hFlt3 protein, as evaluated by using anti-hFlt3 Abs on FACS (Figs. 1 and 2). Consistent with a previous report (41), MEPs and hFlt3$^-$ CMPs had the lowest levels, GMPs and CLPs had the highest levels, and the hCD34$^+$hCD38$^-$ HSC population had a medium level of hFlt3 mRNA. Collectively, functional hLT-HSCs express hFlt3 mRNA

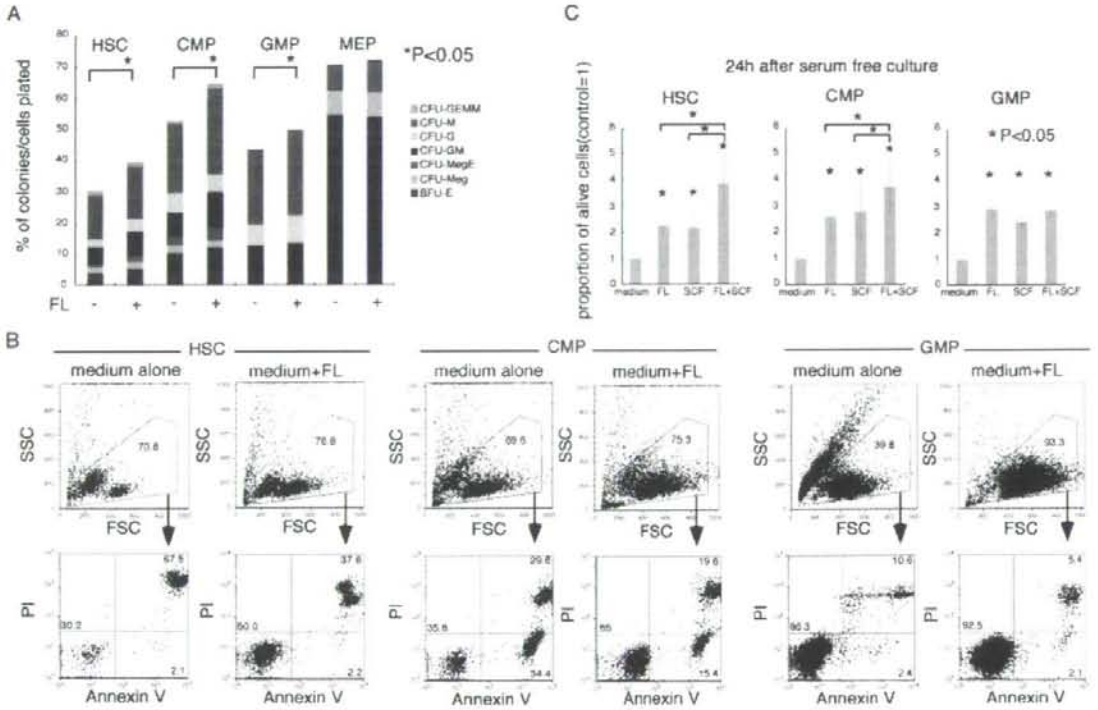

FIGURE 5. Effect of FL and SCF on the survival of purified progenitors. *A*, The effect of additional FL on colony formation of purified progenitors in methylcellulose in the presence of SCF, IL-3, IL-11, GM-CSF, Epo, and Tpo. Results from five independent experiments are shown here. Note that colony numbers are increased by the addition of FL into cultures in all hFlt3-expressing subsets including HSCs, CMPs, and GMPs but not in hFlt3$^-$ MEPs. *B*, An evaluation of apoptotic cell death in cultures of stem and progenitor cells. HSCs, hFlt3$^+$ CMPs, and GMPs were cultured in the serum-free media, with or without FL, and analyzed at 12, 18, 24, 30, 48, and 72 h after initiation of culture. A representative data obtained after 24-h culture is shown. *C*, Anti-apoptotic effects of FL and/or SCF on HSCs and Flt3$^+$ CMPs. Annexin$^-$ PI$^-$ live cells were enumerated after 24-h culture in a serum-free media. Each graph shows n-fold differences in the percentage of live cells relative to the ones without cytokine. Each bar represents the mean value and the SD of five independent samples.

and surface protein, and the distribution of Flt3 is quite different between human and mouse in early hematopoiesis.

In contrast, c-Kit was expressed at high levels in human HSCs and myelo-erythroid progenitors, while at a low level in CLPs (Fig. 1*C*). The expression pattern of c-Kit in human hematopoietic stem and progenitor cells is generally consistent with that in mouse hematopoiesis (4, 6, 7), suggesting that the c-Kit expression program is preserved in mouse and human hematopoiesis.

hFlt3 is expressed in functional hHSCs capable of reconstituting normal hematopoiesis in the NOD/SCID/IL-2 receptor γ-chain null (NOD/SCID/IL2rγ^{null}) mouse model

In the NOD/SCID/IL2rγ^{null} newborn system, hCD34$^+$hCD38$^-$ BM and CB cells are capable of reconstitution of all hematopoietic lineages for a long term (33). The entire hCD34$^+$hCD38$^-$ BM population expressed hFlt3 (Fig. 1*A*), suggesting that functional hBM HSCs possess hFlt3 on their surface. In contrast, hCD34$^+$hCD38$^-$ CB cells contained some hCD90$^-$ cells that did not express hFlt3. To formally test whether Flt3-expressing hCD34$^+$hCD38$^-$ CB cells possess LT-HSC activity, we transplanted hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ CB cells in to NOD/SCID/IL2rγ^{null} newborns. As shown in Fig. 3*B*, NOD/SCID/IL2rγ^{null} mice transplanted with 1×10^3 hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ CB cells reconstituted all hematolymphoid lineages for >6 mo, indicating that hFlt3 is expressed in functional hHSCs in CB as well as in BM.

Fig. 3*C* shows the phenotypic analysis of human progeny from 5×10^3 hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ BM cells 6 (*upper panels*) or 15 wk (*lower panels*) after transplantation into NOD/SCID/IL2rγ^{null} newborns (33). hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ BM cells differentiated into all hematopoietic lineage cells, including hCD33$^+$ granulocytes, hCD14$^+$ monocytes, hCD41$^+$ megakaryocytes, hCD19$^+$ B cells, hCD3$^+$ T cells, hCD56$^+$ NK cells (Fig. 3*C*), and hGPA$^+$ erythrocytes (not shown). Furthermore, transplanted hFlt3$^+$hCD34$^+$hCD38$^-$ HSCs purified from primary recipients developed secondary hFlt3$^+$ HSCs and hFlt3$^{-/+}$ CMPs, hFlt3$^-$ MEPs, and hFlt3$^+$ GMPs recapitulating normal human hematopoietic development. Thus, the hCD34$^+$hCD38$^-$hCD90$^+$ BM population contains cells with long-term SCID reconstitution potential as reported (33, 42), and all cells within this population express hFlt3 on their surface (Fig. 3*D*).

The up- or down-regulation of hFlt3 in the myeloid pathway is associated with GM or MegE differentiation activity, respectively

Fig. 4*A* shows the differentiation potential of purified BM progenitors in vitro in the presence of the myeloid cytokine mixture containing SCF, FL, IL-3, IL-11, Tpo, Epo, and GM-CSF. hFlt3$^+$

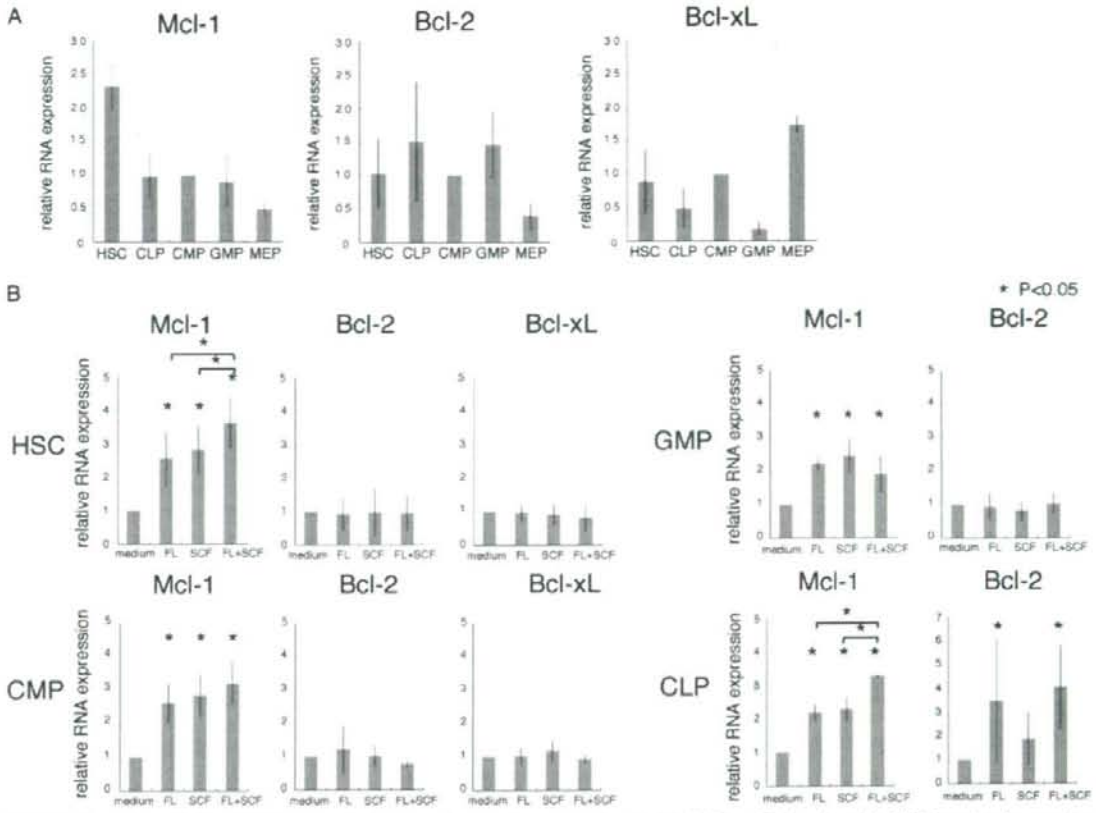

FIGURE 6. *A*, Quantitative RT-PCR assays for human anti-apoptotic genes such as Mcl-1, Bcl-2, and Bcl-x_L in purified HSCs and each progenitor population. Each bar represents an *n*-fold difference in the amount of anti-apoptotic gene expression relative to that in Flt3$^+$ CMPs. Note that Mcl-1 expression level is highest in HSCs, whereas Bcl-2 and Bcl-x_L expression is most pronounced in GMPs and MEPs, respectively. *B*, Changes in anti-apoptotic gene expression in each progenitor after incubation with FL and/or SCF. Significant up-regulation of Mcl-1 mRNA was seen in HSCs, Flt3$^+$ CMPs, GMPs, and CLPs after incubation with FL and/or SCF. Each bar represents the mean value and the SD of sixe independent samples.

CMPs formed a variety of myelo-erythroid colonies including clonogenic CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM), whereas hFlt3$^-$ CMPs did not form CFU-GEMM, but preferentially differentiated into the MegE lineage. Since GMPs (hFlt3$^+$) and MEPs (hFlt3$^-$) exclusively gave rise to GM- and MegE-related colonies, respectively, hFlt3 expression could be associated with GM lineage development. These results suggested that hFlt3$^+$ CMPs might differentiate into MEPs via hFlt3$^-$ CMPs. We thus directly tested the lineage relationship of these purified myelo-erythroid progenitor populations (Fig. 4*B*). hFlt3$^+$ and hFlt3$^-$ CMPs were purified and cultured in vitro. Then, 72 h after the initiation of culture, hFlt3$^+$ CMPs gave rise to hFlt3$^-$ CMPs, hFlt3$^+$ GMPs and hFlt3$^-$ MEPs, whereas hFlt3$^-$ CMPs did not up-regulate hFlt3, differentiating only into hFlt3$^-$ MEPs. Such phenotypically defined secondary myeloid progenitors displayed differentiation activity consistent with their phenotypic definition (Fig. 4*C*). These data suggest that multipotent hFlt3$^+$ CMPs can differentiate into both GMPs and MEPs, whereas hFlt3$^-$ CMPs represent a transitional stage into MEPs.

Flt3 signaling protects human hematopoietic stem and progenitor cells from apoptotic cell death

We wished to elucidate the role of Flt3 signaling in human hematopoiesis. We first tested the effect of Flt3 signaling on the differentiation of HSCs, CMPs, and GMPs. Purified hFlt3$^+$ HSCs, CMPs, and GMPs were cultured in methylcellulose in the presence of the myeloid cytokine mixture, with or without hFL. As shown in Fig. 5*A*, the addition of FL in the culture did not affect the percentage of GM, MegE, or mix colonies in any of these populations. Interestingly, however, the colony numbers significantly increased in all cases when FL was added to the culture. This effect was dose-dependent, and the stimulatory activity of FL reached its peak at a concentration of 5 ng/ml (not shown). The plating efficiencies of hFlt3$^+$ HSCs, CMPs, and GMPs cultured with the cytokine mixture containing FL (20 ng/ml) were significantly higher than those cultured without FL, suggesting that FL signaling may enhance the viability of cells (Fig. 5*A*). We then directly tested the viability of HSCs, CMPs, and GMPs 24 h after the initiation of culture in serum-free media, with or without FL. The live, apoptotic, and dead cells after culture were enumerated by the Annexin/PI staining (43). In this staining, live cells are Annexin$^-$/PI$^-$, whereas Annexin$^+$/PI$^-$ and Annexin$^+$/PI$^+$ cells are apoptotic and dead cells, respectively (Fig. 5*B*). Without FL, a considerable proportion of purified HSCs, CMPs, and GMPs rapidly became Annexin$^+$/PI$^-$ and Annexin$^+$/PI$^+$ cells undergoing apoptotic cell death. The addition of FL significantly blocked apoptotic cell death in all of these populations, indicating that FL plays a critical role in human hematopoietic stem and progenitor cell survival (Fig.

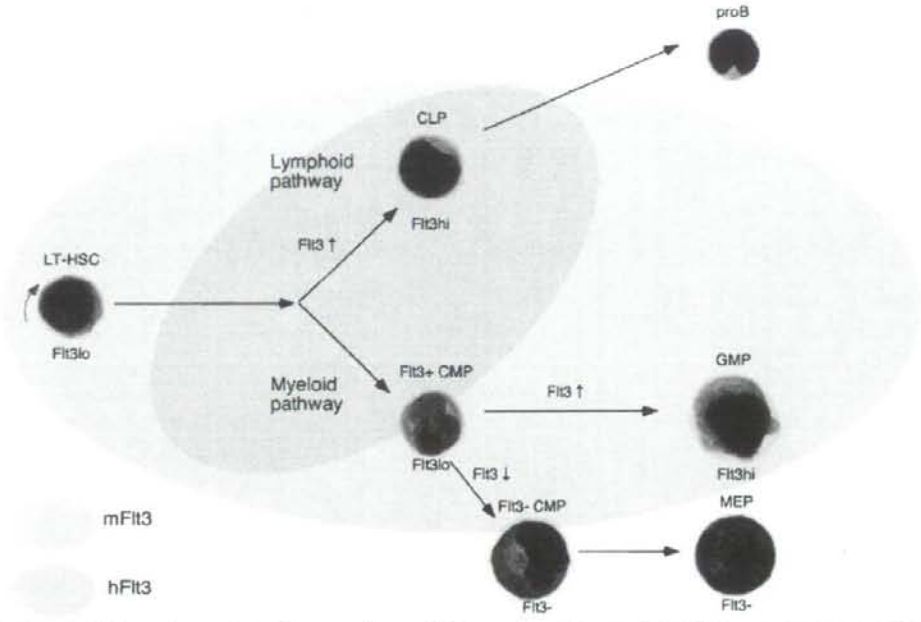

FIGURE 7. Proposed differential expression of human and mouse Flt3 in steady-state hematopoiesis. Cellular morphology of directly sorted each progenitors (May-Giemsa ×1000) is shown here. In human, the most primitive LT-HSC expressed hFlt3 at a low level and its expression is up-regulated at the early GM and the lymphoid progenitor stages, while it is down-regulated in MEPs. In contrast, the mouse LT-HSC lacks mFlt3 expression, and mFlt3 is expressed in cells primed to the lymphoid pathway, including CLPs and a fraction of CMPs.

5*B*). These data strongly suggest that Flt3 signaling does not instruct hematopoietic lineage commitment in hFlt3-expressing myeloid progenitors, but it does promote their survival.

SCF, the ligand for c-Kit, has also been shown to play a critical role in the maintenance of survival in early hematopoiesis. Both c-Kit and Flt3 belong to the class III receptor tyrosine kinase (RTK) family, sharing their major signaling cascade (44). Human HSCs, CMPs, and GMPs expressed both c-Kit and Flt3 at the single cell level (Fig. 1). Thus, we tested the anti-apoptotic effect of SCF in this system. As shown in Fig. 5*C*, in all HSC, CMP, and GMP populations, SCF also displayed anti-apoptotic effects whose impact on cell survival is similar to that of FL. Furthermore, in HSCs and CMPs, the combination of FL and SCF further increased percentages of live cells as compared with those in the presence of either FL or SCF alone, suggesting that SCF and FL signals collaborate to maintain cell survival of HSCs and CMPs.

Flt3 signaling up-regulates Mcl-1, but not Bcl-2 or Bcl-x_L, expression in human hematopoietic stem and progenitor cells

The question: is the mechanism of cell survival enhancement by signaling of RTKs, such as Flt3 and c-Kit? We have shown that in murine hematopoiesis, Mcl-1, a Bcl-2 homologue, is indispensable for hematopoietic stem and progenitor cell survival, and that c-Kit signaling is one of the most critical inducers for Mcl-1 expression in mHSCs (45). We therefore hypothesized that Flt3, as well as c-Kit, signaling may up-regulate Mcl-1 to maintain cell survival in human hematopoiesis as well.

Fig. 6*A* shows the distribution of the transcripts of Bcl-2 family molecules including Mcl-1, Bcl-2, and Bcl-x_L in human stem and progenitor cells. Mcl-1 is expressed at the highest level in HSCs. CMPs and CLPs expressed similar levels of Mcl-1, and MEPs expressed Mcl-1 at the lowest level. This expression pattern of Mcl-1 transcript in human hematopoiesis is consistent with that in murine hematopoiesis (45). In contrast, Bcl-2 was highly expressed in GMPs and CLPs, whereas Bcl-x_L was expressed in MEPs at the highest level.

Purified stem and progenitor populations were incubated with FL and/or SCF in serum-free media. Both FL and SCF dramatically up-regulated the expression of Mcl-1 in a dose-dependent manner, and it reached its peak 30 min after initiation of culture at a concentration of 5 ng/ml (data not shown). Fig. 6*B* shows the relative expression level of Mcl-1, Bcl-2, and Bcl-x_L in the presence of 20 ng/ml FL and/or SCF. We found that both FL and SCF significantly up-regulated the expression of Mcl-1, but not of Bcl-2 or Bcl-x_L, in HSCs, CMPs, and GMPs. These data collectively suggest that one of the important functions of these class III RTKs is to specifically activate Mcl-1 expression. Interestingly, in HSCs, FL and SCF displayed an additive effect on the up-regulation of Mcl-1. Therefore, Flt3 and c-Kit signaling collaborate to protect $Flt3^+$ HSCs and early myeloid progenitors from apoptotic cell death, presumably through activating anti-apoptotic Mcl-1 transcription. In CLPs, however, FL activated not only Mcl-1 but also Bcl-2 transcription.

Discussion

In this study, by using a multicolor FACS and a highly efficient xenograft system, we provide evidence that the distribution of Flt3 RTK is quite different in human and mouse hematopoiesis. First, although mouse LT-HSCs do not express mFlt3, the HSC-enriched $hCD34^+hCD38^-hLin^-$ population, that can reconstitute human hematopoiesis for a long-term in our xenogenic mouse model, uniformly expresses hFlt3 in both BM and CB. It is still unclear whether SCID-repopulating cells directly correspond to hLT-HSCs. However, because the $hCD34^+hCD38^+hLin^-$ cells never

reconstituted in xenogenic hosts for a long-term in our and others' experiments (42), it is highly likely that $hCD34^+hCD38^-hLin^-$ population is highly enriched for hLT-HSCs. Therefore, it is suggested that the negative expression of hFlt3 does not mark LT-HSCs in human, while mFlt3 does in mouse (16, 17). Second, in contrast to mouse hematopoiesis, where mFlt3 expression is restricted within progenitor populations of lymphoid potential including CLPs and a minority of CMPs that can differentiate into B cells (20), hFlt3 is expressed in human CMPs and GMPs, as well as in CLPs. The Flt3 expression is suppressed after cells are committed into the MegE lineage in both human and mouse. The distribution of Flt3 in mouse and human hematopoiesis is schematized in Fig. 7. The significant difference of Flt3 distribution in human and mouse hematopoiesis suggests that the critical role of Flt3 signaling in hematopoietic development could also be different between these species.

We further found that the important function of hFlt3 should include the maintenance of cell survival via the up-regulation of anti-apoptotic Mcl-1 in early hematopoiesis. Previous studies have demonstrated that FL can support in vitro survival of human long-term culture-initiating cells (24, 46, 47). MCL-1 is a non-redundant anti-apoptotic protein, at least in mouse hematopoiesis, because the removal of Mcl-1 from hematopoietic cells in a conditional knockout system caused fatal hematopoietic failure, and because in vitro disruption of *Mcl-1* in mouse HSCs, CMPs, or CLPs rapidly induced their apoptotic cell death (45). The expression level of Mcl-1 was the highest at the HSC stage and gradually declined as HSCs differentiate into myeloid and lymphoid progenitors in mouse hematopoiesis (45). The pattern of Mcl-1 distribution is well preserved in human hematopoiesis (Fig. 6*A*), suggesting that Mcl-1 might also be essential for hHSC survival. In mouse HSCs, Mcl-1 is up-regulated by signals from cytokines including SCF, IL-6, and IL-11, and SCF exerts the most potent effect on the up-regulation of Mcl-1(45). In contrast to mouse LT-HSCs that express c-Kit but not Flt3, functional hLT-HSCs coexpress c-Kit and Flt3 (Fig. 1), and importantly, FL as well as SCF are potent inducers for Mcl-1 transcription (Fig. 6*). The fact that FL and SCF activated only Mcl-1, but not Bcl-2 or Bcl-x_L, in turn suggests that Mcl-1 might be the most critical survival factor controlled by exogenous cytokine signals at the HSC stage. Although it remains unclear whether hFlt3 and/or c-Kit signaling is absolutely required for hHSC survival, our data suggest that, to maintain the Mcl-1 level in hHSCs, the Flt3/FL system could work as an alternative to the SCF/c-Kit system. This is of interest because the SCF/c-Kit system is non-redundant in mouse hematopoiesis (48), where mouse LT-HSCs express only c-Kit, but not Flt3.

The anti-apoptotic effect of hFlt3 signaling was also seen in hFlt3-expressing myeloid progenitor populations. The incubation of CMPs and GMPs with FL significantly prevented their apoptotic cell death in vitro, and FL, as well as SCF, rapidly activated the Mcl-1 transcription in these progenitors. Interestingly, in CLPs, FL activated not only Mcl-1 but also Bcl-2. In lymphopoiesis, Bcl-2 (49, 50), as well as Mcl-1 (51), is critical. FL may collaborate with IL-7 to maintain lymphoid cell survival by up-regulating both Bcl-2 and Mcl-1. Collectively, in humans, Flt3 signaling might support cell survival in early hematopoietic stages with only the exception of the MegE lineage developmental pathway.

Our data also provides an important insight into pathogenesis of AML with *FLT3* mutations. A total of 15–35% of AML patients have either internal tandem duplications (ITDs) in the juxtamembrane domain or mutations in the activating loop of *FLT3* (28, 29), resulting in ligand-independent constitutive signal activation. The *FLT3* mutations are rarely found in acute lymphoblastic leukemia (28, 29). The etiologic link of *FLT3* mutations with AML does not fit the lymphoid-only expression pattern of Flt3 in mouse hematopoiesis. In mouse models, however, the ectopic expression of *FLT3*-ITDs in the bone marrow promotes development of myeloproliferative disorders, but these mutations themselves do not cause leukemia (52). We have found that AML cells with *FLT3*-ITD mutations possess extremely high levels of Mcl-1, and transduction of *FLT3*-ITD into normal HSCs induces rapid up-regulation of Mcl-1 of up to >10-fold higher levels (G. Yoshimoto and K. Akashi, manuscript in preparation). Because the expression of *FLT3* mutations should occur in concert with that of normal Flt3, our data suggest that once *FLT3* mutations are acquired in human hematopoiesis, abnormal survival-promoting signals of Mcl-1 should be expressed in LT-HSCs, and is progressively up-regulated in GMPs. It has been shown that both LT-HSCs and GMPs are the critical cellular target for leukemic transformation. The reinforced survival of CMPs/GMPs by blocking two independent apoptotic pathways (53), or the enforced expression of bcr-abl together with survival-promoting Bcl-2 at the GMP stage (54), results in AML development in mouse models. In human bcr-abl-positive chronic myelogeneous leukemia, GMPs could be the target for blastic transformation by acquisition of β-catenin signaling (55). GMPs can also be converted into leukemic stem cells simply by transducing leukemia fusion genes, such as MLL-ENL (56) or MOZ-TIF2 (57). Thus, these data collectively suggest that the acquisition of *FLT3* mutations in human hematopoiesis might induce the reinforced survival of cells at the HSC and myeloid progenitor stages, where *FLT3* mutations might collaborate with other genetic abnormalities to achieve full AML transformation.

In conclusion, our data show that the distribution of Flt3 is quite different in mouse and human hematopoeisis. hFlt3 targets LT-HSCs and myeloid progenitors except for MEPs. Flt3 signaling might support cell survival in early hematopoiesis including the HSC and the myeloid progenitor stages through up-regulation of Mcl-1. This is a striking example that the expression pattern of key molecules could be significantly different between human and mouse. Accordingly, special considerations are required in using mouse models to understand the role of Flt3 and *FLT3* mutations in human hematopoiesis.

Disclosures

The authors have no financial conflict of interest.

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ORIGINAL ARTICLE

CD34+CD38+CD19+ as well as CD34+CD38−CD19+ cells are leukemia-initiating cells with self-renewal capacity in human B-precursor ALL

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The presence of rare malignant stem cells supplying a hierarchy of malignant cells has recently been reported. In human acute myelogenous leukemia (AML), the leukemia stem cells (LSCs) have been phenotypically restricted within the CD34+CD38− fraction. To understand the origin of malignant cells in primary human B-precursor acute lymphocytic leukemia (B-ALL), we established a novel *in vivo* xenotransplantation model. Purified CD34+CD38+CD19+, CD34+CD38−CD19+ and CD34+CD38−CD19− bone marrow (BM) or peripheral blood (PB) cells from three pediatric B-ALL patients were intravenously injected into sublethally irradiated newborn NOD/SCID/IL2rγ^{null} mice. We found that both CD34+CD38+CD19+ and CD34+CD38−CD19+ cells initiate B-ALL in primary recipients, whereas the recipients of CD34+CD38−CD10−CD19− cells showed normal human hematopoietic repopulation. The extent of leukemic infiltration into the spleen, liver and kidney was similar between the recipients transplanted with CD34+CD38+CD19+ cells and those transplanted with CD34+CD38−CD19+ cells. In each of the three cases studied, transplantation of CD34+CD38+CD19+ cells resulted in the development of B-ALL in secondary recipients, demonstrating self-renewal capacity. The identification of CD34+CD38+CD19+ self-renewing B-ALL cells proposes a hierarchy of leukemia-initiating cells (LICs) distinct from that of AML. Recapitulation of patient B-ALL in NOD/SCID/IL2rγ^{null} recipients provides a powerful tool for directly studying leukemogenesis and for developing therapeutic strategies.

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Introduction

Acute lymphocytic leukemia (ALL) is the most common hematological malignancy in childhood. On the basis of ontogenic classification, pediatric ALL is divided into T-ALL, B-precursor ALL and mature B-ALL. B-precursor ALL accounts for 80–85% of total pediatric ALL cases.[1,2] Recent reports suggest that at least some cases of human leukemia and cancer, including acute myelogenous leukemia (AML), selectively develop from a rare fraction of malignant stem cells.[3–5] Unlike AML, however, whether the malignant clone arises from such a leukemic stem cell fraction has not been clarified in B-precursor ALL.

To identify human leukemia stem cells (LSCs), the *in vivo* leukemia-initiating capacity of purified cells has been evaluated in various xenotransplantation systems using immunocompromized mice. These leukemia-initiating cells (LICs) have been considered equivalent to LSCs, although not all studies have demonstrated other properties of stem cells, that is, differentiation and self-renewal capacities. In AML, the cell surface phenotype defined by the markers CD34 and CD38, that is, CD34+CD38− analogous to normal hematopoietic stem cells (HSCs), have been used to identify LIC-enriched cell population.[6,7] Although the engraftment of B-ALL CD34+ cells in NOD/SCID mice has been reported,[8–10] markers for further enrichment of B-precursor ALL-initiating cells have not been identified. CD38 is expressed by a variety of normal and malignant leukocytes and functions in cell adhesion and signaling. In normal hematopoiesis and in AML, its absence on CD34+ cells highly enriches a primitive self-renewing stem cell population.[6,7] Similarly, Cobaleda *et al.*[10] have reported that in Ph+ ALL, CD34+CD38− cells exclusively initiate leukemia in NOD/SCID recipients. In this study, we aimed to clarify the significance of CD38 expression in B-precursor ALL-initiating cells.

For this purpose, we used the newborn NOD/SCID/IL2rγ^{null} xenotransplantation model. This model takes advantage of the absence of acquired immunity accompanied by multiple defects in innate immunity in a novel NOD/SCID strain carrying a complete null mutation in the cytokine receptor common γ chain.[11] The use of this severely immunocompromised strain overcomes the limitations of existing SCID-repopulating assays using CB17-*scid*, NOD/SCID and NOD/SCID/β2m^{null} mice in engraftment levels of human normal and primary leukemic cells, and differentiation from normal or leukemic stem cells into progeny.[12,13] Especially, when human cells are intravenously injected into newborn recipients, differentiation and self-renewal capacities are efficiently detected both in normal and malignant hematopoiesis, making this model an ideal system for creating mouse models of primary human hematological malignancies.[12,13]

Using the newborn NOD/SCID/IL2rγ^{null} xenotransplantation model, we demonstrate that CD34+CD38+CD19+ cells as well as CD34+CD38−CD19+ cells have the capacities to initiate B-ALL, to infiltrate into non-hematopoietic organs *in vivo* and to self-renew. Leukemia initiation by self-renewing CD34+CD38+CD19+ primary human ALL cells demonstrates a distinct pathogenesis of B-precursor ALL from that of AML, which may provide new insight into the development of

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novel strategies for the treatment of pediatric B-precursor ALL. Furthermore, the use of anti-human CD19 antibody may discriminate normal HSCs and LSCs within CD34+CD38− stem fraction, enabling autologous BM transplantation without LIC contamination in patients with B-precursor ALL.

Materials and methods

Mice

NOD.Cg-*Prkdc*^{scid}*IL2rg*^{tm1Wjl}/Sz (NOD/SCID/IL2rγ^{null}) mice[11] were developed at The Jackson Laboratory (Bar Harbor, ME, USA). The NOD/SCID/IL2rγ^{null} strain was established by backcrossing a complete null mutation of the γ chain locus onto the NOD.Cg-*Prkdc*^{scid} strain. These mice have been bred and maintained under defined flora with irradiated food at the animal facility at RIKEN Research Center for Allergy and Immunology (RCAI). All experimental procedures were performed according to the guidelines established by the Institutional Animal Committee at RCAI.

Cell purification and xenogeneic transplantation

Bone marrow (BM; Cases 1 and 3) or peripheral blood (PB; Case 2) samples were obtained from three pediatric patients with newly diagnosed B-precursor ALL after written informed consent. The patient ages at the time of diagnosis were 6 years, 3 months and 10 months old, respectively, for Cases 1, 2 and 3. The white blood cell count at the time of diagnosis were 260.0, 134.0 and 196.0 ($\times 10^9$ ml^{-1}), respectively, for Cases 1, 2 and 3. MLL rearrangement was identified in Cases 2 and 3. BM and PB mononuclear cells (MNCs) were isolated by density centrifugation using lymphocyte separation medium (ICN Biomedicals, Oh, USA). BMMNCs or PBMNCs were stained with mouse anti-human CD10, CD19, CD3, CD4, CD8, CD34 and CD38 monoclonal antibodies (BD Immunocytometry, San Jose, CA, USA). Samples were analyzed and sorted using FACSAria (Becton Dickinson, San Jose, CA, USA). Nonviable cells were excluded by 7-aminoactinomycin D (BD Immunocytometry) staining. Within the viable CD3−CD4−CD8− BMMNCs or PBMNCs, CD34+CD38−CD19+, CD34+CD38+CD19+ and CD34+CD38−CD10−CD19− populations were sorted and injected into sublethally irradiated (1.5 Gy) NOD/SCID/IL2rγ^{null} mice through the facial vein within 48 h of birth. The purity of each cell population was higher than 97%. As control, normal human HSCs (Lin−CD34+CD38− cells) were purifed from cord blood MNCs and intravenously transplanted into NOD/SCID/IL2rγ^{null} newborns. Cord blood was obtained from Tokyo Cord Blood Bank after written consent was obtained from donors, and experimental plans were evaluated at IRB.

Evaluation of hematopoietic chimerism by flow cytometry

Starting 4 weeks after transplantation, PB was harvested from retro-orbital plexus of the recipients every 2–4 weeks. Recipients were killed when they became moribund and their BM, spleen and PB were evaluated for the repopulation of human normal or leukemic cells with mouse anti-human CD3, CD4, CD8, CD10, CD19, CD20, CD33, CD34, CD38, CD41a, CD45, HLA-DR and surface IgM monoclonal antibodies (BD Immunocytometry). Multicolor flow cytometric analyses were performed using FACSAria or FACSCanto II (Becton Dickinson). Engraftment of human B-ALL was defined by the frequency of the hCD45+hCD19+ cells.

Histological analysis

The liver, kidney and spleen tissues of the recipients were fixed with 4% paraformaldehyde for 1 h, dehydrated with 70% ethanol, embedded in paraffin and 5 μm sections were prepared. Hematoxylin-eosin staining was performed on each tissue section derived from the recipient mice. Immunostaining with mouse anti-human CD19 primary antibody (AbD SeroTec, Oxford, UK) and Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) was performed after dehydration with graded alcohol and antigen retrieval with heated citrate buffer. Each section was examined using light microscopy (Zeiss Axiovert 200, Carl Zeiss, Germany) and laser scanning confocal microscopy (Leica TCS, Leica, Germany) to identify the infiltrating B-ALL cells.

Serial transplantation

For serial transplantation, either sorted human CD45+CD34+CD38+CD19+ cells or magnetic bead-enriched human CD34+ cells (Miltenyi Biotec, Germany) were obtained from the recipient BM and spleen, and 10^4–10^6 purified cells were intravenously transplanted into newborn NOD/SCID/IL2rγ^{null} mice. Human engraftment was evaluated in the serially transplanted recipients at 4–12 weeks post-transplantation.

Statistical analysis

Continuous variables were expressed as mean ± s.d. The data were analyzed by SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Phenotypic characterization of primary human B-ALL cells

It is essential to understand leukemogenesis of B-precursor ALL, the most common hematological malignancy in children. While AML-initiating cells and Ph+ ALL-initiating cells are highly enriched within the CD34+CD38− population, the significance of CD38 expression in B-precursor ALL-initiating cells has not been clarified. To address this question, we first examined the phenotypic characteristics of B-precursor ALL cells in three patient samples. We analyzed the frequency of each fraction expressing CD34, CD38, CD10 and CD19 antigens by multicolor flow cytometry (Figure 1). As expected, considerable heterogeneity in the expression of cell surface antigens was observed in the three patient samples examined. Within the CD3−CD4−CD8− BM or PB MNC populations, consistent with the diagnosis of B-precursor ALL, CD34+ cells accounted for 90.5 ± 3.6%. CD34+CD38− and CD34+CD38+ cells accounted for 5.2 ± 6.0 and 85.3 ± 7.7%, respectively. In contrast with normal BM and cord blood samples, CD19+ cells accounted for 66.9 ± 44.4 and 93.1 ± 11.1% of CD34+CD38− and CD34+CD38+ populations, respectively. The CD34+CD38−CD10−CD19− subfraction was present in low frequency (0.18 ± 0.17%) in all three samples tested. The complete phenotype is shown in Figure 1.

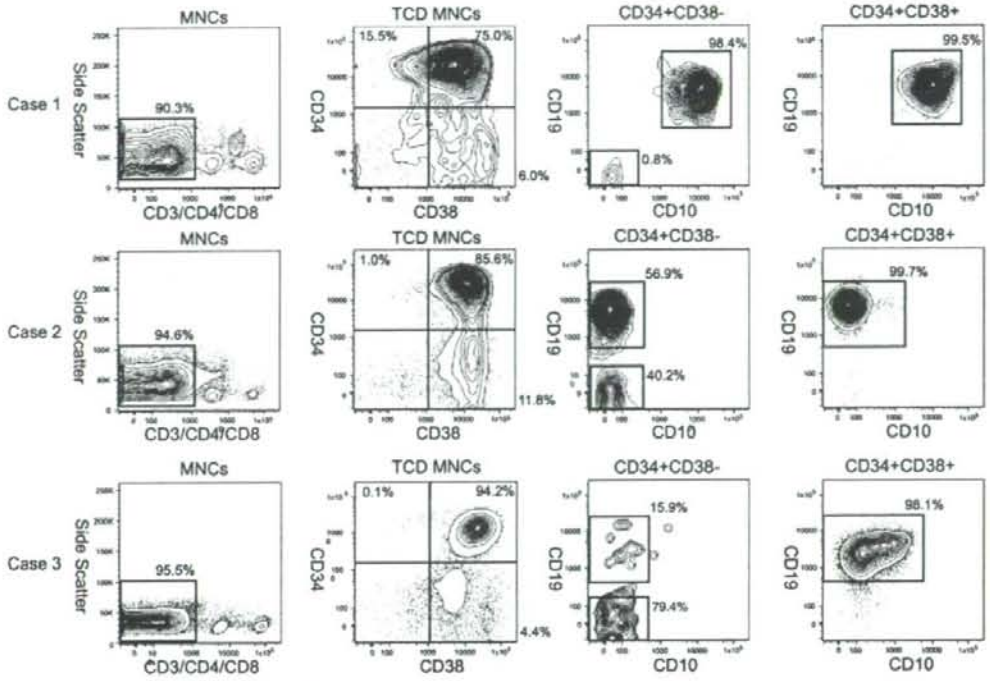

Figure 1 Phenotypic analysis of primary B-precursor ALL cells. BM (Cases 1 and 3) and PB (Case 2) from three cases of B-precursor ALL were analyzed for the expressions of CD34 and CD38 within T-cell-depleted MNCs. Within CD34+CD38− and CD34+CD38+ populations, the expression patterns of CD10 and CD19 were analyzed. Three subfractions (CD34+CD38−CD19+, CD34+CD38+CD19+ and CD34+CD38−CD10−CD19− cells) from each sample were transplanted into newborn NOD/SCID/IL2rγ^{null} mice. ALL, acute lymphocytic leukemia; BM, bone marrow; MNCs, mononuclear cells; PB, peripheral blood; TCD, T-cell-depleted.

Both CD34+CD38+CD19+ and CD34+CD38−CD19+ primary human B-ALL cells have long-term engraftment and leukemia-initiating capacity

On the basis of phenotypic characterization above, we simultaneously purified CD34+CD38+CD19+, CD34+CD38−CD19+, CD34+CD38−CD10−CD19− cells from B-precursor ALL patient BM (Cases 1 and 3) and PB (Case 2), and intravenously transplanted these purified populations into sublethally irradiated NOD/SCID/IL2rγ^{null} newborns. The information on each recipient is summarized in Table 1. The engraftment of human B-ALL cells was monitored by flow cytometric analysis of hCD45+CD19+ cells in the recipient PB. In contrast with previous reports on AML[7] and Ph+ ALL,[10,14] the injection of either CD34+CD38−CD19+ or CD34+CD38+CD19+ cells resulted in efficient engraftment of human B-ALL (Table 1). The engraftment of B-ALL in the PB of recipient mice following injection of CD34+CD38+CD19+ cells was seen for long term, with human B-ALL cells being observed to increase over time for up to 15 weeks post-transplantation. Purified CD34+CD38−CD10−CD19− cells did not initiate B-ALL in the recipients, but showed engraftment of normal human hematopoietic cells.

When the recipient mice exhibited ruffled fur and lethargy, we killed them to analyze the engraftment levels of human B-ALL in the BM and the spleen. As observed in the PB, BM and spleen showed efficient engraftment of ALL both in the recipients transplanted with CD34+CD38+CD19+ cells and those transplanted with CD34+CD38−CD19+ cells (Table 1 and Figure 2). The significance of CD38 expression on leukemia-initiating capacity is totally different between adult AML and pediatric B-precursor ALL.

Transplanted primary human B-ALL cells infiltrate into recipient organs

As we demonstrated leukemia-initiating capacity both in CD34+CD38−CD19+ and CD34+CD38+CD19+ cells, we next performed histological analyses of the recipient organs to examine the infiltration of B-ALL cells in the liver, kidney and spleen. In the liver and the kidney, infiltration of monomorphic MNCs was detected with hematoxylin-eosin staining in the recipients transplanted either with CD34+CD38+CD19+ or CD34+CD38−CD19+ cells derived from all three cases (Figure 3). When compared with the organs from normal human HSC recipient, there are sheets and clusters of monomorphic MNCs in the organs of the B-ALL recipients. The infiltrating cells exhibited cellular morphology similar to that of cells engrafted in the spleen. These cells also expressed CD19 on their surface, suggesting that they are human B-ALL cells. No infiltrating CD19+ cells were detected in the liver and kidney of the normal human HSC recipient. The degree of infiltration in the recipients transplanted with CD34+CD38−CD19+ cells and those transplanted with CD34+CD38+CD19+ cells was not significantly different.

Table 1 Serial transplantation of primary human B-ALL CD34+CD38+CD19+ and CD34+CD38-CD19+ cells

Patient ID	Transplant round	Graft cell type	Graft cell dose	Survival post-transplant (weeks)	% BM hCD45+CD19+	% spleen hCD45+CD19+	% PB hCD45+CD19+
(a)							
1	Primary	34+38+19+	6.5E+04	13	63.7	27.8	ND
		34+38+19+	6.5E+04	13	74.8	33.4	ND
		34+38+19+	1.0E+05	15	93.5	46.9	78.7
	Secondary	34+38+19+	8.0E+04	12	97.8	65.3	92.0
		34+38+19+	3.0E+04	11	ND	ND	95.0
		34+	2.0E+05	8	ND	ND	89.9
		34+	2.0E+05	9	95.7	90.2	42.8
2	Primary	34+38+19+	5.0E+03	6	99.9	ND	69.4
		34+38+19+	5.0E+04	5	ND	ND	73.8
		34+38+19+	5.0E+04	5	99.9	99.4	82.3
		34+38+19+	8.0E+04	5	54.9	10.8	10.7
	Secondary	34+38+19+	3.0E+04	7	86.3	87.4	60.3
		34+	1.0E+04	5	99.6	99.5	92.2
		34+	1.0E+05	4	98.4	89.1	ND
		34+	4.0E+06	4	90.3	83.3	ND
	Tertiary	34+	1.0E+05	5	ND	ND	73.4
		34+	1.0E+05	4	94.9	73.2	75.3
		34+	1.0E+06	4	93.9	72.2	94.8
		34+	1.0E+06	4	96.1	87.4	94.0
3	Primary	34+38+19+	2.5E+04	10	98.1	87.9	72.3
		34+38+19+	2.5E+05	7	98.8	52.7	61.2
		34+38+19+	5.0E+05	11	99.1	77.1	87.7
		34+38+19+	1.0E+04	13	97.9	44.4	23.3
		34+38−19+	6.5E+04	10	90.0	ND	70.0
	Secondary	34+38+19+	1.0E+05	6	91.2	ND	ND
		34+38+19+	1.0E+06	4	ND	94.7	ND
		34+	1.0E+05	9	72.8	ND	71.5
	Tertiary	34+	1.0E+05	9	ND	ND	98.2
		34+	1.0E+05	9	ND	ND	90.3
		34+	1.0E+06	7	99.7	96.1	91.5
		34+	1.0E+06	8	98.7	99.9	56.9
(b)							
1	Primary	34+38−19+	6.5E+04	12	52.4	37.2	ND
		34+38−19+	1.0E+05	13	90.3	42.2	76.0
	Secondary	34+	2.8E+05	4	97.6	99.3	ND
		34+	2.8E+05	4	95.2	99.9	ND
		34+	2.8E+05	4	98.8	ND	ND
		34+	2.8E+06	4	98.2	99.3	ND
		34+	2.8E+06	4	98.5	99.6	ND
		34+	2.8E+06	4	99.5	99.1	ND
2	Primary	34+38−19+	4.0E+04	5	6.0	1.8	1.1
		34+38−19+	4.0E+04	5	7.9	4.8	1.1
		34+38−19+	5.0E+03	7	98.7	77.6	85.6
		34+38−19+	5.0E+03	7	ND	ND	85.7
	Secondary	34+	2.0E+05	4	13.8	ND	ND
		34+	2.0E+05	4	7.6	36.0	ND
		34+	2.0E+05	4	11.0	41.0	ND
		34+	2.0E+05	8	87.8	49.2	47.0
3	Primary	34+38−19+	6.5E+04	10	90.0	ND	70.0

Abbreviations: ALL, acute lymphocytic leukemia; BM, bone marrow; ND, not determined; PB, peripheral blood.
PB, BM and spleen engraftment levels in primary, secondary and tertiary recipients from: (a) primary recipients of CD34+CD38+CD19+ B-ALL cells and (b) primary recipients of CD34+CD38-CD19+ human B-ALL cells.

Primary human B-ALL CD34+CD38+CD19+ cells possess self-renewal capacity

As the ALL-initiating capacity and the ability to infiltrate recipient organs were confirmed in the CD34+CD38+CD19+ as well as the CD34+CD38−CD19+ population, we examined the self-renewal capacity of CD34+CD38+CD19+ and CD34+CD38−CD19+ populations by serial transplantation. From CD34+CD38+CD19+ recipients, we intravenously transplanted 10^4–10^6 sorted CD45+CD34+CD38+CD19+ cells or enriched CD34+ cells from the primary (all three cases) and secondary (Cases 2 and 3) recipients into secondary and tertiary newborn NOD/SCID/

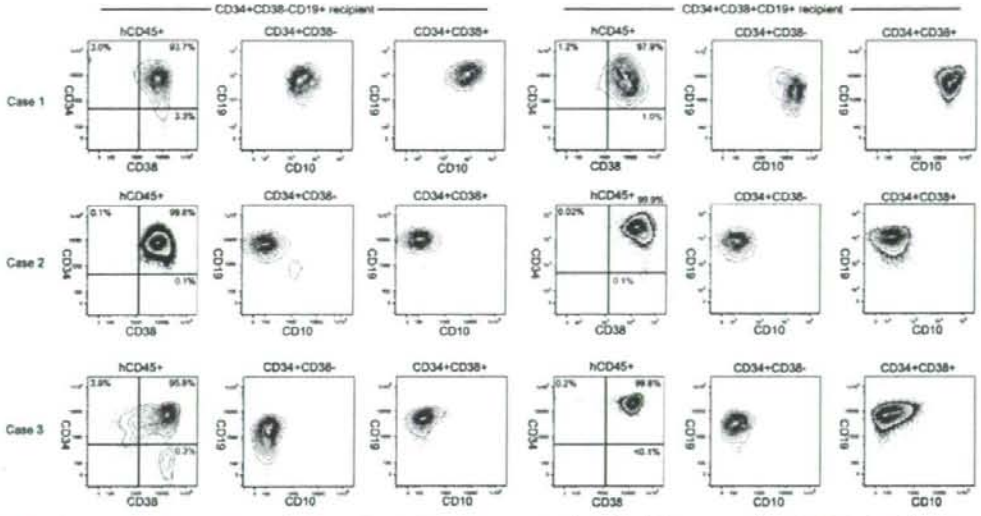

Figure 2 Both CD34 + CD38−CD19 + and CD34 + CD38 + CD19 + B-ALL cells have the ability to reconstitute B-ALL *in vivo*. Flow cytometric analyses of BM from representative recipients of each cell fraction from each case are shown. Left panels: gated on hCD45 + cells; middle panels: gated on hCD34 + CD38− cells; right panels: gated on hCD34 + CD38 + cells. For Case 1, 6.5×10^4 CD34 + CD38 + CD19 + and CD34 + CD38−CD19 + were transplanted. For Case 2, 5.0×10^4 CD34 + CD38 + CD19 + and 4.0×10^4 CD34 + CD38−CD19 + cells were transplanted. For Case 3, 2.5×10^4 CD34 + CD38 + CD19 + and 6.5×10^4 CD34 + CD38−CD19 + cells were transplanted. ALL, acute lymphocytic leukemia; BM, bone marrow.

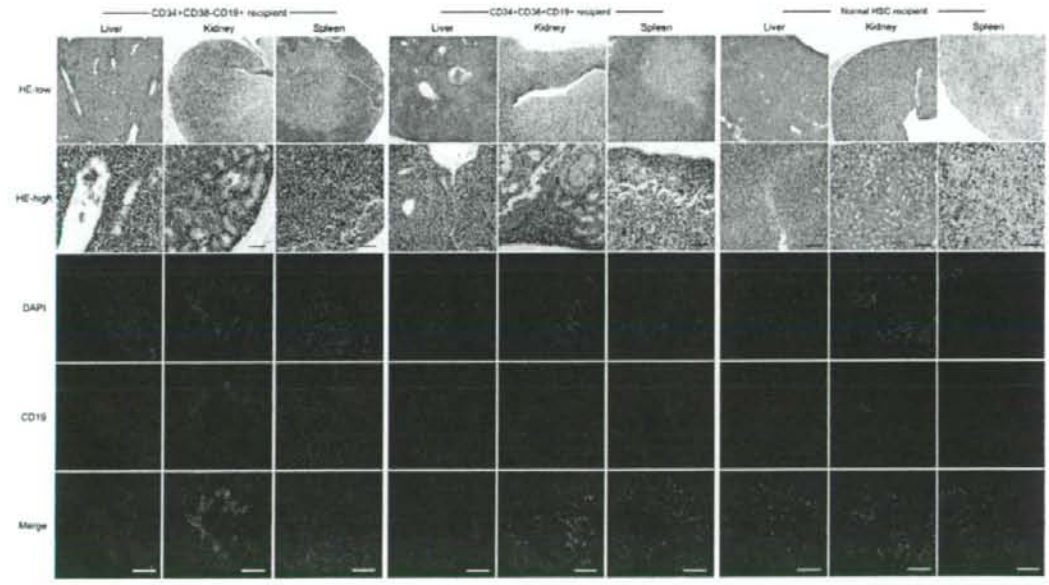

Figure 3 Both CD34 + CD38−CD19 + and CD34 + CD38 + CD19 + B-ALL cells infiltrate into recipient organs. Hematoxylin-eosin staining and anti-human CD19 antibody labeling of the liver, kidney and spleen of a recipient transplanted with CD34 + CD38 + CD19 + cells and a recipient transplanted with CD34 + CD38−CD19 + cells. Similarly stained and labeled liver, kidney and spleen sections from a recipient of normal human cord blood CD34 + CD38− cells are included as controls. Nuclei were stained with DAPI. ALL, acute lymphocytic leukemia; DAPI, 4,6-diamidino-2-phenylindole. Scale bars represent 20 μm in HE staining and 50 μm in CD19 immunostaining.

IL2rγ^{null} recipients. In all the secondary and tertiary recipients, high levels of hCD45 + CD19 + engraftment were seen (Table 1a). Similarly, when 2×10^5–2.8×10^6 enriched CD34 + cells from CD34 + CD38−CD19 + primary recipients (Cases 1 and 2) were transplanted into secondary recipients, high levels of hCD45 + CD19 + engraftment were found (Table 1b). These findings suggest that CD34 + CD38 + CD19 + and CD34 + CD38−CD19 + cells not only initiate leukemia, but also possess self-renewal capacity.

CD34 + CD38−CD10−CD19− B-ALL cells exhibit normal multilineage differentiation capacity

In normal hematopoiesis and in AML, the CD34 + CD38− population is highly enriched for self-renewing stem cells. Transplantation of either CD34 + CD38−CD19 + or CD34 + CD38−CD10−CD19− cells derived from B-ALL BM resulted in the development of hCD19 + cells in the recipient PB. However, 7/7 recipients transplanted with CD34 + CD38−CD19 + cells died of leukemia, whereas 0/3 transplanted with CD34 + CD38−CD10−CD19− cells developed disease. While almost all the engrafted CD19 + cells in the PB of recipients transplanted with CD34 + CD38−CD19 + cells express CD34 on their surface and lack CD20 and surface IgM expression, the engrafted CD19 + cells in the PB of recipients transplanted with CD34 + CD38−CD10−CD19− cells express CD20 and surface IgM, not CD34, on their surface (Figure 4). In addition, human myeloid and platelet development is also detected only in the recipients transplanted with CD34 + CD38−CD10−CD19− cells (Figure 4). CD34 + CD38−CD10−CD19− cells derived from B-ALL BM cells are highly enriched with normal HSCs, suggesting that the expression of CD19 distinguish LICs from normal HSCs within the CD34 + CD38− population.

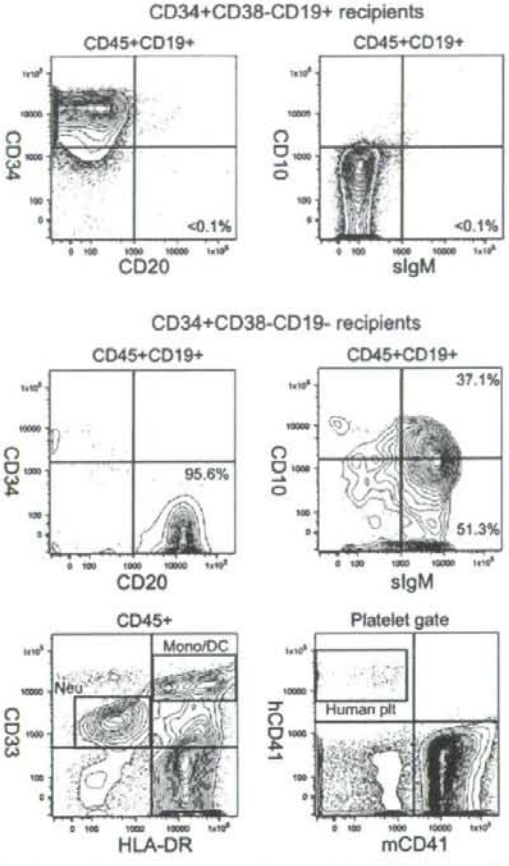

Figure 4 CD34 + CD38−CD10−CD19− B-ALL cells show multilineage normal hematopoietic differentiation *in vivo*. Representative flow cytometric analysis of PB mononuclear cells demonstrating that the majority of human CD45 + CD19 + cells in a representative recipient of CD34 + CD38−CD19 + B-ALL cells express CD34 but lack CD20, CD10 and surface IgM expression. In contrast, mature human B cells, myeloid cells and platelets can be detected in a representative recipient transplanted with 2.0×10^3 CD34 + CD38−CD10−CD19− cells at 4 months post-transplantation. ALL, acute lymphocytic leukemia; Mono/DC, monocytes and dendritic cells; Neu, neutrophils; PB, peripheral blood; plt, platelets.

Discussion

We have recently established the newborn NOD/SCID/IL2rγ^{null} mouse transplantation model that supports significantly higher engraftment levels of human normal HSCs and primary AML stem cells compared with the NOD/SCID/β2m^{null} mice.[12,13] In this study, we describe the efficient engraftment of primary human pediatric B-precursor ALL using the newborn NOD/SCID/IL2rγ^{null} mouse transplantation model. This is the first report that purified CD34 + CD38 + CD19 + ALL cells efficiently engraft, initiate leukemia, and self-renew *in vivo*. Although only three individual patient samples were analyzed, the consistent engraftment in primary and secondary recipients by CD34 + CD38 + CD19 + cells provides new insights into the leukemogenesis of ALL.

Previous studies have described the engraftment of purified primary B-ALL cells in adult NOD/SCID recipients. Cobaleda *et al*.[10] demonstrated that CD34 + CD38− Ph + ALL cells, but not CD34 + CD38 + cells, engrafted and initiated leukemia in adult NOD/SCID recipients. In contrast, in pediatric B-precursor ALL, we found that the CD34 + CD38 + CD19 + ALL cells, as well as CD34 + CD38−CD19 + cells, are able to efficiently engraft, infiltrate into non-hematopoietic organs and self-renew *in vivo*. B-precursor ALL and Ph + ALL have distinct biological and clinical characteristics, as the former is the most common leukemia in the pediatric population and the latter is more common in adults. The finding that the hierarchical structure of B-precursor ALL, as defined by the surface phenotype of LSCs, is distinct from that of Ph + ALL may reflect the biological differences between these two types of lymphoid malignancy. In particular, CD34 + CD38 + CD19 + cells predominantly reproduced themselves, rather than giving rise to heterogenous cell fraction such as CD34 + CD38−CD19 + or CD34− ALL cells. As FACS discrimination gate border between CD38− and CD38 + cells within CD34 + fraction is not clearly delineated, it is not possible to totally exclude the possibility of contamination even with high levels of purity achieved with cell sorting. Additionally, limiting dilution and serial transplantation studies are required to fully define the differences between these CD34 + CD38−CD19 + and CD34 + CD38 + CD19 + populations in B-precursor ALL. In addition, whether there are differences in the LIC phenotype and the function between these two subtypes of B-ALL remains to be determined. LICs in

other subtypes of ALL (for example, Ph+ ALL and mature B-ALL) and various subtypes of B-precursor ALL, based on genetic abnormalities, need to be examined in the NOD/SCID/IL2rγ^{null} newborn transplantation model to address this question.

In this study, we have successfully demonstrated that in human primary B-ALL, CD38 expression is irrelevant in defining a leukemogenic population, but rather the presence or absence of CD19 segregates the populations with malignant or normal repopulating capacity within the CD34+CD38− cell population. The role of CD19 as a marker to identify the clonogenic B-ALL precursor cells has been examined in the past. In t(4;11)-positive and t(9;22)-positive high-risk pediatric ALL, the leukemia-specific translocations have been identified in CD34+CD19− as well as CD34+CD19+ ALL cells.[13] The reconstitution of the myeloid lineage occurring from the purified CD19−, but not CD19+, cells has been reported in TEL–AML1 fusion-positive and Ph+ ALL.[15] Similarly, only CD34+CD19− and CD34+CD10− cells were found to engraft when sorted populations from t(9;22) and t(4;11)-negative B-precursor ALL were transplanted into NOD/SCID recipients.[8] Here, we report the reconstitution of mature human B cell and human platelets in addition to myeloid cells from CD34+CD38−CD10−CD19− primary human B-ALL cells. The discrepancy in the engraftment and proliferation of normal myeloid as well as lymphoid lineages in these studies, compared with that in our findings, may be due to the superior sensitivity of engraftment and capacity for normal human hematopoietic development in the newborn NOD/SCID/IL2rγ^{null} transplantation system. Although a previous publication has raised a concern for the possibility of LSC contamination in autologous stem cell graft in ALL,[16] our finding suggests that by CD34+CD38−CD10−CD19− purification, the potential contamination of autologous stem cell graft by B-ALL LSCs may be avoidable.

The finding that it is possible for both CD34+CD38+CD19+ and CD34+CD38−CD19+ cells to act as the LICs in B-precursor ALL may be an important pathophysiological difference between AML and ALL. Delineation of the molecular basis of these differences between CD34+CD38− and CD34+CD38+ B-ALL-initiating cells may allow us to develop targeted therapy specific for primitive LICs that elude current antileukemia treatment strategies.

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