after TLR9 stimulation, and cytotoxicity was suppressed by NKG2D Ab treatment. These results were consistent with activated NK cells reported by Blasius et al. (39). However, CD11c$^+$B220$^+$CD122$^+$ cells were morphologically distinct from NK cells, and NK cells did not acquire B220 expression after adoptive transfer. Recently, Guimont-Desrochers et al. (34) obtained similar results demonstrating that CD11c$^+$B220$^+$CD122$^+$ cells are distinct from activated NK cells. Thus, controversy remains in describing the relationship between CD11c$^+$B220$^+$CD122$^+$ cells and activated NK cells, and further studies are needed to define the differences between these cells.

The role of NK cells in xenograft rejection in NOD-*scid* mice has been described previously. Kollet et al. (15) reported an 11-fold higher rate of xenoengraftment after transplantation of human HSCs for NOD-*scid* $\beta2m^{null}$ mice that lacked NK activity compared with that for NOD-*scid* mice. Additionally, McKenzie et al. (17) showed enhancement of xenoengraftment after HSC transplantation when the NK cells were eliminated by treatment with a CD122 Ab. In those studies, the observed higher xenoengraftment level cannot be attributed solely to NK cells because CD11c$^+$B220$^+$CD122$^+$ cells could have been present in their eliminated fraction. Our current results indicate that CD11c$^+$B220$^+$CD122$^+$ cells are more effective than NK cells at inducing xenograft rejection, and we conclude that CD11c$^+$B220$^+$CD122$^+$ cells, but not NK cells, are the main effector cells for xenograft rejection. CD11c$^+$B220$^+$CD122$^+$ cells produce higher levels of IFN-γ than NK cells upon IL-15 stimulation (35, 36). Chaudhry et al. (40) reported that CD11c$^+$B220$^+$NK1.1$^+$ cells (called NKDCs), which are the same as CD11c$^+$B220$^+$CD122$^+$ cells, and NK cells were absent in IL-15$^{-/-}$ mice, and they observed that CD11c$^+$B220$^+$NK1.1$^+$ cells were restored more rapidly than NK cells by exogenous IL-15 treatment. Their in vitro analysis showed that CD11c$^+$B220$^+$NK1.1$^+$ cells stimulated with IL-15 retained cytotoxic capacity and potent IFN-γ secretion. Moreover, tumor metastasis in the lung caused by transplanted B16F10 melanoma cells was inhibited by syngeneic CD11c$^+$B220$^+$NK1.1$^+$ cell transplantation. In contrast, CD11c$^+$B220$^+$NK1.1$^+$ cells from IFN-γ−deficient mice did not cause this type of inhibition. Furthermore, Lin et al. (41) transplanted pig cells into T cell-depleted, IFN-γ–deficient mice and found that the engraftment of pig cells was significantly enhanced in the IFN-γ–deficient mice compared with the control T cell-depleted, wild-type mice. Those findings support our current results that CD11c$^+$B220$^+$CD122$^+$ cells from IFN-γ–deficient mice do not suppress xenoengraftment. Thus, xenograft rejection by CD11c$^+$B220$^+$CD122$^+$ cells may depend on the amount of IFN-γ, and IFN-γ deficiency may contribute to the high acceptance rate of xenografts in NOG mice. However, we have no evidence that the IFN-γ produced by CD11c$^+$B220$^+$CD122$^+$ cells modulates the rejection of xenografts. We speculate that IFN-γ can promote xenograft rejection through at least two scenarios. In the first scenario, CD11c$^+$B220$^+$CD122$^+$ cells self-activate in an autocrine manner through secretion of IFN-γ and enhance cytotoxicity mediated by perforin/granzyme, FasL, and the TRAIL pathway against targeted xenografts that lack species-specific MHC class I molecules, which interact with killer inhibitory receptors (42). In the second scenario, IFN-γ produced by CD11c$^+$B220$^+$CD122$^+$ cells induces the activation of macrophages, and these macrophages are subsequently recruited to the graft site through the upregulation of monocyte-attracting chemokines, resulting in a direct attack on the xenograft. The relative importance of IFN-γ–dependent xenograft rejection by CD11c$^+$B220$^+$CD122$^+$ cells remains to be elucidated.

In conclusion, we demonstrated that the high-level acceptance of xenografts in NOG mice is due to a lack of CD11c$^+$B220$^+$CD122$^+$ cells, and we suggest that IFN-γ produced by CD11c$^+$B220$^+$CD122$^+$ cells plays an important role in xenograft rejection. These data are useful for clarifying the immunological mechanisms leading to rejection of xenotransplants. Further studies are needed to confirm the exact pathway involved in CD11c$^+$B220$^+$CD122$^+$ cell-dependent mechanisms of xenograft rejection.

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Disclosures

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Supplementary figure legends

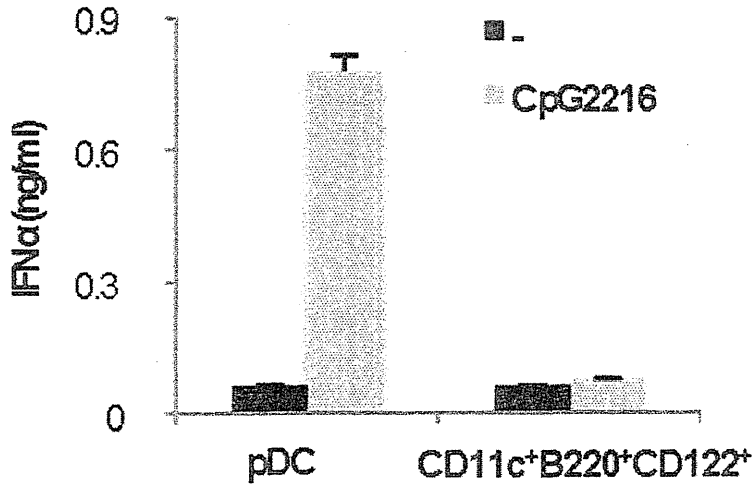
Figure S1. $CD11c^{+}B220^{+}CD122^{+}$ cells do not produce IFNα.

$CD11c^{+}B220^{+}CD122^{+}$ cells or $CD11c^{+}B220^{+}CD122^{-}$ pDCs were isolated from the $CD11c^{+}B220^{+}$ fraction or $CD11c^{-}B220^{-}$ fraction of NOD-*scid* mouse spleen cells using the MoFlo cell sorter. These fractions were cultured in RPMI 1640 medium (Invitrogen) containing 10% FCS in 96-well flat-bottomed plates at 37°C in 5% CO_2. To induce IFNα, cells were stimulated with 1μM CpG ODN 2216 (Sigma-Aldrich) for 48 h. Culture supernatants were collected and IFNα was assayed using the Verikine Mouse IFNα ELISA Kit (PBL Interferon Sources, Piscataway NJ).

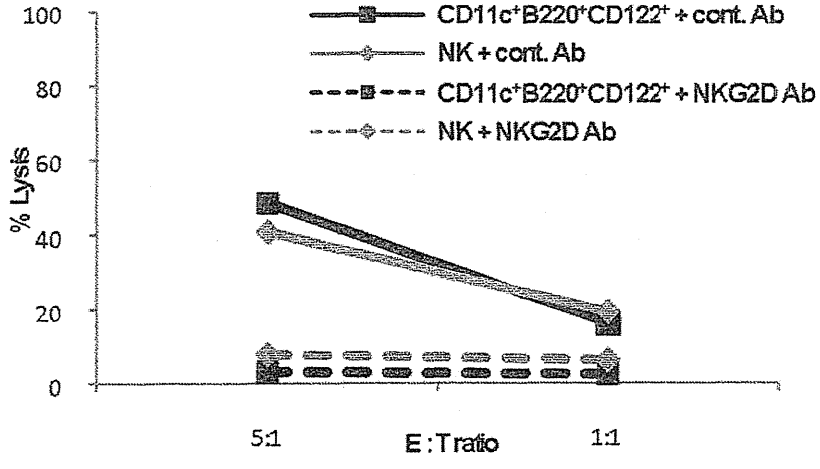
Figure S2. Cytotoxicity is reduced *via* inhibition of an NKG2D receptor on $CD11c^{+}B220^{+}CD122^{+}$ and NK cells.

Non-RI cytotoxic assay was performed using the Cellular DNA Fragmentation ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacture's instructions. Sorted $CD11c^{+}B220^{+}CD122^{+}$ or NK cells from the spleens of NOD-*scid* mice were stained with anti-mouse NKG2D and isotype control antibody (Biolegend), respectively, and co-cultured with BrdU-labeled Yac-1 cells at various ratios for 4 h. After co-culturing, the supernatant was removed and amount of BrdU-labeled fragmented DNA was measured by ELISA. The percentage of lysis was calculated as described in *Cytotoxicity measurements* in the Materials and Methods.

Supplementary Figure S1



Supplementary Figure S2



Development of a Multi-Step Leukemogenesis Model of MLL-Rearranged Leukemia Using Humanized Mice

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Abstract

Mixed-lineage-leukemia (MLL) fusion oncogenes are intimately involved in acute leukemia and secondary therapy-related acute leukemia. To understand MLL-rearranged leukemia, several murine models for this disease have been established. However, the mouse leukemia derived from mouse hematopoietic stem cells (HSCs) may not be fully comparable with human leukemia. Here we developed a humanized mouse model for human leukemia by transplanting human cord blood-derived HSCs transduced with an MLL-AF10 oncogene into a supra-immunodeficient mouse strain, NOD/Shi-*scid*, IL-2Rγ$^{-/-}$ (NOG) mice. Injection of the MLL-AF10-transduced HSCs into the liver of NOG mice enhanced multilineage hematopoiesis, but did not induce leukemia. Because active mutations in ras genes are often found in MLL-related leukemia, we next transduced the gene for a constitutively active form of K-ras along with the MLL-AF10 oncogene. Eight weeks after transplantation, all the recipient mice had developed acute monoblastic leukemia (the M5 phenotype in French-American-British classification). We thus successfully established a human MLL-rearranged leukemia that was derived *in vivo* from human HSCs. In addition, since the enforced expression of the mutant K-ras alone was insufficient to induce leukemia, the present model may also be a useful experimental platform for the multi-step leukemogenesis model of human leukemia.

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Introduction

Chromosomal translocations that result in MLL (mixed-lineage leukemia)-fusion oncogenes are associated with acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome, and secondary therapy-related acute leukemia [1]. More than 60 partner genes that form MLL-fusion oncogenes have been identified [2], and the partner usually characterizes the specific pathological phenotype of the MLL-rearranged leukemia. For example, MLL-rearranged AMLs, more than 60% of which involve the MLL-AF9, MLL-AF10, or MLL-AF6 fusion gene, often exhibit the morphological subtypes acute myelomonoblastic leukemia and monoblastic leukemia, which are classified as French-American-British (FAB) M4 and M5, respectively [3]. Although patients with MLL-rearranged leukemia usually show a poor prognosis and are sometimes resistant to therapy [3,4], the development of efficient therapies targeting the MLL-fusion genes has been slow, in part because much is still unknown about the cellular and molecular mechanisms underlying this disease.

To gain insight into the etiology and pathogenesis of MLL-rearranged leukemia, several mouse models have been developed. Mice with a knocked-in MLL-AF9 fusion gene develop AML as they age [5], although in humans the congenital MLL-AF9 rearrange typically affects infants. Leukemia has also been generated in mice by exogenously expressing an MLL-fusion gene in the animals or by transplanting mouse hematopoietic stem cells (HSCs) that have been retrovirally transduced with an MLL-fusion gene [6]. However, the phenotype and pathogenesis of the leukemia produced in these murine models sometimes do not match those observed in human leukemia associated with the same genetic lesion. For example, the MLL-ENL fusion gene is frequently associated with B-precursor ALL in humans, but generates AML in mice [7]. Thus, there is a gap in our understanding about mouse versus human leukemogenesis, and further leukemia modeling studies using human primary leukemia cells are needed [8].

In vivo studies in which primary human leukemia cells were transplanted into immunodeficient mice, provided significant advances in our understanding of the pathogenesis of human leukemia [9,10]. However, these models, in which the leukemia cells had already developed in human patients before being transplanted into mice, are not suitable for studying physiological leukemogenesis, including the initiation and progression processes since these processes do not occur in the model. Therefore, a new experimental model for analyzing leukemogenesis in which

primary human HSCs are converted into leukemia cells by genetic hits is needed.

In the past two decades, a number of studies have tried to graft human HSCs into immunodeficient mice, but these attempts to reconstitute human blood cells in mice have failed [11]. However, the recent development of a new immunodeficient mouse strain has opened up the possibility for generating better xenotransplantation systems. NOD/SCID mice with a targeted mutation of the IL-2-receptor γ chain gene (NOD/Shi-*scid* IL-2Rγ$^{-/-}$ (NOG) and NOD/LtSz-scid IL-2Rγ$^{-/-}$ (NSG) mice) lack T, B, and NK cells and some innate immunity functions [11,12]. When human HSCs are transplanted into these mice, human T, B, NK, and dendritic cells develop normally and are maintained in the mice for at least 10 months[13 17]. Thus, these immunodeficient mice may be good recipients for examining, not only the normal development of human HSCs, but also their abnormal development, including leukemogenesis. Two previous studies modeled the initiation and progression of human acute leukemia by ectopically expressing MLL-fusion genes in human HSCs, and transplanting these cells into special immunodeficient mice [18,19]. Dick et al demonstrated that ectopic expression of AF9 or MLL-ENL in human HSCs caused leukemia in NOD/SCID mice in which the NK cells were depleted by an anti-NK cell antibody [18]. Similarly, Mulloy et al successfully modeled acute leukemia by transducing MLL-AF9 into human HSCs, and transplanting these cells into NOD/SCID mice that expressed transgenic human SCF, GM-CSF, and IL-3 genes [19]. However, in a similar model using NSG mice, the enforced expression of MLL-AF4 in HSCs did not induce leukemia [20].

Despite intensive studies on MLL-ENL, MLL-AF4, and MLL-AF9, there has been no report investigating the *in vivo* oncogenic potential of MLL-AF10 in human HSCs, even though MLL-AF10 is the second most common MLL rearrangement (13%) in pediatric MLL-rearranged AML [3]. Here, using NOG mice, we demonstrated that MLL-AF10 could enhance the multilineage hematopoiesis of human HSCs, even though the MLL-AF10 in mouse HSCs preferentially enhances myeloid differentiation [21,22]. More importantly, the co-transfection of MLL-AF10 with an active form of the K-ras gene induced acute monoblastic leukemia with the FAB M5 phenotype. These results provide a novel leukemia model using human cells showing the requirement of two genetic hits for leukemogenesis.

Methods

All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University, which was granted by Tohoku University Ethics Review Board (No. 2010MA165) and the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication 85-23, revised 1996).

CD34$^+$ Hematopoietic Stem Cell Isolation

Cord blood from full-term human deliveries was obtained from the Miyagi Cord Blood Bank (Miyagi, Japan) and RIKEN Bioresource Center Cell Bank (Tsukuba, Japan), following the institutional guidelines approved by the Tohoku University Committee on Clinical Investigations. Mononuclear cells were isolated from the cord blood by density gradient centrifugation using Lymphocyte Separation Medium (MP Biomedicals, Solon, OH, USA) after removing the phagocytes with silica (Immuno Biological Laboratories, Takasaki, Japan). The cells were washed and suspended in PBS containing 2%FCS. CD34$^+$ stem cells were obtained by magnetic cell sorting (MACS) (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, CD34$^+$ cells were labeled with a biotin-conjugated anti-human CD34 monoclonal antibody (Serotec, Oxford, UK) after blocking the Fc receptor. The cells were then incubated with anti-biotin Microbeads (Miltenyi Biotech). The magnetically labeled CD34$^+$ cells were purified twice on LS columns (Miltenyi Biotech). The purity of the CD34$^+$ fraction was >95%. The purified CD34$^+$ cells were suspended in Cell Banker (Juji Field, Tokyo, Japan) and cryopreserved at −80°C in a deep freezer until use.

Plasmid Construction

The retroviral vector, pDANsam-IRES-EGFP, which is based on Murine Stem Cell Virus with enhanced green fluorescent protein (EGFP) as a marker under an internal ribosomal entry site (IRES), was kindly provided by Dr. Masafumi Onodera (Tsukuba University) [23]. PLAT-F, a package cell line that produces a pseudotype virus with an RD114 envelope, was previously established and provided by Dr. Toshio Kitamura (Institute of Medical Science, University of Tokyo) [24]. The human MLL-AF10 cDNA [22], which was kindly provided by Dr. M.L. Cleary (Stanford University), was inserted into a cloning site in the pDANsam-IRES-EGFP vector to make the pDANsam-MLL-AF10 plasmid. To construct the pDANsamIRES VENUS vector, the EGFP cDNA of the retroviral vector was replaced with Venus cDNA [25], which was provided by Dr. Hiroyuki Miyoshi (RIKEN, Tsukuba). The human flag-tagged K-ras^{G12V} cDNA, which was previously described [26], was inserted into the Xho I site in the cloning site of pDANsamIRES Venus to make pDANsam-K-ras^{G12V}.

Gene Delivery into CD34$^+$ Cells by Retrovirus

The CD34$^+$ cells isolated from cord blood were cultured in X-VIVO15 (Cambrex Bioscience, Walkersville, MD, USA), supplemented with 1% human serum albumin (HSA) (Kaketsuken, Kumamoto, Japan) and stimulated with a cytokine cocktail [100 ng/ml stem cell factor, 100 ng/ml Flt-3 ligand, 100 ng/ml thrombopoietin, and 100 ng/ml IL-6 (Peprotech)] in a 24-well plate (2×10^5 per well) for 48 h. The stimulated CD34$^+$ cells were then harvested and placed into non-tissue culture-treated 6-well plates (Becton Dickinson) that had been coated with 20 μg/ml CH-296, a recombinant fibronectin fragment (Retronectin, Takara, Japan). (3×10^5 cells per well) in the presence of the respective virus supernatant. The virus supernatants were diluted 1:2 with X-VIVO15 containing 1% HSA and the cytokine cocktail described above. Every 12 h, the medium was replaced with fresh virus supernatant. After 48 h of culture, the frequency of GFP- and/or Venus-expressing CD34$^+$ cells was examined by FACS.

Cell Transplantation into Mice

NOD/Shi-*scid*, IL-2Rγ$^{-/-}$ (NOG) mice were obtained from the Central Institute for Experimental Animals (CIEA, Kawasaki, Japan) and maintained in an animal facility at Tohoku University Graduate School of Medicine under specific pathogen free conditions. All procedures were performed according to the protocols approved by Tohoku University Ethics Review Board (No. 2010MA165). Neonatal (1 3 days) NOG mice were irradiated with 30 cGy of X-rays, and the cultured CD34$^+$ cells ($1.0-1.5\times10^5$) in 70 μl PBS were intrahepatically injected into the mice later the same day. From one cord blood sample, donor cells could be prepared for transplantation into 3 recipient mice. Therefore, 2 to 4 different cord blood samples were used for each mouse group. Since even NOG mouse sometimes failed to engraft human HSCs, 9 recipient mice that had no human CD45$^+$

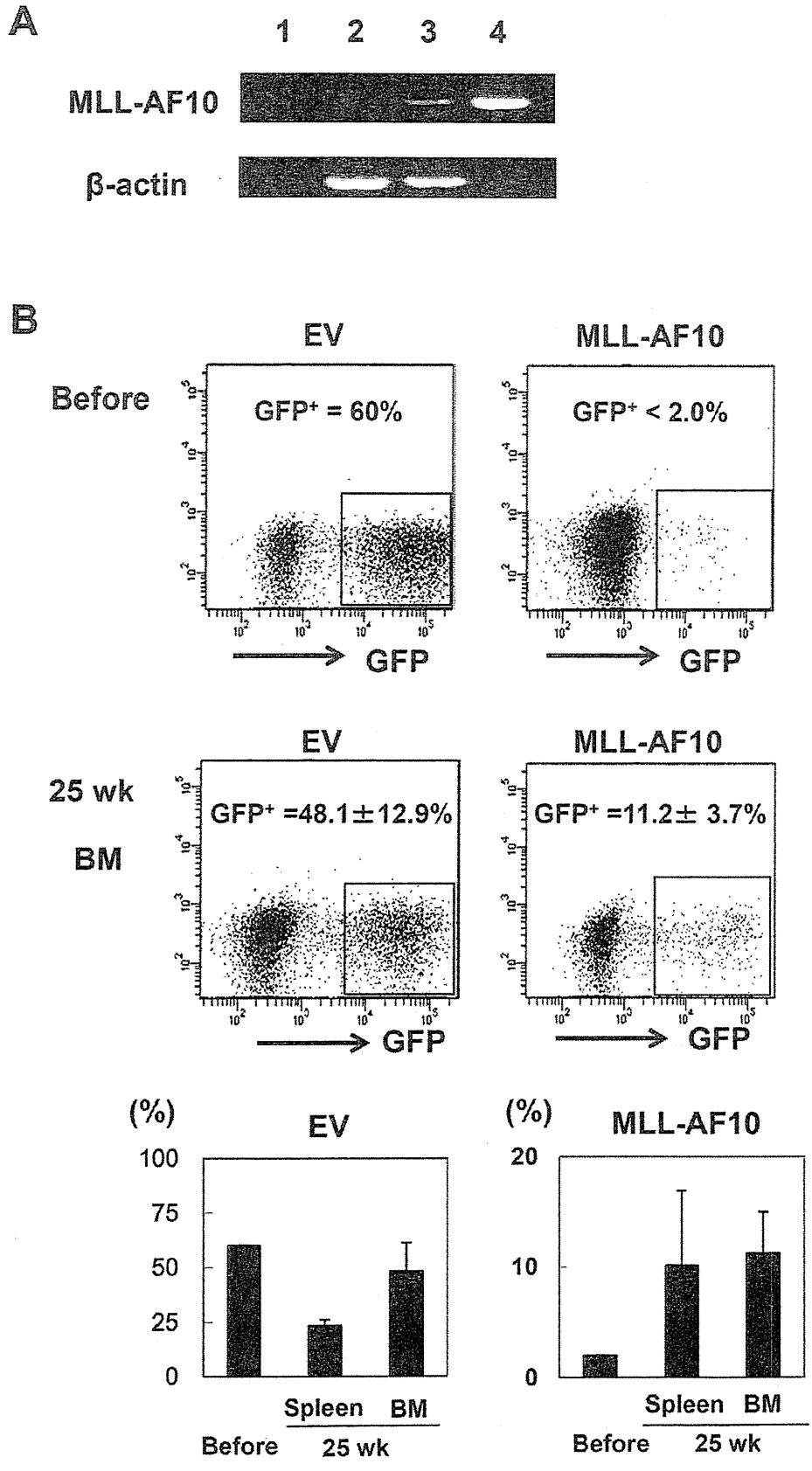

A
1
2
3
4
MLL-AF10
β-actin
B
EV
MLL-AF10
Before
GFP+ = 60%
GFP+ < 2.0%
GFP
GFP
EV
MLL-AF10
25 wk
BM
GFP+ =48.1± 12.9%
GFP+ =11.2± 3.7%
GFP
GFP
(%)
EV
100
75
50
25
0
Spleen
BM
Before
25 wk
(%)
MLL-AF10
20
10
0
Spleen
BM
Before
25 wk

Figure 1. Enforced expression of MLL-AF10 augmented multilineage hematopoiesis, but was insufficient to induce leukemogenesis in vivo. (A) Representative RT-PCR results confirming the long-term expression of the MLL-AF10 transcript in the BM cells of mice 25 weeks after transplantation (lane 1; water, lane 2; cells from a mouse in the EV-transfused group, lane 3; cells from a mouse in the MLL-AF10-transfused group, and lane 4; positive control (MLL-AF10 plasmid)). (B) Flowcytometric analysis of the frequency of GFP^+ cells. The indicated vector (EV, left or MLL-AF10, right)-transduced human $CD34^+$ cells, whose *in vitro* GFP expression is shown in the upper panels (Before) of the flowcytometric analysis, were transplanted into NOG mice. Twenty-five weeks later, the GFP-expressing cells gated on human $CD45^+$ hematopoietic cells in the BM was measured (lower panels of the FACS profiles). The data shown are representative of 3 independent experiments. The graphs show the frequency of GFP^+ cells in human $CD34^+$ cells just before transplantation (Before) and the mean ± SD of the frequency of GFP^+ cells in the BM and spleen of mice receiving transplants of EV-transduced HSCs (n = 8) or of MLL-AF10-transduced HSCs (n = 6) 25 weeks after transplantation, in one representative experiment of three. Similar results were obtained in the 3 independent experiments.
doi:10.1371/journal.pone.0037892.g001

hematopoietic cell in the BM and spleen at the sacrifice were excluded from this study.

Antibodies and Flow Cytometric Analysis

Surface markers were detected with the following fluorescent human specific antibodies from BD Biosciences, San Jose, CA, USA: CD33-PE, HLA-DR-PE, CD14-PE, CD19-APC, CD11b-APC, CD15-APC, and CD45-APC-Cy7. To monitor the reconstitution of human blood in NOG mice, peripheral blood was periodically taken from the tail vein. To examine the spleen, bone marrow (BM), and liver, single-cell suspensions were prepared from the spleen and liver by mincing with metal mesh and from the BM by flushing the tibiae and femurs with PBS containing 2% FCS, using a 27-gauge needle. The cells were stained with the relevant antibodies for 15 min on ice, then washed with cold PBS containing 2% FCS, and stained with the appropriate secondary antibodies when necessary. After the final wash, the cells were subjected to flowcytometric analysis with a FACSCanto II cytometer (BD Biosciences). To distinguish between the EGFP (510 nm) and Venus (535 nm) fluorescences, an apporopiate combination of mirrors and optical filters was used. The proportion of each lineage was calculated using FACS Diva software (BD Biosciences).

Reverse Transcription-PCR (RT-PCR)

Total RNA was prepared from cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The concentration of total RNA was measured by a NanoDrop 1000 (Nanodrop Technologies Inc., Wilmington, DE, USA), and first-strand cDNA was synthesized using Superscript II (Invitrogen) with an oligo (dT) primer. The cDNA for MLL-AF10, Flag-K-ras, or β-actin was amplified with specific primers using Ex-Taq polymerase (Takara). The primer sets were as follows; MLL-AF10: forward, 5-ccaaaagaaaaggaaaaacca and reverse, 5-gacttcagcattcctaaaatgtca; Flag-K-ras: forward, 5-gactacaaagacgatgacg and reverse, 5-ccctgtcttgtctttgc; β-actin: forward, 5-gctcgtcgtcgacaacggctc and reverse, 5-caaacatgatctgggtcatcttctc.

DNA Extraction and Southern Blot Analysis

Genomic DNA was extracted from spleens using the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, USA). For Southern blot analyses, 10 μg genomic DNA was digested with BglII (Toyobo, Osaka, Japan) overnight at 37°C and separated by electrophoresis on a 0.7% agarose gel for 12 h at 40 V. The DNA was transferred overnight to a Hybond N^+ membrane (GE Healthcare, Chalfont St. Giles, UK). The DNA was fixed onto the membrane with a UV Stratalinker 1800 (Stratagene). The membrane was probed with 10^6 cpm/ml of [C^{32}P]dCTP (Perkin Elmer)-labeled EGFP cDNA. This EGFP probe was derived from a 700-bp ScaI fragment of the pDANsam-IRES-EGFP vector, and radiolabeled using a random prime labeling kit (Takara). The membrane was then washed and exposed to a BAS-III imaging plate (Fuji Photo Film Co., LTD, Japan), and the image was produced and analyzed using a BAS-III software (Fuji Photo Film Co., LTD).

Histopathology

The BM, spleen, and liver were examined macroscopically, and the spleen was weighed. Tissues were fixed in 10% formalin for at least 48 hours. Hematoxylin and eosin staining or May-Giemsa staining was performed on 3-μm sections using a routine protocol. For CD45 immunostaining, after dewaxing and antigen retrieval by microwave, the sections were incubated for 1 h with mouse anti-human CD45 (Dako Japan, Tokyo). Immunodetection was performed using HRP as the visualization enzyme and DAB as the substrate chromogen. All slides were examined by light microscopy using an Olympus IX50 microscope (Olympus, Tokyo, Japan) and a SPOT camera.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using either a paired or unpaired Student's t-test. Statistical significance was defined as a P-value <0.01 (**).

Results

Enforced Expression of MLL-AF10 Augmented Multilineage Hematopoiesis, but was Insufficient for Leukemogenesis in vivo

To examine the pathogenesis of the MLL-AF10 fusion gene during the differentiation of human blood cells from HSCs, MLL-AF10-infected cord blood $CD34^+$ cells were transplanted intrahepatically into sublethally irradiated neonatal NOG mice. None of the recipient mice showed any sign of disease, such as body weight loss or the appearance of abnormal peripheral leukocytes, 25 weeks after transplantation (data not shown). The expression of the transduced MLL-AF10 gene in the reconstituted human blood cells was confirmed (Figure 1A), indicating that the enforced expression of MLL-AF10 in human HSCs could not induce any hematological disorders including leukemia in this model. However, the MLL-AF10 appeared to augment hematopoiesis. As shown in Figure 1B, when empty control vector (EV) was transdused into human $CD34^+$ HSCs, the frequency of the GFP^+ population did not change significantly (60% before transplantation and 24% and 48% after transplantation in the spleen and BM, respectively) (Figure 1B). In contrast, when MLL-AF10 was exogenously expressed, the GFP^+ population markedly increased from 2% (before) to 11% (25 weeks after transplantation) (Figure 1B). Therefore, the MLL-AF10-positive blood cells may have a growth advantage *in vivo* compared to the MLL-AF10-negative normal blood cells ($CD45^+GFP^-$ cells).

We next characterized the lineage composition of the human $CD45^+GFP^+$ cells in the BM and spleen of both groups. The GFP^+ MLL-AF10-expressing cells did not show a skewed lineage differentiation compared to the EV-transfected hematopoietic

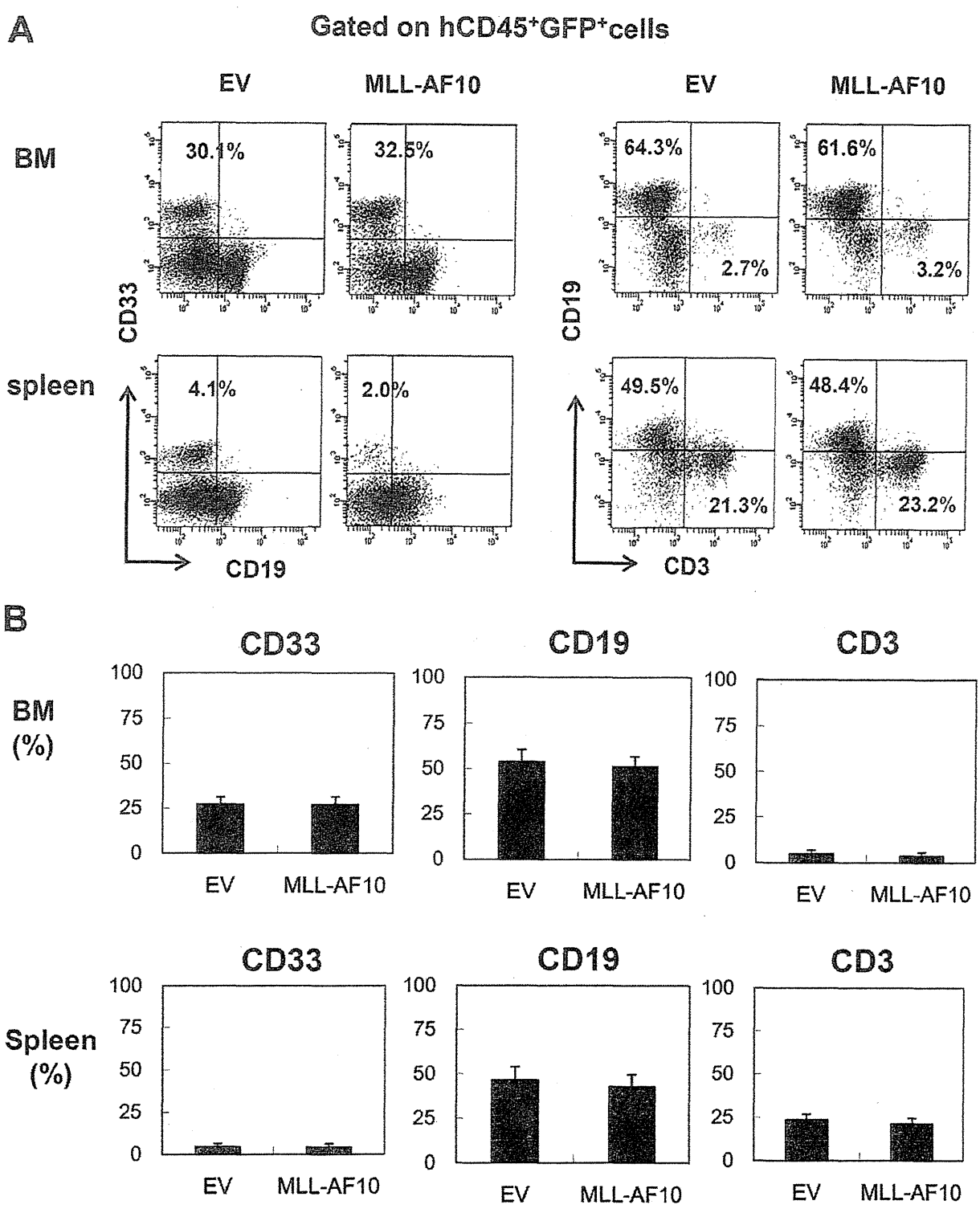
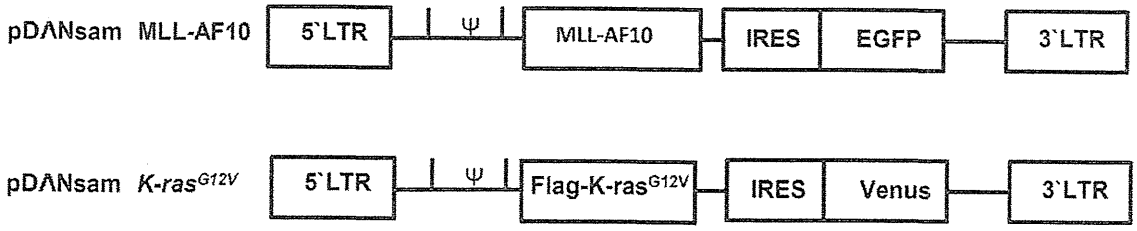
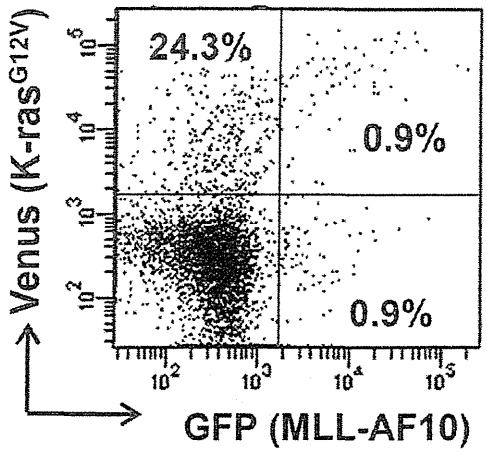


Figure 2. Flowcytometric analysis confirming multilineage engraftment. (A) Representative flowcytometric results of EV- or MLL-AF10-transduced human hematopoietic cells. The human CD45$^+$ GFP$^+$ cells were analyzed for their lineage distributions to B cells (CD19$^+$), T cells (CD3$^+$), and myeloid cells (CD33$^+$). (B) Multilineage differentiation of MLL-AF10-transduced cells. The data shows cells gated on the CD45$^+$GFP$^+$ cell population. The graph represents the mean ± SD of the frequencies of CD33$^+$ myeloid cells, CD19$^+$ B cells, and CD3$^+$ T cells in the BM (upper) and spleens (lower) of mice engrafted with EV-transduced (n = 8) or MLL-AF10-transduced (n = 6) CD34$^+$ HSCs. No difference in the graft composition between the EV- and MLL-AF10-expressing CD34$^+$ HSCs was found. Similar results were obtained in 3 independent experiments.
doi:10.1371/journal.pone.0037892.g002

A



B



Summary of transplantation

	Efficiency	Cell No. (x10^3)
GFP+Venus+	0.8 - 0.9 %	1.0
GFP+Venus-	0.8 - 0.9 %	1.0
GFP-Venus+	22 - 24 %	31-33
GFP-Venus-	74- 76 %	97-115
Total	100%	130 -150

Figure 3. Co-transduction of activated K-ras and MLL-AF10 into CD34$^+$HSCs. (A) Schematic structure of the MLL-AF10-GFP and Flag-K-ras^{G12V}-Venus vectors. (B) Infectious efficiency of the MLL-AF10-GFP and Flag-K-ras^{G12V}-Venus co-transfection. The data and the summary shown in the flowcytometric analysis is representative of the transduced CD34$^+$ HSCs in 2 experiments.
doi:10.1371/journal.pone.0037892.g003

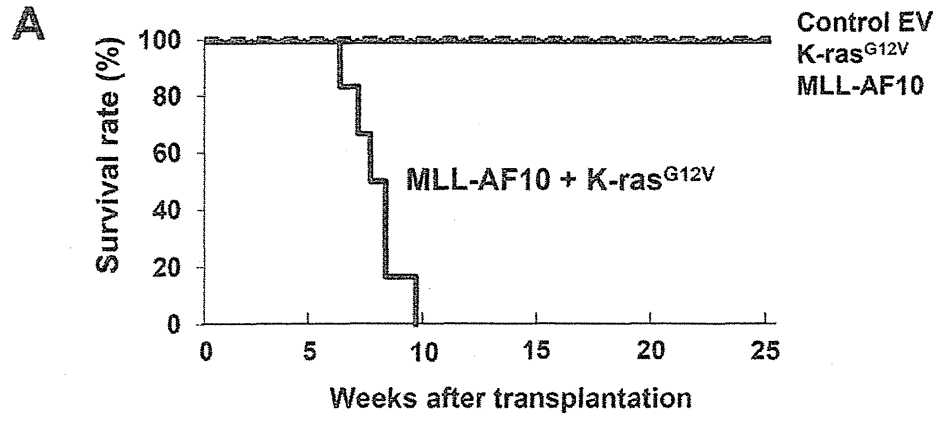
cells (Figures 2A and 2B). In contrast to the previous reports with mouse HSCs, which showed skewed myeloid differentiation by the exogenous expression of MLL-AF10 [21,22], the enforced expression of MLL-AF10 in human HSCs enhanced the hematopoietic repopulation of HSCs without affecting cell differentiation.

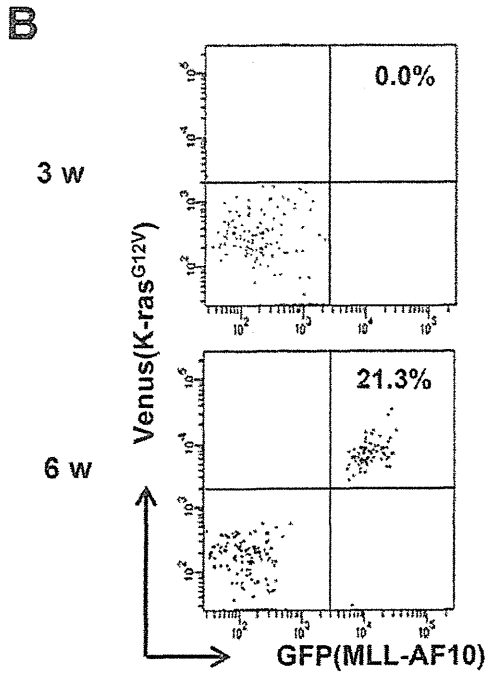
Co-transduction of Activated K-ras and MLL-AF10 into CD34$^+$ HSCs

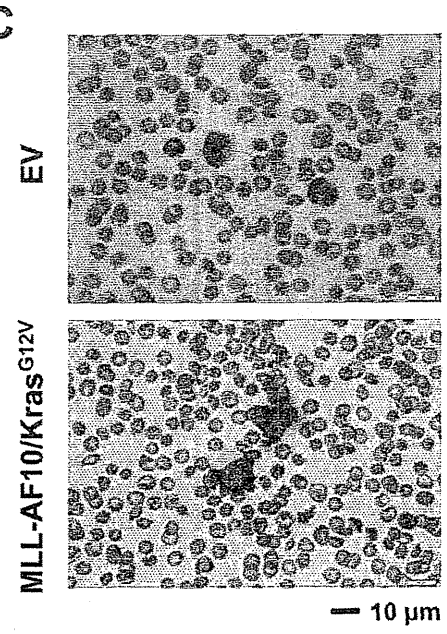
As described above, transduction of the MLL-AF10 gene alone was not sufficient to induce leukemogenesis from human HSCs in the present model. Similarly, Menendez et al recently demonstrated that the MLL-AF4 gene alone cannot induce leukemia from human CD34$^+$ HSCs in a humanized mouse model [20]. These observations prompted us to generate a two-hit model of leukemogenesis using MLL-AF10 and one additional oncogene. We thus used the K-ras^{G12V} oncogene because K-ras^{G12V} is a very well-characterized oncogene, and because ras mutations are found in about 30% of the cases of pediatric MLL-rearranged AML [3]. To evaluate two hits in one cell, we needed to distinguish the MLL-AF10 and K-ras^{G12V} expressions. We thus retrovirally transfected CD34$^+$ HSCs with MLL-AF10-EGFP and K-ras^{G12V} that was co-expressed with the Venus fluorescent protein, which can be distinguished from EGFP by flowcytometry (Figure 3A). The MLL-AF10 and K-ras co-infected (GFP$^+$Venus$^+$) cells were quite rare (0.9%) (Figure 3B). Nevertheless, we injected the CD34$^+$ HSCs, which contained 74% non-transduced, 0.9% MLL-AF10-alone-transduced, 24% K-ras^{G12V}-alone-transduced, and 0.9% co-transduced cells, intrahepatically into NOG mice (Figure 3B).

Cooperation of MLL-AF10 with Activated K-ras Induced Acute Monoblastic Leukemia

By 8 weeks after transplantation, several mice in the MLL-AF10/K-ras^{G12V} co-transduced group showed a rough coat, slow movement, and weight loss, while no mice in the other groups (EV, MLL-AF10 alone, and K-ras^{G12V} alone) demonstrated any disease manifestations (data not shown). As the mice in the co-transduced group got sick, most of their peripheral leukocytes became double positive for GFP and Venus (Figure 4B). In addition, morphologically identifiable leukemia cells, characterized by abnormal nuclei and cytoplasmic vacuoles, were found in the peripheral blood (Figure 4C). The morphology of the leukemia cells was compatible with that of the M5 type of FAB classification, which is relatively common in MLL-rearranged AML. Furthermore, splenomegaly in the mice engrafted with the MLL-AF10/K-ras^{G12V} co-transduced human HSCs was observed 8 weeks after


A
Survival rate (%)
100
80
60
40
20
0
0
5
10
15
20
25
Weeks after transplantation
MLL-AF10 + K-ras^{G12V}
Control EV
K-ras^{G12V}
MLL-AF10


B
3 w
6 w
0.0%
21.3%
Venus(K-ras^{G12V})
GFP(MLL-AF10)


C
EV
MLL-AF10/Kras^{G12V}
10 μm

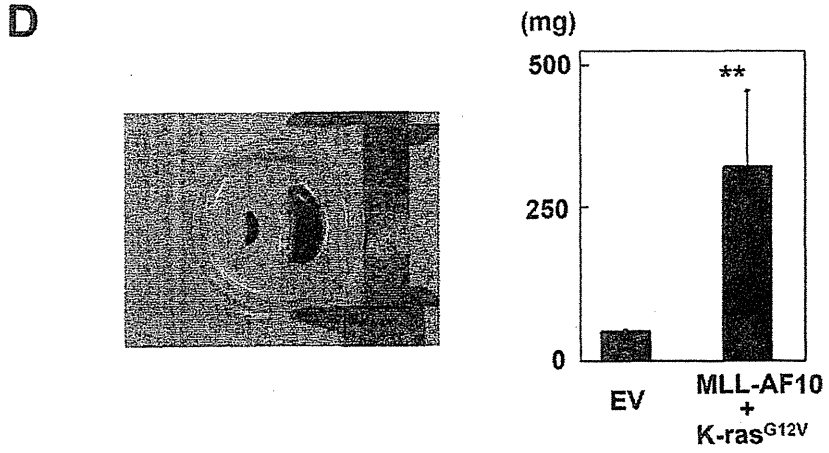

D
(mg)
500
250
0
**
EV
MLL-AF10
+
K-ras^{G12V}

Figure 4. Cooperation of MLL-AF10 with activated K-ras induced acute monoblastic leukemia. (A) Kaplan-Meier survival analysis of mice receiving transplants of human HSCs transfected with EV (n = 8), K-ras ^{G12V} (n = 12), MLL-AF10 (n = 6), or MLL-AF10 plus K-ras^{G12V} (n = 6) vectors. (B) GFP and Venus expression in peripheral blood cells at the indicated weeks after transplantation with human HSCs co-transfected with the MLL-AF10 and K-ras^{G12V} genes. (C) May-Giemsa staining of the peripheral blood of mice engrafted with human HSCs co-transfected with the MLL-AF10 and K-ras^{G12V} genes. Morphologic leukemia cells were found in the peripheral blood of these mice 50 days after transplantation. (D) Splenomegaly in the MLL-AF10/K-ras^{G12V} mice. Spleens from mice engrafted with EV-transduced HSCs (left) and MLL-AF10/K-ras^{G12V} co-transduced HSCs (right) are shown. The graph shows the mean ± SD of the spleen weights from mice receiving transplants of EV-transduced HSCs (n = 6) or of MLL-AF10/K-ras^{G12V} co-transduced HSCs (n = 6). ** represents $p<0.01$.
doi:10.1371/journal.pone.0037892.g004

transplantation (Figure 4D), and the average weight of their spleens was 5 times greater than in the other groups (Figure 4D). By 10 weeks after transplantation, 100% of the mice that had received MLL-AF10/K-ras^{G12V} co-transduced HSCs were dead, while all the mice in the other 3 groups survived and remained healthy 25 weeks after transplantation (Figure 4A).

We next investigated by FACS the composition of the blood cells in the BM, spleen, and liver of the MLL-AF10/K-ras^{G12V}-expressing HSC-treated mice. Most of the hematopoietic cells in these tissues were GFP$^+$ Venus$^+$ double positive (Figure 5A). The double-positive cells were confirmed to express both the MLL-AF10 and K-ras^{G12V} genes by RT-PCR with specific primers for the exogenously transduced genes (Figure 5B). Interestingly, no cells expressing MLL-AF10 alone (GFP$^+$ Venus$^-$) or K-ras^{G12V} alone (GFP$^-$Venus$^+$) were observed in any mice in the co-transduced group, even though MLL-AF10 alone could effectively promote multilineage hematopoiesis (Figures 1 and 2). More importantly, in the group receiving K-ras^{G12V} alone-transduced HSCs, there were no Venus$^+$ K-ras^{G12}-expressing blood cells in any tissues examined, even in the BM (data not shown). These results indicate that the co-expression of MLL-AF10 and K-ras^{G12V} (GFP$^+$Venus$^+$) was necessary for the *in vivo* induction of leukemia from human HSCs.

Additional FACS data showed that the GFP$^+$Venus$^+$ human CD45$^+$ blood cells in the all recipient mice transfused with the co-transfected HSCs had a uniform surface marker profile, CD33$^+$CD11b$^+$HLA-DR$^+$CD14$^+$CD15$^+$ (Figure 5C), which was fully compatible with the FAB M5 phenotype. This finding suggested that the abnormal blood cells observed in the mice receiving co-transduced HSCs were leukemia cells. We next examined the cellular clonality of the abnormal blood cells by Southern blotting. As shown in Figure 5D, an EGFP probe that recognizes both EGFP and Venus (because the nucleotide sequences between EGFP and Venus are 98% identical) revealed one or two bands in the DNA of the abnormal leukocytes from individual mice in the co-transduced group, indicating the monoclonality of the abnormal leukocytes.

Taken together, we have successfully established a human leukemia model in which acute monoblastic leukemia cells are derived from human normal HSCs *in vivo*.

Pathological Phenotypes of the Leukemia

Pathological examination of our human leukemia model showed that the spleens were extensively infiltrated with human hematopoietic cells, and the architecture of the red pulp and the white pulp was disrupted (Figures 6A and 6B). The tibia bones looked very pale (data not shown), and the BM was occupied by uniform blood cells, which expressed human CD45 (Figures 6A and 6B). The periportal regions of the liver were also massively infiltrated with human hematopoietic cells (Figure 6A and 6B). Hepatosplenomegaly is a common symptom in patients with acute monoblastic leukemia with rearranged MLL genes, and leukemia cell infiltration into the intrahepatic periportal regions is also a common pathological manifestation of human MLL-rearranged monoblastic leukemia. Therefore, our model may be a good representation of human MLL-rearranged monoblastic leukemia.

Discussion

A detailed understanding of leukemogenesis requires the development of experimental murine models that can accurately mimic this process. Some studies have sought to recapitulate the harboring of MLL fusion genes in human AML using mouse HSCs [6]. However, the leukemia occurring in mice often does not faithfully recapitulate human leukemia, in part because of the biological differences between human and mouse HSCs [8,27]. In two improved models, the MLL-ENL or MLL-AF9 gene was recently shown to be capable of initiating human leukemogenesis in NK-cell-depleted NOD/SCID mice [18] or human cytokine transgenic NOD/SCID mice [19]. However, overexpression of the MLL-AF4 fusion gene in human CD34$^+$ cells is not sufficient to initiate leukemia [20]. Similar to the latter report, we found that MLL-AF10 was insufficient to induce leukemogenesis at least by 25 weeks after transplantation in the present model. In our experiments, the infectious efficiency of the MLL-AF10 vector in human HSCs was low (<2.0%). Although the efficiency of MLL-ENL and MLL-AF9 in the former report was unclear [18], differences in the infectious efficiency of the vectors used among these studies might have been responsible for the different leukemogenic effects. In another model with MLL-AF9 [19], human SCF, GM-CSF, and IL-3 were transgenically expressed in the recipient NOD/SCID mice. These cytokines may provide the MLL-AF9-transdused HSCs with additional signals for cell growth and survival, which might work as an oncogenic promoter. Comparing our model with this model, ectopic expression of K-ras^{G12V} in our model may in part compensate the cytokine (SCF, GM-CSF, and IL-3) signals in the MLL-AF9 model since stimulation with SCF and cytokines directly activates the ras signaling pathway in leukemogenesis [28]. However, it is also likely that each MLL-rearranged gene (MLL-ENL, MLL-AF4, MLL-AF9, and MLL-AF10) has a different leukemogenic efficacy, because the different partners of MLL may confer different biological functions to the produced MLL-fusion molecule [2].

Accumulating evidence points to a multistep pathogenesis for leukemia development and progression [29,30]. In the multi-genetic step models of leukemogenesis, particularly in the two-hit model of leukemia, the initial genetic hit often leads to abnormal cell differentiation (Type II mutation), while subsequent mutations may activate specific signaling pathways that are involved in cell growth, such as the ras/MAP kinase pathway (Type I mutation) [3,29,31]. Indeed, MLL-rearranged AML in neonatal bloodspots was shown to be of prenatal origin, supporting the idea that MLL rearrangement is one of the earliest events (first hit) in leukemogenesis [32]. In this line, the effect of MLL-AF10 as the first hit is thought to be differentiation arrest or the promotion of a specific hematopoietic lineage, because two previous studies demonstrated that MLL-AF10 expression in mouse HSCs results in skewed myeloid hematopoiesis [21,22]. However, in human HSCs, MLL-AF10 did not give rise to skewed hematopoiesis, but

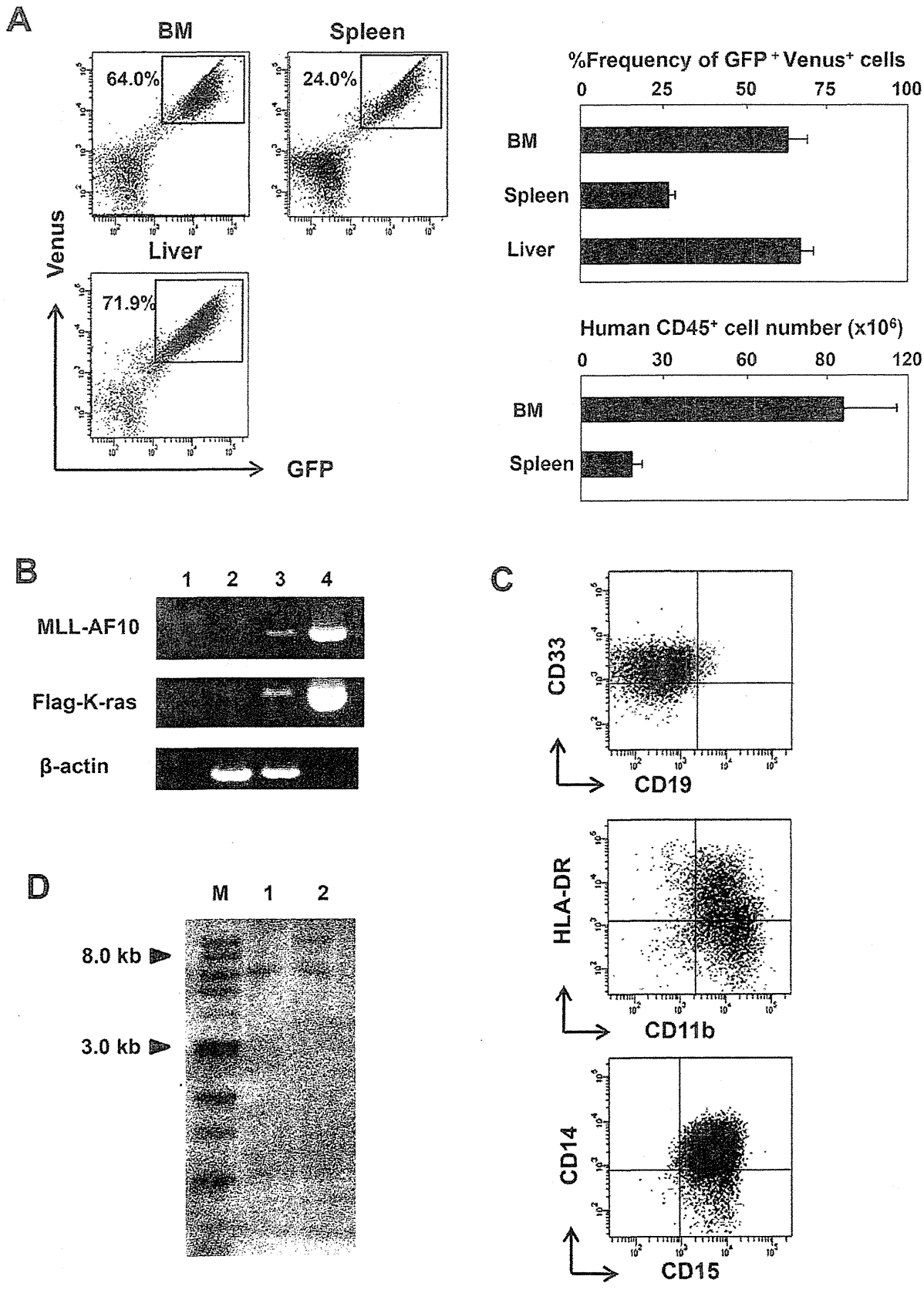

A
BM
Spleen
64.0%
24.0%
Liver
71.9%
Venus
GFP
%Frequency of GFP+Venus+ cells
0
25
50
75
100
BM
Spleen
Liver
Human CD45+ cell number (x10^6)
0
30
60
80
120
BM
Spleen
B
1
2
3
4
MLL-AF10
Flag-K-ras
β-actin
C
CD33
CD19
HLA-DR
CD11b
CD14
CD15
D
M
1
2
8.0 kb
3.0 kb

Figure 5. Immunophenotype and clonality of the MLL-AF10/K-ras-induced leukemia. (A) Frequencies of GFP$^+$/Venus$^+$ cells or human CD45$^+$ cells in the BM, spleen, and liver at 8 weeks after transplantation with human HSCs co-transfected with the MLL-AF10 and K-ras^{G12V} genes were examined by flowcytometric analysis. The flowcytometry data shown are representative of 6 to 8 mice per group in one representative experiment of two (left). The average of %frequencies of the GFP$^+$ and Venus$^+$ cells in whole cells in the indicated organs is shown with the standard deviation (right, upper; n = 6). The absolute cell number of human CD45$^+$ cells in the indicated organs is shown with the standard deviation (right, lower; n = 6). (B) Representative RT-PCR results confirming the stable, long-term expression of the MLL-AF10 and Flag-K-ras^{G12V} transcripts in human hematopoietic cells in the BM of mice 8 weeks after transplantation. (C) Lineage distribution of the GFP$^+$ and Venus$^+$ cells in the BM of a mouse engrafted with HSCs expressing MLL-AF10 and activated K-ras. (D) Southern blot analysis of DNA prepared from the human blood cells in the spleen of mice receiving transplants of MLL-AF10/K-ras^{G12V} co-transduced HSCs. Independent leukemia samples derived from two mice (lane 1; mouse 1 and lane 2; mouse 2) were examined. DNA was digested with Bgl II and probed with an EGFP probe. M: marker.
doi:10.1371/journal.pone.0037892.g005

enhanced the growth (or survival) of hematopoietic cells (Figures 1B and 2). Therefore, the biological impact of MLL-AF10 as the first hit remains controversial.

The active mutation of ras genes is a known additional hit in MLL-rearranged leukemia [27,29,33], and was so in our model. The hematopoietic cells undergoing the first hit must survive until the additional hit occurs during leukemogenesis. Since no HSCs transdused with K-ras^{G12V} alone could be found in the BM of the humanized mice even 25 weeks after transplantation (data not shown), it is unlikely that the ras mutation was the first hit in our model. Based on the present results, MLL-AF10 as the first hit might alter the self-renewal regulation of HSCs because of its promotion effect on multi-lineage hematopoiesis (Figures 1B and 2), and the HSCs (or leukemic precursor cells) might then receive the additional hit (K-ras^{G12V}), leading to leukemia. A recent report similarly demonstrated that co-transduction of BMI1 and Bcr-Abl oncogenes in HSCs induced leukemia, in which ectopic expression of BMI1 probably functioned as the additional hit, in NOD/SCID mice [34]. Although further examination will be required to test the additional hit hypothesis in leukemogenesis, our leukemia model may be a useful and unique experimental system with which to examine the multi-hit model of leukemogenesis.

Previous studies in which primary human leukemia cells were transplanted into immunodeficient mice provided important information about leukemia biology, in particular, about the biological significance of leukemic stem cells. Ishikawa et al recently demonstrated using NSG mice that the CD34$^+$CD38$^-$ cell population of the donor human leukemia most easily expands *in vivo* and survives by associating with the osteoblast-like stroma cells [9,10]. These findings shed light on how the leukemic stem cells are maintained *in vivo*. However, evaluating the transplantability or tumorigenic potential of each subpopulation from primary leukemia into immunodeficient mice might be insufficient to define leukemic stem cells. Intriguingly, a recent report

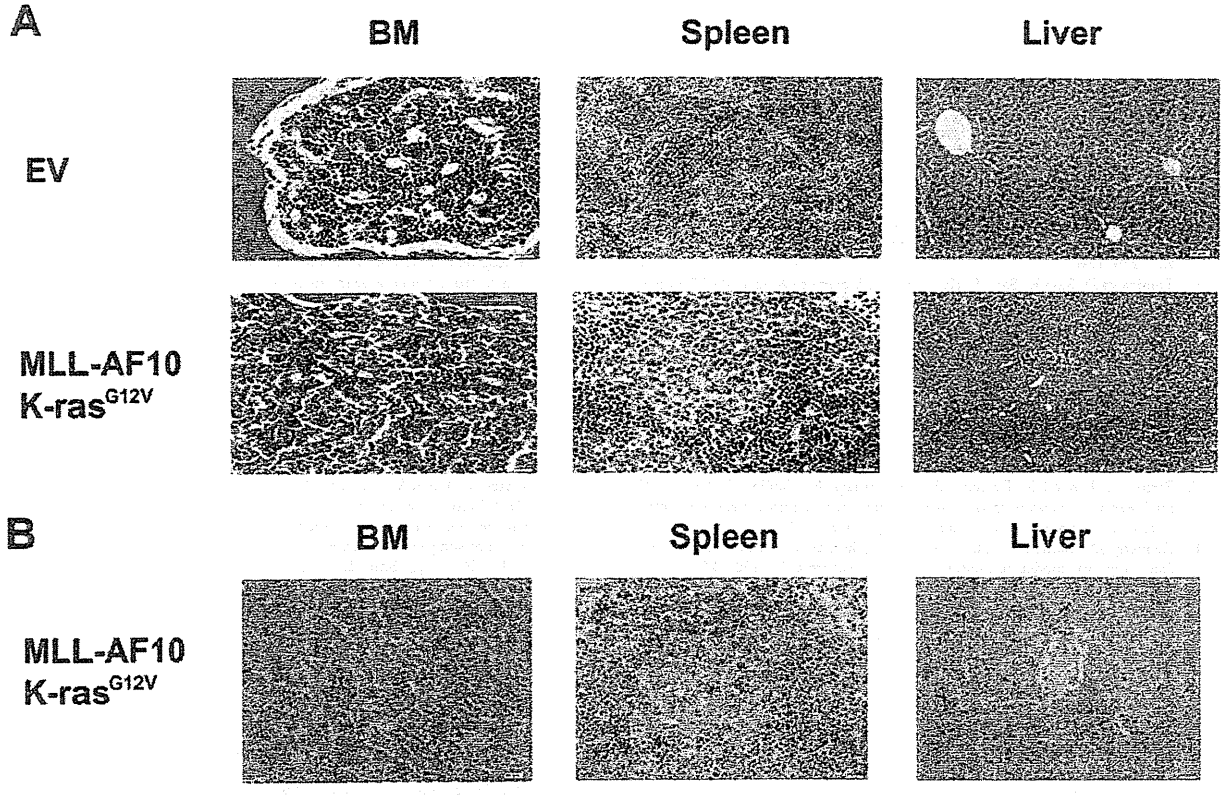


Figure 6. Pathological phenotypes of the leukemia. (A) Hematoxylin and eosin staining showing the infiltration of leukemic cells in the indicated organs of mice engrafted with HSCs expressing the MLL-AF10 and K-ras^{G12V} genes compared to control mice. (B) Immunostaining by a human CD45 mAb in the BM, spleen, and liver in mice engrafted with HSCs expressing the MLL-AF10 and K-ras^{G12V} genes.
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demonstrated using primary human melanoma cells that most of the cells (putative non-stem cells) that were not transplantable in NOD/SCID mice, which have NK cells, could easily survive and expand, like stem cells, in NSG mice, which lack NK cells [35]. This indicates the possibility that the high transplantability of the putative stem cell population as defined in the NOD/SCID mice reflected simply their resistibility to NK-cell-killing. Therefore, the observations in a certain transplantation model using immunodeficient mice may not necessarily reflect the physiological pathogenesis of human leukemic stem cells.

In this context, the leukemia that arose *in vivo* from human HSCs as demonstrated here may better reflect the physiological biology of human leukemic stem cells. Furthermore, the primary leukemia cells from different patients with leukemia showing even the same phenotypic markers should be different. Thus, the *in vivo* results from mice receiving transplants of different donor cells may be difficult to interpret, even if the leukemia cells from individual donors show the same phenotype. In contrast, the M5 leukemia established here was uniform in terms of cell morphology, symptoms (including selective tissue infiltration), and even survival prognosis. Importantly, the high reproducibility by which the same gene combination (MLL-AF10 and K-ras^{G12V}) induced the same leukemia in individual mice may represent a considerable advantage of these mice for the *in vivo* modeling of human leukemia over previous models.

To examine whether the leukemia is transplantable in a second recipient, we transplanted the leukemic cells from the spleen and BM to healthy adult NOG mice at least 20 times. Unexpected, we, however, could not find any GFP$^+$ cells in any second recipients even 10 months after transplantation (data not shown). Since GFP$^+$Venus$^+$ leukemic cells in the BM of the first hosts contained less than 0.1% CD34$^+$CD38$^-$ putative AML stem cells (data not shown), leukemic stem cells might have lost their self-renewal ability and been run out in the first host. To understand the unexpected phenomenon, Further investigation will be required.

Over the last decade, the outcome in pediatric AML has improved significantly, with up to 60% of children suffering from MLL-rearranged AML currently surviving [3]. However, improving the outcome of pediatric AML using current treatment protocols is hampered by treatment-related deaths and long-term side effects. In addition, most patients with MLL-rearranged ALL still indicate a poor prognosis. Therefore, to improve the outcome in pediatric MLL-rearranged leukemia, the development of leukemia-specific targeting drugs is an important strategy [36]. We hope that this newly established model using humanized mice will contribute to future studies aimed at revealing the molecular mechanisms for MLL-rearranged gene related leukemogenesis and developing new therapies against MLL-related malignancies.

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Author Contributions

Conceived and designed the experiments: NI KM SK ST KS. Performed the experiments: KM MS YW TT. Analyzed the data: TU YS. Contributed reagents/materials/analysis tools: YA SK MM. Wrote the paper: NI KM.

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REVIEW

Current advances in humanized mouse models

Ryoji Ito, Takeshi Takahashi, Ikumi Katano and Mamoru Ito

Humanized mouse models that have received human cells or tissue transplants are extremely useful in basic and applied human disease research. Highly immunodeficient mice, which do not reject xenografts and support cell and tissue differentiation and growth, are indispensable for generating additional appropriate models. Since the early 2000s, a series of immunodeficient mice appropriate for generating humanized mice has been successively developed by introducing the *IL-2Rγ*^{null} gene (e.g., NOD/SCID/γc^{null} and Rag2^{null}γc^{null} mice). These strains show not only a high rate of human cell engraftment, but also generate well-differentiated multilineage human hematopoietic cells after human hematopoietic stem cell (HSC) transplantation. These humanized mice facilitate the analysis of human hematology and immunology *in vivo*. However, human hematopoietic cells developed from HSCs are not always phenotypically and functionally identical to those in humans. More recently, a new series of immunodeficient mice compensates for these disadvantages. These mice were generated by genetically introducing human cytokine genes into NOD/SCID/γc^{null} and Rag2^{null}γc^{null} mice. In this review, we describe the current knowledge of human hematopoietic cells developed in these mice. Various human disease mouse models using these humanized mice are summarized.
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Keywords: animal model; immunodeficient mice; immunology; hematology; humanized mice

INTRODUCTION

'Humanized' mouse models in which various types of human cells and tissues are engrafted and function, as they would in humans are considered extremely useful in basic and applied human disease research.[1–4] For this purpose, highly immunodeficient mice, which do not reject xenografts and support the differentiation and growth of cells and tissues, are indispensable. The support system that maintains human cells and tissues is also a key factor. In addition, technical modifications necessary for the generation of humanized mice are important.

The discoveries of nude and severe combined immunodeficiency (SCID) mice were key advances in the development of immunodeficient mice for xenotransplantation.[5,6] The development of non-obese diabetic (NOD)/SCID mice *via* the introduction of the *Prdkc*^{scid} gene into a NOD inbred strain also contributed to the generation of humanized mice.[7,8] NOD/SCID/β2m^{null} and NOD/Rag1^{null}Pfp^{null} mice were subsequently derived from NOD/SCID and NOD/Rag1^{null} mice.[9,10]

Since the early 2000s, immunodeficient mice appropriate for generating humanized mice have been successively developed by introducing the mutant *IL2rα* gene into NOD/SCID and RAG1/2^{null} mice by backcross mating, thus resulting in NOD/SCID/γc^{null} mice[11,12] and Rag1/2^{null}γc^{null} mice.[13–16] These mice show multiple immunodeficiencies, including defects in T, B and natural killer (NK) cells, and reduced macrophage (Mϕ) and dendritic cell (DC) function.[11] In these mice, extremely high human cell engraftment rates and increases in well-differentiated human multilineage hematopoietic cells from human hematopoietic stem cells (HSCs), as compared with parent immunodeficient mice, were observed.[17–19] Humanized mice that retain various human immune cells are often termed human immune system mice[3] or human hematolymphoid system mice.[20] The production of humanized mice that are reconstructed with human cells would facilitate analysis of the underlying mechanisms of human disease pathogenesis. Indeed, various humanized models have been developed using these mice.

Various technical modifications have been used when generating humanized mice. These include modifications in the HSC injection route, age of mice, and HSC and irradiation sources, each of which may affect the efficacy of human cell engraftment. In terms of the injection route and age, intrahepatic or intravenous injection (through the facial vein) into newborn mice and intravenous injection (*via* the tail vein) into adult mice were generally used.[11,14,21] Brehm *et al.*[16] examined various parameters, including injection route, injection age and immunodeficient mouse strains. By comparing engraftment rates of human cells from HSCs, they concluded that intrahepatic injection of HSCs into newborn mice enhanced engraftment as compared with adult mice. With respect to the source of HSCs, CD34$^+$ cells from cord blood or fetal liver were typically used. However, CD34$^+$ cells from granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood (PB) or bone marrow (BM) served as additional sources. Lepus *et al.*[22] reported that CD34$^+$ cells from fetal liver were more efficient than those from cord blood or G-CSF-mobilized PB. In addition, Matsumura *et al.*[23] reported that CD34$^+$ cells from cord blood were more effective than those from G-CSF-mobilized PB and BM. Busulfan treatment can also be used in place of irradiation, and results in more effective differentiation of B cells from HSCs as compared with

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irradiation.[24] The use of this drug would be beneficial for research, since special equipment is not required. The engraftment rate of human cells from HSCs varies between reports. A possible explanation for this difference is the quality of HSCs, which is influenced by different isolation techniques and the HSC source (i.e., cord blood or fetal liver).

Despite these efforts, some human cell lineages, such as erythrocytes and neutrophils, have not yet been developed in humanized mice. The differentiation of HSCs into immature human T and B cells and poor interactions between these cells were suggested based on cell phenotype analysis and the rare production of antigen-specific immunoglobulin G (IgG) class antibodies.[25] These results suggest that current immunodeficient mice may be insufficient, and human factors that support the differentiation and maturation of cells and mediate cell-to-cell interactions must be introduced. To this end, a new series of immunodeficient mice has been generated by introducing human cytokine genes into NOD/SCID/γc^{null}, $Rag2^{null}\gamma c^{null}$ mice.

In this review, we describe the current knowledge of human hematopoietic cells developed in NOD/SCID/γc^{null} and $Rag1/2^{null}\gamma c^{null}$ mice and the strains derived from them. In addition, the humanized mouse models used in studies of various human diseases will be summarized.

IMMUNODEFICIENT MICE

Since the discovery of nude and SCID mice,[5,6] humanized mice have been generated by using various immunodeficient mice. SCID mice that received human T and B cells by transplantation of fetal liver and thymus, which were termed *SCID-hu* mice by McCune *et al.*,[26,27] provided an attractive humanized model for research in various fields; however, engraftment rates were not high. In 1998, Goldman *et al.*[13] reported the enhanced engraftment of human cells in $Rag2^{null}$ mice possessing the IL-2Rγ^{null} gene. In the 2000s, a series of immunodeficient mice was developed by combining the IL-2Rγ^{null} gene with conventional SCID and $Rag1/2^{null}$ mice. These strains showed extremely high engraftment rates and differentiation of human cells, resulting in remarkable advances in the development of human disease models. These stains include the NOG (NOD/Shi-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/Jic) mice reported in 2002;[11] RG (BALB/cA-$Rag2^{null}Il2r\gamma^{null}$ (BRG) and C57BL/6-$Rag2^{null}Il2r\gamma^{null}$ (B6RG)) mice reported in 1997, 1998 and 2004;[13,14,28] and NSG (NOD/LtSz-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/J) mice reported in 2005.[12] Recently, immunodeficient BALB/c-$Rag1^{null}Il2rg^{null}$,[16] NOD-$Rag1^{null}Il2rg^{null}$,[15] and NOD/SCID-$JaK3^{null}$ mice have been established as alternatives to NOG/NSG mice. Data accumulated to date suggest that NOG/NSG mice are the best recipients for humanized tissue and human cell engraftment occurs in the following order: NSG=NOG>NRG>BRG>NOD/SCID>B6RG.[15,16] The disadvantages of SCID and NOD/SCID mice include the frequent occurrence of thymic lymphoma and the leakiness, in which T and B cells develop in aged mice.[29,30] However, NOG mice show no leakiness or spontaneous thymic lymphoma.[31,32] This may be attributed to inactivation of IL-2Rγ, which is shared by important cytokines, such as IL-2, IL-4, IL-7, IL-15 and IL-21, each of which is important for T- and B-cell growth.[33] These results indicate that NOG/NSG mice are better recipients of human cells and tissues.

The most attractive feature of these humanized mice is the development of multilineage hematopoietic cells by transplantation of human HSCs. In particular, T-cell subpopulations, including CD4 and CD8 single positive cells, which could not be differentiated in NOD/SCID mice, successfully developed in these mice.[14,18,21] These results suggest the usefulness of humanized mice for investigating human immune responses. Indeed, the utility of these humanized mice is well accepted by researchers worldwide. However, these humanized mice are insufficient, since human cells are not fully functional. For example, no or rare antigen-specific IgG production occurs after multiple injections of antigens.[25]

To overcome this issue, various improved strains based on these immunodeficient mice have been developed or are being developed by several groups, including our group.[34,35] In Figure 1, the history of the development of immunodeficient humanized mice and their respective improvements are summarized. These mice have been established in NOG/NSG or BRG backgrounds. Background strain selection is crucial for the improvement of immunodeficient mice. In 1996, we reported human granulocyte–macrophage colony-stimulating factor (hGM-CSF) and interleukin-3 (IL-3) cosecreting transgenic (Tg) B6-SCID mice.[36] These strains were able to maintain human tumor cell lines possessing hGM-CSF and human IL-3 (hIL-3) receptors on the surface;[37] human cells, however, could not engraft in these mice. We recently established hGM-CSF/IL-3 Tg NOG mice by the conversion of strain B6 to NOG by backcross mating. High human cell engraftment rates were observed in hGM-CSF/IL-3 Tg NOG mice. We also compared engraftment of HSCs in NOG, BRG and B6RG mice. Higher engraftment rates were obtained in NOG and BRG, but not B6RG mice. In particular, we were surprised to detect only a few human cells in B6RG mice (unpubl. data). Similar results were reported by Traggiai *et al.*,[14] indicating that immunodeficient mice in a B6 background are not sufficient for generating humanized mice. Recent reports on signal regulatory protein (Sirpα) suggested that NOD mice are superior to other strains, because Sirpα in NOD strains is more similar to that in humans, compared with Sirpα in other mice strains.[38,39] Therefore, BRG transgenic mice with human Sirpα have been established to enhance human cell engraftment efficacy.[20]

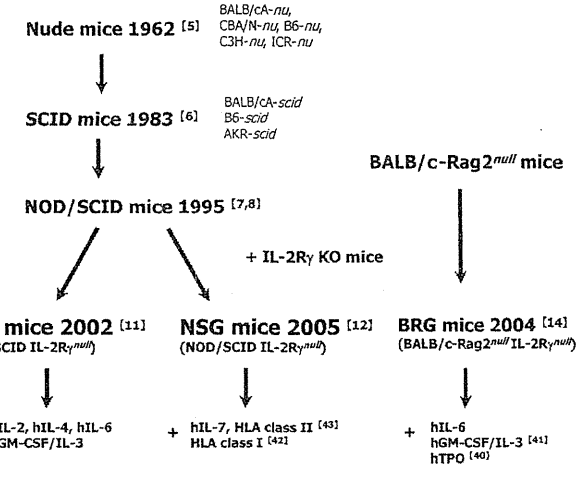

Figure 1 The history of the development on immunodeficient mice for humanized mice model. In retrospect, nude mice or SCID mice were the first immunodeficient strains. Subsequently, their congenic strains were generated to improve engraftment capacities. NOD/SCID mice established in 1995 have been a milestone in this field, because of the severer phenotype than nude and SCID mice. In early 2000s, NOG, NSG and BRG mice were established by introducing the IL-2Rγ^{null} allele into NOD/SCID or BALB/cA $RAG2^{null}$ mice. Due to the complete loss of murine immune systems, human hematopoiesis has been enormously enhanced in these mice. Currently, these strains were further improved by introducing human genes for various cytokines or HLA class I and II, so as to recapitulate a human *bona fide* hematopoiesis and immune system. The superscripts represent the respective references. BRG, BALB/cA-$Rag2^{null}Il2r\gamma^{null}$; HLA, histocompatibility leukocyte antigen; NOD, nonobese diabetic; NSG, NOD/LtSz-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/J; SCID, severe combined immunodeficiency.

Improved strains were generated by injecting human DNA into pronuclear stage embryos, injecting genetically modified embryonic stem cells into blastocysts (knock-in (KI)), or introducing human genes by backcross mating with established transgenic mice. Specifically, human cell differentiation from HSCs in improved mice produced using the KI strategy has been analyzed by Flavell's group at the Yale University.[20] They reported elevated HSC and myeloid cell numbers in thrombopoietin (TPO) KI mice and increased alveolar Mϕ in hGM-CSF/IL-3 KI mice.[40,41] We also developed transgenic NOG mice that secreted human IL-2 or IL-4 by injecting DNA into NOG embryos. The suppression of graft-versus-host disease (GVHD) and dominant conversion to Th2 cells in hIL-4 Tα NOG mice and remarkable differentiation of human NK cells in hIL-2 Tα NOG mice, respectively, were observed (unpubl. data). Shultz's group generated human leukocyte antigen (HLA) class I transgenic NSG mice by backcross mating the respective transgenic mice into NSG mice. The successful generation of antigen-specific cytotoxic T cells[42] was observed. Antigen-specific IgG was produced in HLA class II transgenic NSG mice.[43]

Real humanized mice are expected to be generated in the near future by improving immunodeficient mice.

HEMATOLYMPHOID HUMANIZED MICE

The reconstitution of the human hematopoietic system is one of the most advanced areas in humanized mouse research. The use of NOG/NSG or BRG mice has greatly improved human hematopoiesis, as shown by the development of multiple human cell lineages, including B and T lymphocytes, NK cells, myeloid DC, plasmacytoid DC, Mϕ and erythroblasts. Here, we discuss the current status and perspectives in this field.

Lymphoid cells

Two major subsets of lymphoid cells, i.e., B and T cells, were developed in NOG mice by simply transferring human HSCs after irradiation.

B cells. Human B cells are detected in the PB 1 month after HSC transplantation and gradually increase in number over the following 3 months. In the BM, B-cell differentiation in humanized mice seems to consistently resemble that in humans, since several distinct precursor populations exist:[25] $CD19^-CD38^+CD10^+CD34^+$ early-B cells, $CD19^+IgM^-CD20^-CD34^+$ pro-B cells, $CD19^+IgM^-CD20^-CD34^-$ pre-B cells and $CD19^+IgM^+IgD^-$ immature B cells. The pro-B and pre-B populations were also characterized by the expression of intracellular V_{preB} and Cμ chains. In the spleen, one of the remarkable B-cell phenotypes in humanized mice is high CD5 expression, which is markedly different from genuine human B cells.[23] The significance of this upregulated CD5 expression remains controversial, i.e., whether these are human B-1 cells or transitional 1 B cells.[23,25]

Immunization of humanized mice with various exogenous substances induces antigen-specific IgM responses, suggesting that the B-cell repertoire is diverse and can cover a myriad of antigens.[21,23] Since antigen-specific IgG responses in conventional humanized mice are very weak,[14,21,23] it has been speculated that these B cells have some intrinsic defects in the class switch machineries. Several *in vitro* experiments, however, demonstrated that these B cells do produce IgG in response to stimulation through their antigen receptors and CD40 in the presence of IL-21.[25] In addition, recent reports have shown that new mouse strains that express HLA-DR mounted an antigen-specific IgG response upon immunization.[43] Collectively, B cells in humanized mice maintain the ability to mediate humoral immune reactions.

T cells. Human T cells can develop in humanized NOG/NSG or BRG mice in the thymus and accumulate in the spleen. This is one of the most important points of distinction from NOD/SCID mice, which only partially support the differentiation of human T cells. Typically, 3–5 months is necessary for T cells to colonize the spleen.[44] In the thymus, human thymocytes show typical surface phenotypes,[44] i.e., $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^-CD8^+$ and $CD4^+CD8^-$ stages, suggesting that they follow the normal differentiation pathway. The thymus is indispensable for T-cell development, and selection is largely mediated by mouse major histocompatibility complex (MHC) molecules, given that Foxn1-deficient NOG mice (NOG *nu/nu*) cannot support T-cell development, and $CD4^+$ and $CD8^+$ T cells do not fully develop in NOG I-A_β^{null} or NOG $\beta2m^{null}$ backgrounds,[25] respectively.

Although development seems to be relatively normal, the functionality of T cells in humanized mice remains controversial. For example, in studies using Epstein–Barr virus (EBV), humanized mice showed antiviral T-cell responses in which interferon-γ producing $CD8^+$ T cells were differentiated and protected mice from lymphoma development,[14,45] thus supporting the normal functions of human $CD8^+$ T cells. However, *in vitro* experiments have suggested that human T cells have a limited ability to respond to antigenic stimulation.[25] Although the mechanisms underlying abnormal T-cell functions remain unclear, mismatches between mouse MHC and the HLA of donor human cells may be involved. Indeed, HLA-A- or HLA-DR-expressing NSG mice showed normal cytotoxic reactions (cytotoxic T-lymphocyte responses) to viral infection or IgG responses against exogenous antigens, respectively.[43,46,47]

NK cells. Human NK cells also develop from the early stage (4 weeks) of HSC transfer. However, the number of NK cells is not very high (a low percentage of human $CD45^+$ cells), indicating the necessity for growth and differentiation factors in immunodeficient mice.[48] Huntington *et al.*[49] reported that the hIL-15/hIL-15Rα complex induces extensive proliferation and differentiation of $CD16^+KIR^+$ NK cells. Chen *et al.*[50] also reported that administering IL-15 and the Flt-3/Flk-2 ligand by plasmid DNA injection into HSC-transferred mice leads to an increased number of NK cells. In IL-2 Tg NOG mice (Katano *et al.*, manuscr. in prep.), human NK cells predominantly develop prior to B and T cells after HSC transfer, and consist of the largest population in human $CD45^+$ cells. However, their function has not been well characterized.

As an alternative method for studying human NK cell functions, *ex vivo* isolated human NK cells from peripheral blood mononuclear cells (PBMCs) have been transplanted into NOG mice. Although the inoculated NK cells were not maintained in these mice for a long time, the cells exerted effective antibody-dependent cellular cytotoxicity and suppressed the growth of a Burkitt's lymphoma cell line (Daudi), following concomitant administration of an anti-CD20 antibody (rituximab).[51]

Myeloid cells

In conventional humanized mice, although human myeloid cells were shown to be differentiated, the efficiency was poor. Recently, however, the development of various lineages of myeloid cells has been improved by introducing human cytokines.

Monocytes. Human monocytes/Mϕ can be detected in the blood, lymphoid organs (spleen and BM) and some tissue organs (lung and liver) in conventional humanized mice. The frequency of human $CD14^+$ cells among the total human $CD45^+$ cell population is usually not more than 1%–2% in the spleen, while the frequency can reach 8% or 5% in