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Activation of Notch1 promotes development of human CD8$^+$ single positive T cells in humanized mice

Yoichi Haji [a,b], Makiko Suzuki [a], Kunihiko Moriya [a], Takanori So [a], Katsuto Hozumi [c], Masamichi Mizuma [b], Michiaki Unno [b], Naoto Ishii [a,*]

[a] Department of Microbiology and Immunology, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
[b] Department of Surgery, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
[c] Department of Immunology, Tokai University School of Medicine, Isehara 259-1193, Japan

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ABSTRACT

Notch1 mutations are found in more than 50% of human T cell acute lymphoblastic leukemia (T-ALL) cells. However, the functions of Notch1 for human T cell development and leukemogenesis are not well understood. To examine the role of Notch1, human hematopoietic stem cells (HSCs), which had been transduced with a constitutively active form of Notch1 (ICN1), were transplanted into severely immunodeficient NOD/Shi-*scid*-IL2rγ^{null} (NOG) mice. We found that the great majority of the ICN1-expressing hematopoietic cells in the bone marrow expressed surface markers for T cells, such as CD3, CD4, and CD8, and that this T cell development was independent of the thymus. Accordingly, phenotypically mature CD8$^+$ single positive (SP) T cells were observed in the spleen. Furthermore, T-ALL developed in one NOG recipient mouse out of 26 that had been secondary transferred with the T cells developed in the first NOG mice. These results indicate that Notch1 signaling in HSCs promotes CD8$^+$ SP T cell development, and that T cell leukemogenesis may require additional oncogenic factors other than Notch1 activation.

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1. Introduction

Notch signaling contributes to the maintenance of hematopoietic stem cells (HSC) [1–3], and to several cell fate decisions in the lymphoid lineage. In particular, roles of Notch1 in lymphopoiesis have been well studied. The best characterized function of Notch1 signaling is the lineage commitment and maturation of T cells in the thymus in murine [4–7]. Several gain- and loss-of-function studies have provided the importance of Notch1 for the T cell lineage commitment. Inducible inactivation of Notch1 blocked T cell development while increased ectopic B cell development in the thymus in mice [1]. In contrast, a constitutive active form of Notch1 promoted extrathymic T cell development at the expense of B cell development in the bone marrow in mice [8]. These mouse studies have revealed that Notch1 signaling in hematopoietic cells induces not only T cell lineage specification, but also maturation for the CD4/CD8 double-negative (DN) stage to the double-positive (DP) stage [7,9]. Furthermore, Notch1 signaling is continuously required at several stages of T cell development, which is the early T cell differentiation steps and maturation such as T cell receptor rearrangement [10,11] and CD8$^+$ T cell commitment rather than CD4 in the mouse system [12]. In spite of the accumulating evidence for Notch1 roles in mice, almost all of our knowledge about Notch1 roles in human T cell development is based on *in vitro* results, in which, for example, OP9-Delta like 1 coculture systems and fetal thymic organ culture have been commonly used [13–15]. Therefore, little is known about the *in vivo* relationship between Notch1 function and human T cell development although De Smedt et al. showed that human HSCs transfected with an intracellular domain of Notch1 (ICN1) gene developed to CD4/CD8 DP T cells in the bone marrow of immunodeficient NOD/Scid mice. However, any single-positive (SP) T cells could not be found in the peripheral tissues in the humanized mouse model [16].

Notch1 has also been well described in T-cell leukemogenesis. Unregulated forced expression of Notch1 causes T cell leukemia in mice [17,18]. Moreover, several recent reports by using mouse models revealed that the environment that promotes Notch

Abbreviations: HSC, hematopoietic stem cell; DN, CD4$^+$ and CD8$^+$ double-negative; DP, CD4$^+$ and CD8$^+$ double-positive; ICN1, intracellular domain of Notch1; SP, single positive; T-ALL, T cell acute lymphoblastic leukemia; NOG, NOD/Shi-*scid*-IL2rγ^{null}; mAb, monoclonal antibody; EGFP, enhanced green fluorescent protein.

* Corresponding author. Address: Department of Microbiology and Immunology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Fax: +81 22 717 8097.
E-mail address: ishiin@med.tohoku.ac.jp (N. Ishii).

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signaling induces T cell leukemogenesis even if HSCs did not have any mutations of Notch genes [19]. In addition to the mouse models, Notch1 gene mutations causing overexpression of Notch1 are found in more than 50% of human T cell acute lymphoblastic leukemia (T-ALL) cells [20]. Therefore, Notch1 signaling may contribute to leukemogenesis of T cells in both murine and humans.

Here, we examined the *in vivo* role of Notch1 signaling in human T cell development and leukemogenesis by transferring human HSCs, which had been transduced with an ICN1 gene, into severely-immunodeficient NOD/Shi-*scid*-IL2rγ^{null} (NOG) mice. Almost all the ICN1-expressing human HSCs differentiated into T cells in the bone marrow, and accordingly $CD8^+$ SP T cells were observed in the spleen of the recipient mice. Furthermore, T-ALL developed in one mouse that had been secondary transferred with the T cells developed in the first NOG mice. The present humanized mouse model reveals novel *in vivo* roles of Notch1 in human T cell development and leukemogenesis.

2. Materials and methods

2.1. $CD34^+$ HSC isolation from human cord blood

The 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. $CD34^+$ HSCs were isolated as described previously [21,22]. In brief, mononuclear cells from the cord blood were incubated with a biotin-conjugated anti-human CD34 monoclonal antibody (Serotec, Oxford, UK), and then incubated with anti-biotin Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The magnetically labeled $CD34^+$ cells were purified with an AutoMACS pro separator (Miltenyi Biotech). The purity of the $CD34^+$ fraction was >95%.

2.2. Plasmid

The retroviral vector, pDΛNsam-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 (National Center for Child Health and Development) [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 ICN1 cDNA was inserted into a cloning site in the pDΛNsam-IRES-EGFP vector to make the pDΛNsam-ICN1 plasmid.

2.3. Gene transfer and transplantation of $CD34^+$ HSCs

ICN1-trasnduced $CD34^+$ HSCs were prepared and transferred into NOG mice as previously described [22,25]. In brief, human cord blood-derived $CD34^+$ HSCs stimulated with a cytokine cocktail were placed onto 96-well plates (3×10^4 cells/well) that had been coated with CH-296 (Retronectin, Takara, Japan) in the virus supernatant. After 48 h of culture, the whole cultured $CD34^+$ HSCs (1–2×10^5 cells) were transplanted intrahepatically to irradiated newborn NOG mice. Before transplantation, the cellular transduction efficiency of the vector was 29.2 ± 2.7% (n = 15). From one cord blood sample, donor $CD34^+$ cells could be prepared for transplantation into only 2 or 3 recipient mice. 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).

2.4. Antibodies and flow cytometric analysis

Surface markers were detected with the following human specific antibodies: CD1a-Phycoerythrin, CD3-Phycoerythrin, CD4-Allophycocyanin, CD7-V450, CD8-Phycoerythrin, CD19-Allophycocyanin, CD34-Allophycocyanin, CD38-Phycoerythrin-Cy7, CD45-Allophycocyanin-Cy7, Annexin-V-Allophycocyanin were purchased from BD Biosciences (San Jose, CA, USA). CD4-Pacific Blue and CD8-Pacific Blue, CD45-Brilliant Violet510 were purchased from BioLegend (San Diego, CA, USA). To analyze human lymphocytes in transplanted NOG mice, single-cell suspensions were prepared by conventional methods from the spleen, BM and the peripheral blood 12–16 weeks after transplantation. The cells were stained with the relevant antibodies for 30 min on ice, and washed with cold PBS containing 2% FCS. Then, the cells were subjected to flow cytometric analysis with a FACS Canto II cytometer (BD Biosciences). The proportion of each cell lineage was calculated using FACS Diva software (BD Biosciences).

2.5. Real-time PCR

GFP^+ and GFP^- human hematopoietic cells (human $CD45^+$) were purified from the bone marrow or spleen using a FACSAria II cell sorter (BD Biosciences). Total RNA was prepared from cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was synthesized using SuperScript Reverse Transcriptase and Random Primers (Invitrogen). The cDNA was then amplified over 40 cycles on a 7500 Real-time PCR system using SYBR Premix EXtaq, ROX Reference DyeII (TaKaRa Bio, Shiga, Japan), and the primer sets. All samples were normalized to GAPDH. The PCR primers were as follows: Notch1: forward agaagttccggttcgaggag, reverse catccaggtgctgctgag, ICN1: forward tagactgacgcggccgcatgcg, reverse ttctggttgtcgtccatgag, GAPDH: forward cgctctctgctcctcctgtt, reverse ccatggtgtctgagcgatct, Hes1: forward aagtgtgctggggaagtacc, reverse gtgcgcacctcggtattaac, Hey1: forward tcggctctaggttccatgtc, reverse ctgggtaccagccttctcag, pTα: forward ctacttctcctggcccttg, reverse accatctgctgctttccatc.

2.6. LAM-PCR

To identify the ICN1 insertion site in the chromosome, LAM-PCR was performed as previously described [26]. In brief, Tsp509I enzyme (BioLegend) was used to digest genomic DNA from leukemic T cells for subsequent ligation to an oligonucleotide linker cassette. Then, nested PCR was performed at the site of viral long terminal repeat (LTR) and the linker cassette. PCR products were sequenced with an ABI 3100 Genetic Analyzer (Applied Biosystems). Resulting alignment data was checked by NCBI/BLAST (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7. Statistical analysis

Statistical analysis was performed using Student's *t*-test. *P*-values <0.05 were considered significant.

3. Results

3.1. Transduced ICN1 functions as an active form of Notch1

To examine the *in vivo* role of Notch1 signaling in human T cell development, human $CD34^+$ HSCs were retrovirally transfected with the ICN1 gene encoding a constitutive active form of Notch1

(Fig. 1A), and transplanted into NOG mice. Since whole HSCs containing both GFP$^+$ and GFP$^-$ cells were transplanted, we could examine differentiation of both GFP$^+$ (ICN1-expressing) and GFP$^-$ human hematopoietic cells in the same individual mouse (Fig. 1B). As shown in Fig. 1C and D, the transcription level of ICN1 correlated well with the GFP fluorescent intensity. Furthermore, GFP$^+$ human hematopoietic cells strongly expressed *hes1*, *hey1* and *pTα* genes (Fig. 1E), all of which are the targets of Notch1, indicating that the transduced ICN1 were functional as active Notch1.

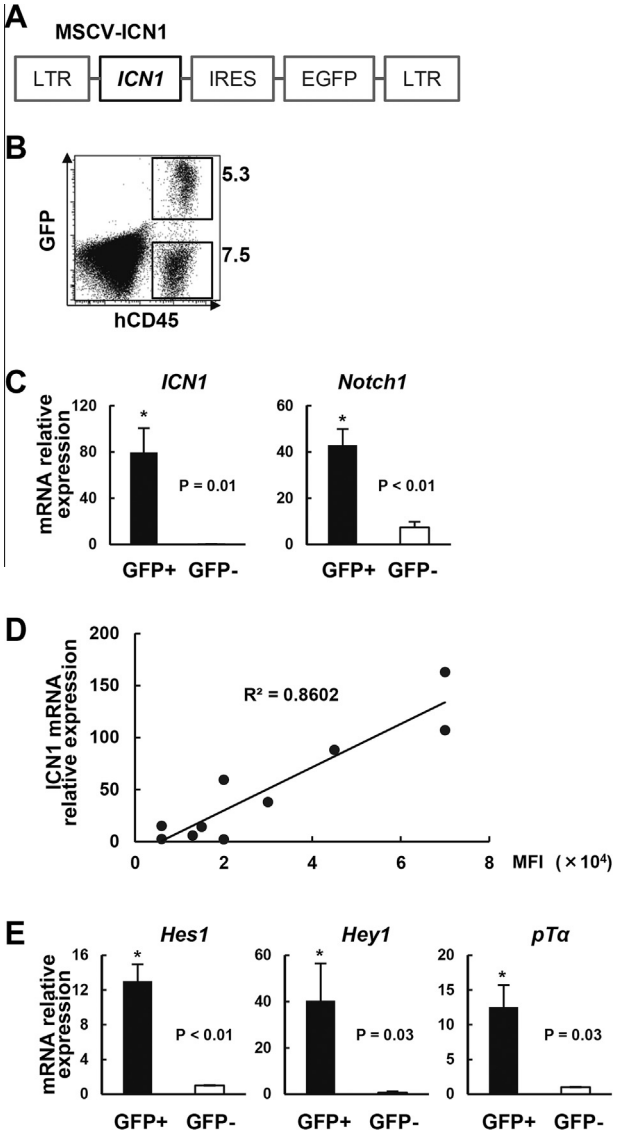

Fig. 1. Structure and *in vivo* analysis of an active form of Notch1 (ICN1). (A) Structure of the ICN1 retroviral vector. The ICN1 cDNA was inserted in the MSCV vector carrying an GFP marker. (B) Flow cytometric analysis of GFP$^+$ cells from the bone marrow of recipient NOG mice at 12 weeks after transfer with human HSCs transduced with the ICN1 vector. (C) ICN1 expression in GFP$^+$ cells in the bone marrow. GFP$^+$ and GFP$^-$ hCD45$^+$ cells were sorted from the bone marrow in the recipient NOG mice ($n = 6$). Notch1 transcription levels in the GFP$^+$ and GFP$^-$ populations were evaluated with ICN1-specific primers (left) and Notch1 primers that detect both ICN1 and the intrinsic wild-type Notch1 gene (right) by semi-quantitative real-time PCR. (D) Direct correlation between GFP protein and Notch1 transcript expression levels. Bone marrow cells with various GFP expression levels were purified from 6 recipient NOG mice, which had been independently transplanted with ICN1-expressing human HSCs. RNA was extracted from the sorted bone marrow cells and subjected to semi-quantitative real-time PCR analysis for ICN1 transcript. Mean fluorescence intensity (MFI) reflecting the GFP expression level was calculated with a FACSAriaII cell sorter. (E) *Hes1*, *Hey1*, and *pTα* mRNA expression in GFP$^+$ hCD45$^+$ cells ($n = 4$). All values were normalized to GAPDH and are presented as the GFP$^+$ population relative to cDNA from the GFP$^-$ population. Error bars indicate the standard error of mean.

3.2. ICN1 induces CD8$^+$ SP T cell development in the bone marrow

We examined the effect of ICN1 on T cell development from human HSCs in the humanized NOG mice. Flowcytometric analysis demonstrated that GFP$^+$ human hematopoietic cells differentiated preferentially into CD3$^+$ T cells while GFP$^-$ cells mainly consisted of CD19$^+$ B cells (Fig. 2A and B). To quantitatively evaluate the effect of Notch1 signals on T cell commitment, we compared the frequency of CD3$^+$ T cells among the three populations (GFP-negative, GFP-low, and GFP-high), and found that higher Notch1 expression induced more T cell development (Fig. 2C).

Further flowcytometric analysis demonstrated that ICN1-expressing cells in the bone marrow contained DN, DP, and CD8$^+$ SP T cells (Fig. 2D). Although a small population of CD4$^+$ SP cells were observed (Fig. 2D, left), the CD4$^+$ cells showed a CD1a$^+$CD3$^-$ phenotype, indicating that the cells were immature T cells. In contrast, almost all of ICN1-expressing human hematopoietic cells in the spleen were CD1a$^-$CD3$^+$CD8$^+$ SP T cells (Fig. 2D), which is fully compatible with a mature CD8$^+$ SP T cell phenotype. Since high ICN1 expression was suggested to suppress SP T cell differentiation from DP T cells in several mouse models [7,8], we compared CD8$^+$ SP T cell differentiation between GFP-low and GFP-high populations. Unlike the mouse models, human cells with high ICN1 expression could also differentiate into CD8$^+$ SP T cells in our model (Fig. 2E). In the recipient mice producing GFP$^+$CD3$^+$CD8$^+$ SP T cells, we could not find any reconstituted thymus even using a microscope, and could not detect any human GFP$^+$ cells in the tissues where the thymus should be located (data not shown). Therefore, Notch1 activation in human hematopoietic cells promotes CD8$^+$ SP T cell development, probably independent of the thymus, in our model.

To investigate function of the reconstituted human T cells, bone marrow cells including T cells were cultured in the presence or absence of CD3 and CD28 monoclonal antibodies (mAbs). However, almost all T cells immediately died and could not respond to stimulation with CD3/CD28 mAbs (Fig. 2F). These results indicated that T cells that developed under unregulated Notch1 signaling did not have normal function.

3.3. Active form of Notch1 alone may be insufficient for T cell leukemogenesis

Since constitutive Notch1 signaling has been suggested to contribute to T cell leukemogenesis, bone marrow cells containing ICN1-expressing human T cells from the first hosts were transplanted into second NOG recipient mice. Among independent 26 secondary transplantations, T-ALL developed in one recipient mouse by 13 months after first transplantation. The bone marrow in leukemic mice was occupied by GFP$^+$hCD45$^+$ cells (Fig. 3A). The leukemia cells had a uniform surface marker profile, CD1a^{+-} CD3$^+$CD7$^+$CD4$^+$CD8$^+$CD33$^-$CD34$^-$ (Fig. 3B), which was fully compatible with human T-ALL with Notch1 gene mutations. Analysis of TCRβ gene rearrangement in the leukemic cells showed monoclonality (data not shown). However, even by 14 months after first transplantation, we could find only the one case with T-ALL, suggesting insufficiency of ICN1 alone for development of T-ALL.

In the case with leukemia, we considered that, in addition to Notch1 activation, retroviral vector-insertion mutagenesis may have functioned as a second hit for leukemogenesis. We thus performed LAM-PCR analysis to identify the vector insertion site, which might have been near or in a certain oncogene locus. The integration site found was located on the chromosome 2p21. Genes

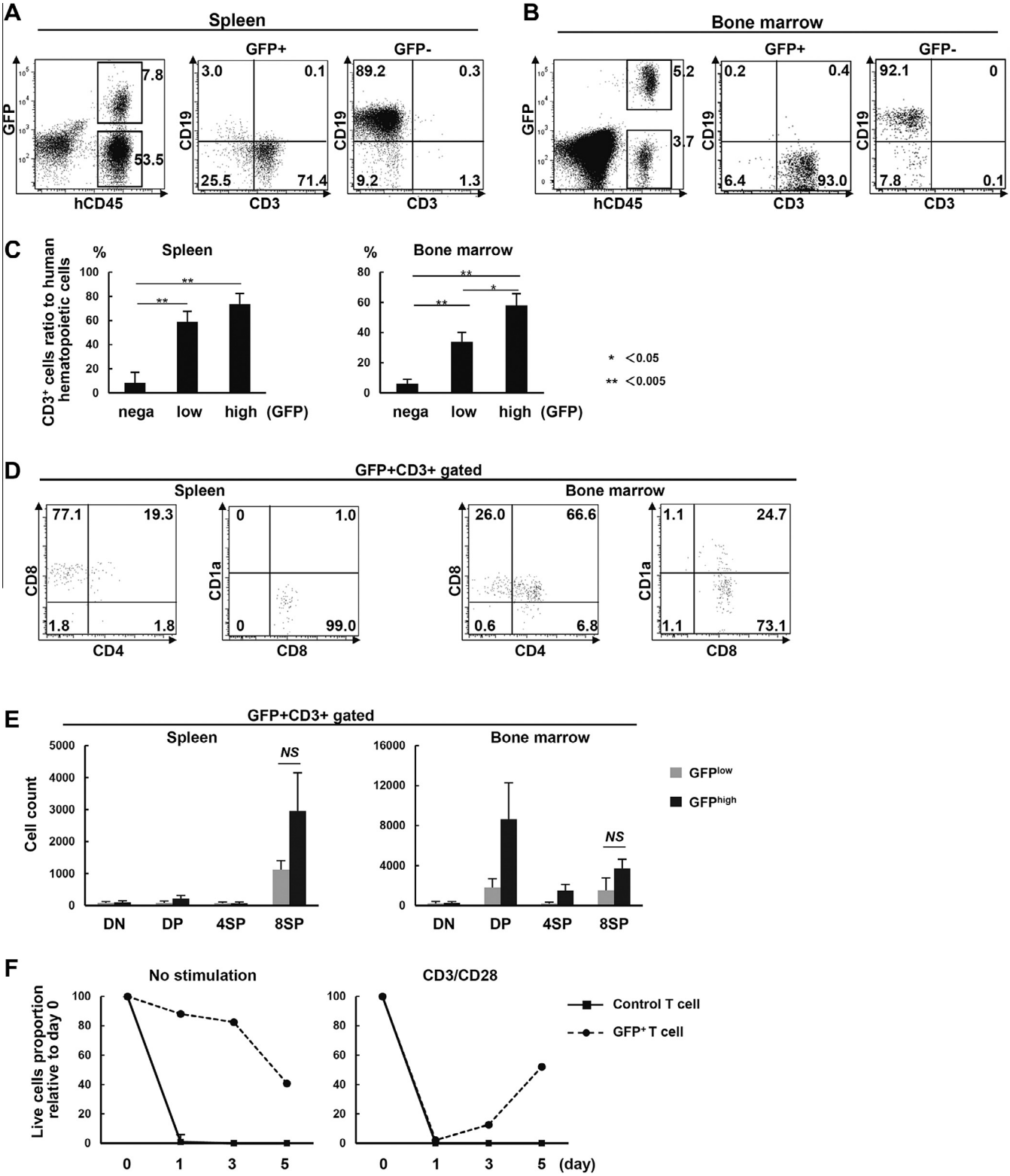

Fig. 2. ICN1 expression in human HSCs promotes T cell development *in vivo*. (A–C) Differentiation of $CD3^+$ T and $CD19^+$ B cells derived from GFP^+ and GFP^- $hCD45^+$ hematopoietic cells in the spleen (A and C, left) and bone marrow (B and C, right) were examined. (A and B) The data shown in the flowcytometric analysis is representative of at least 30 recipient mice. Numbers in plots indicate the percentage relative to total cells. (C) T cell differentiation is induced by ICN1 in an ICN1 dose dependent manner. GFP-negative (neg), GFP-low (MFI = 10^3 – 10^4, low), and GFP-high (MFI > 10^4, high) cell populations in the spleens (n = 16, left) and bone marrows (n = 19, right) from individual mice were independently gated and examined for the frequency of $CD3^+$ T cells in each population. (D) Expression profiles of CD4, CD8, and CD1a on GFP^+CD3^+ T cells in the spleen and bone marrow. (E) Cell numbers in GFP-low and GFP-high $CD3^+$ T cells at the indicated stages of T cell development. NS; not significant (F) Defective function of GFP^+ T cells. Whole bone marrow cells (3×10^5, n = 4) or human peripheral blood mononuclear cells (PBMC, 1×10^5) from healthy donors were cultured in the presence (right) or absence (left) of plate-bound CD3 (10 μg/ml) and soluble CD28 (1 μg/ml) mAbs for the indicated days. Live cell number of GFP^+ T cells in the bone marrow cell culture (GFP^+ T cell) and $CD3^+$ T cells in PBMC culture (control T) was calculated, and the %ratio of the live cell number on the indicated day to that on day 0 is demonstrated. Live cells were defined as cells negative for both Annexin V and propidium iodide stainings and calculated.

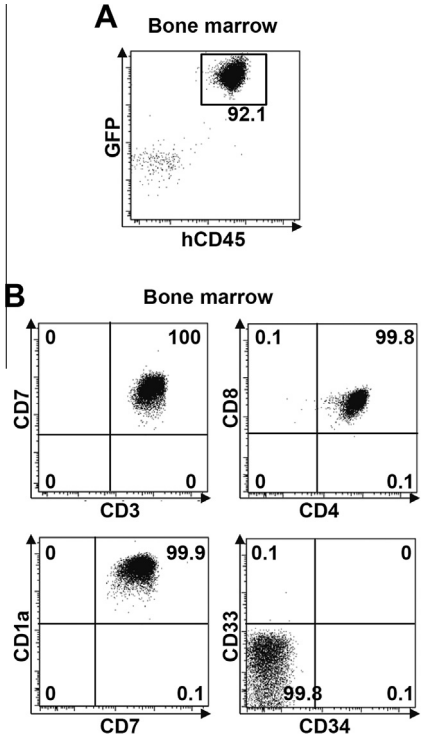

Fig. 3. Human T-ALL development in a humanized mouse. (A) Bone marrow cells almost consist of human GFP$^+$ hematopoietic cells. Bone marrow cells in the leukemic recipient mice were subjected to FACS analysis at 13 months after transfer with the GFP$^+$ bone marrow cells in the first host. (B) The phenotype of the leukemic cells were compatible with human T-ALL. Leukemic cells were analyzed for the cell surface expression of CD3, CD4, CD8, CD7, CD1a, CD33 and CD34.

within 10 kb of the integration sites were AI191600, BU689563, AW009875, and AF150238, all of which are expressed sequence tags and function-unknown putative genes.

4. Discussion

The present study demonstrated that active form Notch1-transduced human HSCs differentiated into phenotypically mature CD8$^+$ SP T cells in the bone marrow of humanized mice, probably independently of the thymus. In addition, T-ALL developed from ICN1-transduced human HSCs *in vivo* although only one case was found. Our humanized mouse model revealed, for the first time to our knowledge, *in vivo* contribution of Notch1 to human SP T cell development and human T cell leukemogenesis.

The role of Notch1 in DP to SP T cell development remained unresolved. Several previous studies revealed that Notch1 activation induces HSCs to develop up to DP T cells in both mouse and human systems [7–9]. In a previous humanized mouse model with NOD/Scid mice, CD8$^+$ SP T cells were not reconstituted from human HSCs transduced with ICN1 [16]. Although transplantation of mouse ICN1-expressing HSCs produced CD8$^+$ SP T cells in the bone marrow of recipient mice, use of thymectomized recipients or recipients lacking both MHC class I and II markedly diminished CD8$^+$ SP T cells [8,12]. Therefore, MHC-dependent thymic positive selection seems to be essential in mice. However, Notch signaling has been reported to promote CD8$^+$ T cell development rather than CD4 in *in vitro* human systems [27,28]. The present study demonstrated that Notch1 activation promote thymus-independent CD8$^+$ SP T cell differentiation. MHC requisite for CD8$^+$ SP T cell development between mouse and human might be different. However, human T cells reconstituted in our model immediately died *in vitro* and could not respond to *ex vivo* stimulation with CD3/CD28 mAbs, indicating defective function of the CD8$^+$ SP T cells. Therefore, functional maturation of CD8$^+$ SP T cells may require thymic microenvironment other than Notch signaling.

T-ALL is one of the human malignant diseases with aberrant Notch1 expression. In this study, we could find one case with T-ALL, which developed by 13 months after transplantation, although we observed 26 recipient mice, which had received secondary transplantation, by 14 months after transplantation. 14 months might be too short to develop leukemia in human systems although mouse T-ALL could be induced by abnormal Notch signaling for a shorter time [17,18,29]. Alternatively, the one case with T-ALL might have had an additional oncogenic hit by vector-insertional mutagenesis since K-ras, c-myc, and an active form of IL-7 receptor have been reported to cooperate with Notch activation in T-ALL leukemogenesis [29–31]. However, the four genes found by LAM-PCR were originally discovered as expressed sequence tags, and their function are still unknown. Therefore, further studies are necessary to address the oncogenic functions of the putative genes.

The successful *in vivo* development of CD8$^+$ SP T cells and T-ALL leukemogenesis from human HSCs suggest a useful animal model examining the functional roles of lymphopoiesis-associated genes, such as Notch1, in lymphopoiesis and leukemogenesis. The humanized mice models are useful not only for functional studies on lymphopoiesis but also for development of therapeutic strategies for leukemia.

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