Fig. S2. Colocalization of wild-type Ikaros and adult T-cell leukemia-type Helios.

Fig. S3. Dominant-negative inhibition of Hel-6, Hel-v1, and Hel-v2 in the suppressive activities of wild-type Helios and Ikaros.

Fig. S4. Downregulation of the expression of Helios mRNA in HTLV-1-positive T cell lines.

Fig. S5. Overexpression of abnormal Helios isoforms lacking exon 6 in adult T-cell leukemia samples.

Fig. S6. Relative value of Helios transcripts skipping exon 3 to all is upregulated in primary adult T-cell leukemia cells.

Fig. S7. Upregulated expression of Hes1 in primary adult T-cell leukemia cells.

Table S1. Clinical characteristics of adult T-cell leukemia patients and HTLV-1 carriers.

Table S2. Primer list and probe sequences.

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Forefront studies on HTLV-1 oncogenesis

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Almost 40 years ago, Takatsuki et al. recognized the existence of a peculiar T cell leukemia in Kyoto, Japan that they named Adult T Leukemia (ATL). They reported a series of 13 patients in 1976 (Uchiyama et al., 1977). In 1980, the group of Gallo reported the discovery of a human oncogenic retrovirus that they named Human T cell Leukemia Virus type 1 (HTLV-1) in cells obtained from two US patients classified as mycosis fungoides and Sezary syndrome (Poiesz et al., 1980), but who were, in retrospect, probably suffering from ATL [for an historical perspective see (Takatsuki, 2005)]. Shortly after, the groups of Hinuma (Miyoshi et al., 1981) and of Yoshida (Yoshida et al., 1982) uncovered the presence of HTLV-1 in cells obtained from ATL patients. In 1985–1986, two groups independently reported that a neurological disease named HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP) was also caused by HTLV-1 (Gessain et al., 1985; Osame et al., 1986). Since then, other inflammatory diseases (uveitis, infective dermatitis) have also been linked to this viral infection. Other members of the HTLV family (i.e., HTLV-2, HTLV-3, and HTLV-4 have also now been reported, none of them being clearly associated so far with an oncogenic process or a neurodegenerative disease (Kalyanaraman et al., 1982; Calattini et al., 2005; Wolfe et al., 2005).

Almost 10 years ago, ours colleagues Kuan Teh Jeang and Mitsuaki Yoshida organized a special issue on HTLV infection in Oncogene. In setting up this issue, we cannot forget the memory our friend Teh.

We called upon the expertise of different research groups from Europe, Japan, and USA. However, we regret that the format of this issue prevented us from soliciting many other colleagues. The following reviews will deal with many fascinating aspects of viral cycle, but summarizes also new approaches that should allow a better integrated research.

A first group of articles provides information about HTLV-1 epidemiology and associated-pathogenesis. The article from Gessain and Cassar provides an updated view on HTLV-1 distribution, based on data obtained from 1.5 billion individuals originating from endemic areas (Gessain and Cassar, 2012). Iwanaga et al. focused their review on ATL epidemiology and show its peculiar characteristic [age at onset, risk factor, proviral load, etc. (Iwanaga et al., 2012)]. Yamano and Sato provide an interesting perspective on HAM/TSP physiopathology, and remind us that optimal therapeutic treatments are still lacking for those patients (Yamano and Sato, 2012). The review by Kamoi ad Mochizuki summarizes our current knowledge on HTLV-1 uveitis, which is the most common cause of uveitis in endemic areas (Kamoi and Mochizuki, 2012). Going deeper in the pathological mechanisms linked to HTLV-1 infection, Yamagishi and Watanabe summarize recent data showing that ATL cells express abnormally low levels of a cellular oncosuppressor miRNA and display some epigenetic changes on the promoter of genes critical for cell cycle (Yamagishi and Watanabe, 2012).

A second group of articles summarizes the interaction between the virus and the host's cells. Before causing diseases, HTLV-1 has to enter the cell. However, the mechanisms of HTLV-1 transmission and cell entry have remained elusive for a long period of time. Pique and Jones have summarized recent insights about those mechanisms both at the cell level but also between individuals (Pique and Jones, 2012). HTLV-1 associated diseases are linked to the fact that HTLV-1 evades both adaptative and innate immune responses. Kannagi et al. provide us with an exciting review, which explains us how the virus evades the interferon response, but also that dysfunction of the CTL response might be a risk factor for disease development in infected carriers (Kannagi et al., 2012).

A third group of articles reports data on individual viral proteins that play important roles in the viral cycle and/or in pathogenesis. Nakano and Watanabe remind us the important role played by Rex, which uses cellular pathways to export unspliced or singly spliced viral mRNAs in the cell cytoplasm, therefore allowing expressing of structural proteins (Nakano and Watanabe, 2012). Currer et al., Zhao and Matsuoka focused their attention on Tax and HBZ, two viral proteins that play important roles in the control of viral transcription and oncogenesis (Currer et al., 2012; Zhao and Matsuoka, 2012). Finally, Bai and Nicot provide an overview on 4 auxiliary viral proteins (p12, p8, p30, and p13), which are required for establishing a persistent infection *in vivo* (Bai and Nicot, 2012).

Finally, Duc Dodon and colleagues remind us that studying HTLV-1 pathogenesis requires animal models (Dodon et al., 2012). Rabbits, rats, transgenic mice, and monkeys have been used in the past. However, recent approaches using humanized mice might represent an interesting alternative for studying HTLV-1 associated diseases.

REFERENCES

Bai, X. T., and Nicot, C. (2012). Overview on HTLV-1 p12, p8, p30, p13, accomplices in persistent infection and viral pathogenesis. *Front. Microbiol.* 3:400. doi: 10.3389/fmicb.2012.00400

Calattini, S., Chevalier, S. A., Duprez, R., Bassot, S., Froment, A., Mahieux, R., et al. (2005). Discovery of a new human T-cell lymphotropic virus (HTLV-3) in Central Africa. *Retrovirology* 2:30. doi: 10.1186/1742-4690-2-30

Currer, R., Van Duyne, R., Jaworski, E., Guendel, I., Sampey, G., Das, R., et al. (2012). HTLV tax: a fascinating multifunctional co-regulator of viral and cellular pathways. *Front. Microbiol.* 3:406. doi: 10.3389/fmicb.2012.00406

Dodon, M. D., Villaudy, J., Gazzolo, L., Haines, R., and Lairmore, M. (2012). What we are learning on HTLV-1 pathogenesis from animal models. *Front. Microbiol.* 3:320. doi: 10.3389/fmicb.2012.00320

Gessain, A., Barin, F., Vernant, J. C., Gout, O., Maurs, L., Calender, A., et al. (1985). Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* 2, 407–410. doi: 10.1016/S0140-6736(85)92734-5

Gessain, A., and Cassar, O. (2012). Epidemiological aspects and world distribution of HTLV-1 infection. *Front. Microbiol.* 3:388. doi: 10.3389/fmicb.2012.00388

Iwanaga, M., Watanabe, T., and Yamaguchi, K. (2012). Adult T-cell leukemia: a review of epidemiological evidence. *Front. Microbiol.* 3:322. doi: 10.3389/fmicb.2012.00322

Kalyanaraman, V. S., Sarngadharan, M. G., Robert-Guroff, M., Miyoshi, I., Golde, D., and Gallo, R. C. (1982). A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. *Science* 218, 571–573. doi: 10.1126/science.6981847

Kamoi, K., and Mochizuki, M. (2012). HTLV-1 uveitis. *Front. Microbiol.* 3:270. doi: 10.3389/fmicb.2012.00270

Kannagi, M., Hasegawa, A., Takamori, A., Kinpara, S., and Utsunomiya, A. (2012). The roles of acquired and innate immunity in human T-cell leukemia virus type 1-mediated diseases. *Front. Microbiol.* 3:323. doi: 10.3389/fmicb.2012.00323

Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., et al. (1981). Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. *Nature* 294, 770–771. doi: 10.1038/294770a0

Nakano, K., and Watanabe, T. (2012). HTLV-1 Rex: the courier of viral messages making use of the host vehicle. *Front. Microbiol.* 3:330. doi: 10.3389/fmicb.2012.00330

Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitani, H., Igata, A., et al. (1986). HTLV-I associated myelopathy, a new clinical entity. *Lancet* 1, 1031–1032. doi: 10.1016/S0140-6736(86)91298-5

Pique, C., and Jones, K. S. (2012). Pathways of cell-cell transmission of HTLV-1. *Front. Microbiol.* 3:378. doi: 10.3389/fmicb.2012.00378

Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., and Gallo, R. C. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. U.S.A.* 77, 7415–7419. doi: 10.1073/pnas.77.12.7415

Takatsuki, K. (2005). Discovery of adult T-cell leukemia. *Retrovirology* 2:16. doi: 10.1186/1742-4690-2-16

Uchiyama, T., Yodoi, J., Sagawa, K., Takatsuki, K., and Uchino, H. (1977). Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood* 50, 481–492.

Wolfe, N. D., Heneine, W., Carr, J. K., Garcia, A. D., Shanmugam, V., Tamoufe, U., et al. (2005). Emergence of unique primate T-lymphotropic viruses among central African bushmeat hunters. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7994–7999. doi: 10.1073/pnas.0501734102

Yamagishi, M., and Watanabe, T. (2012). Molecular hallmarks of adult T cell leukemia. *Front. Microbiol.* 3:334. doi: 10.3389/fmicb.2012.00334

Yamano, Y., and Sato, T. (2012). Clinical pathophysiology of human T-lymphotropic virus-type 1-associated myelopathy/tropical spastic paraparesis. *Front. Microbiol.* 3:389. doi: 10.3389/fmicb.2012.00389

Yoshida, M., Miyoshi, I., and Hinuma, Y. (1982). Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci. U.S.A.* 79, 2031–2035. doi: 10.1073/pnas.79.6.2031

Zhao, T., and Matsuoka, M. (2012). HBZ and its roles in HTLV-1 oncogenesis. *Front. Microbiol.* 3:247. doi: 10.3389/fmicb.2012.00247

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Immunity
Article

The Satb1 Protein Directs Hematopoietic Stem Cell Differentiation toward Lymphoid Lineages

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SUMMARY

How hematopoietic stem cells (HSCs) produce particular lineages is insufficiently understood. We searched for key factors that direct HSC to lymphopoiesis. Comparing gene expression profiles for HSCs and early lymphoid progenitors revealed that Satb1, a global chromatin regulator, was markedly induced with lymphoid lineage specification. HSCs from Satb1-deficient mice were defective in lymphopoietic activity in culture and failed to reconstitute T lymphopoiesis in wild-type recipients. Furthermore, Satb1 transduction of HSCs and embryonic stem cells robustly promoted their differentiation toward lymphocytes. Whereas genes that encode Ikaros, E2A, and Notch1 were unaffected, many genes involved in lineage decisions were regulated by Satb1. Satb1 expression was reduced in aged HSCs with compromised lymphopoietic potential, but forced Satb1 expression partly restored that potential. Thus, Satb1 governs the initiating process central to the replenishing of lymphoid lineages. Such activity in lymphoid cell generation may be of clinical importance and useful to overcome immunosenescence.

INTRODUCTION

To maintain the immune system, hematopoietic stem cells (HSCs) differentiate to lymphoid-primed multipotent progenitors (LMPPs) and then to lymphoid-specified progenitors in a process accompanied by the loss of erythroid-megakaryocyte and myeloid potential (Adolfsson et al., 2005; Lai and Kondo, 2008). Accumulating evidence has suggested that combinations of transcription factors coordinately and sequentially regulate lymphopoiesis. Five transcription factors, PU.1, Ikaros, E2A, EBF, and Pax5 are hierarchically involved in the early steps of B-lineage differentiation (Medina et al., 2004). Whereas EBF and Pax5 specifically act in B-lineage-determined progenitors, PU.1 and Ikaros are expressed in earlier hematopoietic progenitors and involved in multiple lineage decision processes (Scott et al., 1997; Yoshida et al., 2006). E2A, an indispensable factor for B lymphopoiesis, can also affect T lymphocyte formation by regulating Notch1 expression (Ikawa et al., 2006). Furthermore, recent reports have shown that E2A proteins are expressed in primitive hematopoietic progenitors and play a critical role in early lymphoid specification (Dias et al., 2008; Yang et al., 2008; Semerad et al., 2009). However, whether the initiation of lymphoid differentiation is regulated entirely by transcription factors in a hierarchical manner remains unclear.

The immune system changes qualitatively and quantitatively with ontogeny and age (Miller and Allman, 2005; Montecino-Rodriguez and Dorshkind, 2006). Indeed, lymphocyte progenitors expand substantially in the fetal liver (FL), but their production shifts to bone marrow (BM) and becomes stable after birth. With age, replenishment of the adaptive immune system declines (Rossi et al., 2005; Sudo et al., 2000). Qualitative changes in lymphopoietic activity of HSCs are reflected in in vitro cell-culture experiments. If key inducers in early lymphoid lineages can be identified, they will be useful for expanding lymphocytes in culture for clinical purposes. Additionally, manipulating the expression of relevant genes might boost the immune system of immunocompromised and elderly people.

We have developed a method to sort early lymphoid progenitors (ELPs) from Rag1-GFP reporter mice (Igarashi et al., 2002; Yokota et al., 2003a). ELPs expressing Rag1 are present in the Sca1^{+}c-kit^{hi} HSC-enriched fraction; they displayed high B and T lymphopoietic potential, but limited myeloerythroid potential and self-renewal ability. In contrast, Rag1 Sca1^{+}c-kit^{hi} HSCs effectively reconstitute and sustain the lymphohematopoietic system for long periods in lethally irradiated recipients. We conducted gene array comparisons between those two fractions

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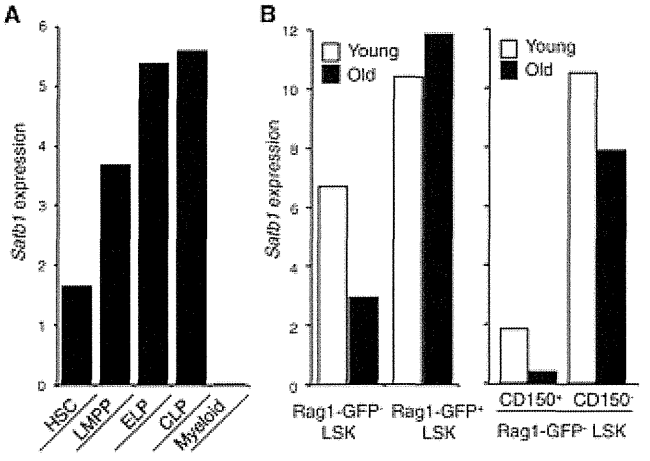

Figure 1. *Satb1* Expression Levels Change with Differentiation and Aging of HSCs

HSCs, LMPP, ELP, CLP, and the myeloid progenitor-enriched fractions were sorted from BM of 8- to 10-week-old Rag1-GFP knockin or WT mice according to cell surface markers and GFP expression (see Experimental Procedures), and transcripts for *Satb1* were quantitatively evaluated with real-time RT-PCR. (B) The LSK Rag1-GFP$^-$ and LSK Rag1-GFP$^+$ fractions (left panel) or the CD150$^+$ LSK Rag1-GFP$^-$ and CD150$^-$ LSK Rag1-GFP$^-$ fractions (right panel) were sorted from 6-week-old or 2-year-old Rag1-GFP knockin mice, respectively. Then *Satb1* expression was evaluated with real-time RT-PCR. The *Satb1* expression values were normalized by *Gapdh* expression and shown in each panel. Each data represents two independent examinations that showed essentially the same results (Figure 1; see also Figure S1 and Table S1).

with the goal of discovering molecules involved in the transition of HSCs to lymphoid lineages.

Herein, we showed that special AT-rich sequence binding 1 (Satb1), a nuclear architectural protein that organizes chromatin structure, plays an important role in lymphoid lineage specification. In parallel with or ahead of key transcription factors, the expression of Satb1 increased with early lymphoid differentiation. In functional assays, lymphopoietic activity was compromised in Satb1-deficient hematopoietic cells, but the induced expression of Satb1 strongly enhanced lymphocyte production from HSCs. Furthermore, exogenous Satb1 expression primed lymphoid potential even in embryonic stem cell (ESC)-derived mesoderm cells and aged BM-derived HSCs. Global analysis of potential Satb1 target genes identified a number that may have critical roles in early lymphopoiesis. The findings demonstrate that the earliest steps in lymphopoiesis are regulated by an epigenetic modifier and indicate how modulation of the process might be used to induce or rejuvenate the immune system.

RESULTS

Profiling Gene Expression of Rag1$^+$ ELP in Fetal Liver

We sorted the Rag1^{lo} c-kit^{hi} Sca1$^+$ ELP fraction and the Rag1 c-kit^{hi} Sca1$^+$ HSC-enriched fraction with high purity from E14.5 FL of Rag1-GFP knockin heterozygous embryos and performed gene arrays. We found that transcripts of *Trbv14* and *Ighm* genes were upregulated even in very early lymphoid progenitors (see Table S1 available online). Furthermore, we detected increased expression of *Il7r*, *Notch1*, and *Flt3* genes encoding cell surface receptors important for B or T lymphocyte differentiation in the ELP fraction. In addition to discovering many signal transduction kinases with unknown functions in lymphopoiesis, our search identified *Lck* and *Xlr4b* genes as being involved in lymphoid differentiation signals. Transcripts for some of these lymphoid-related genes had already been detected in the Rag1$^-$ HSC-enriched fraction (see the microarray data; accession number CBX73). These results suggest that lymphoid-lineage specification begins even before the emergence of Rag1^{lo} ELP. Additionally, the microarray data identified new candidate genes that might be important for early lymphoid development.

Expression of *Satb1* Increases with Early Lymphoid Specification and Declines with Age

Our major goal was to find key genes involved in the specification of lymphoid fates. Because the microarray data showed that expression of various lymphoid-related genes was activated before the ELP stage, we hypothesized the existence of a modulator that synchronously regulates multiple genes. Among the list in Table S1, *Satb1* attracted attention because it was originally identified as a protein binding to the enhancer region of the *Igh* gene and later shown to play a critical role in T cell development (Alvarez et al., 2000; Dickinson et al., 1992). Additionally, recent studies had demonstrated that it serves as a master regulator for many genes, including cytokines, cytokine receptors, and transcription factors (Cai et al., 2006; Han et al., 2008; Notani et al., 2010; Yasui et al., 2002).

To explore possible relationships between Satb1 and early lymphopoiesis, we examined its expression in primitive hematopoietic progenitors. The HSC-enriched Rag1-GFP$^-$ Flt3$^-$ lineage marker-negative (Lin$^-$) Sca1$^+$ c-kit^{hi} (LSK) fraction, the LMPP-enriched fraction, the ELP-enriched fraction, the common lymphoid progenitor (CLP)-enriched fraction, and the myeloid progenitor-enriched Lin$^-$ c-kit^{hi} Sca1$^-$ fraction were sorted from BM of 8- to 10-week-old mice. Transcripts for *Satb1* were then quantitatively evaluated with real-time RT-PCR. *Satb1* expression increased substantially when HSC differentiated into LMPP and ELP (Figure 1A). This trend matched that of other early lymphoid lineage-related genes including those that encode PU.1 (*Sfpi1*), Ikaros (*Ikzf1*), E2A (*Tcf3*), and Notch1 (Figure S1). Importantly, in contrast to its expression in the lymphoid lineage, *Satb1* expression was shut off when HSC differentiated to committed myeloid progenitors. These results suggest that *Satb1* is potentially involved in early lymphoid differentiation.

Lymphopoietic activity becomes compromised during aging. Accumulating evidence suggests that the earliest lymphoid progenitor pools proximal to HSC are deficient in aged BM (reviewed by Miller and Allman, 2005). Indeed, the Rag1$^+$ ELP population markedly decreases with age (data not shown). The downregulation of genes mediating lymphoid specification and function is likely a major cause (Rossi et al., 2005). Because *Satb1* has been listed in microarray panels as a downregulated gene in aged HSC (Chambers et al., 2007; Rossi et al., 2005), we sorted Rag1-GFP$^-$ LSK and ELP-enriched Rag1-GFP$^+$ LSK from BM of 6-week-old or 2-year-old Rag1-GFP heterozygous mice and examined their expression. In agreement with previous studies, our real-time RT-PCR identified an approximate 50% reduction of *Satb1* transcripts in aged Rag1-GFP$^-$ LSK cells (Figure 1B, left panel). The few ELP recovered from aged mice

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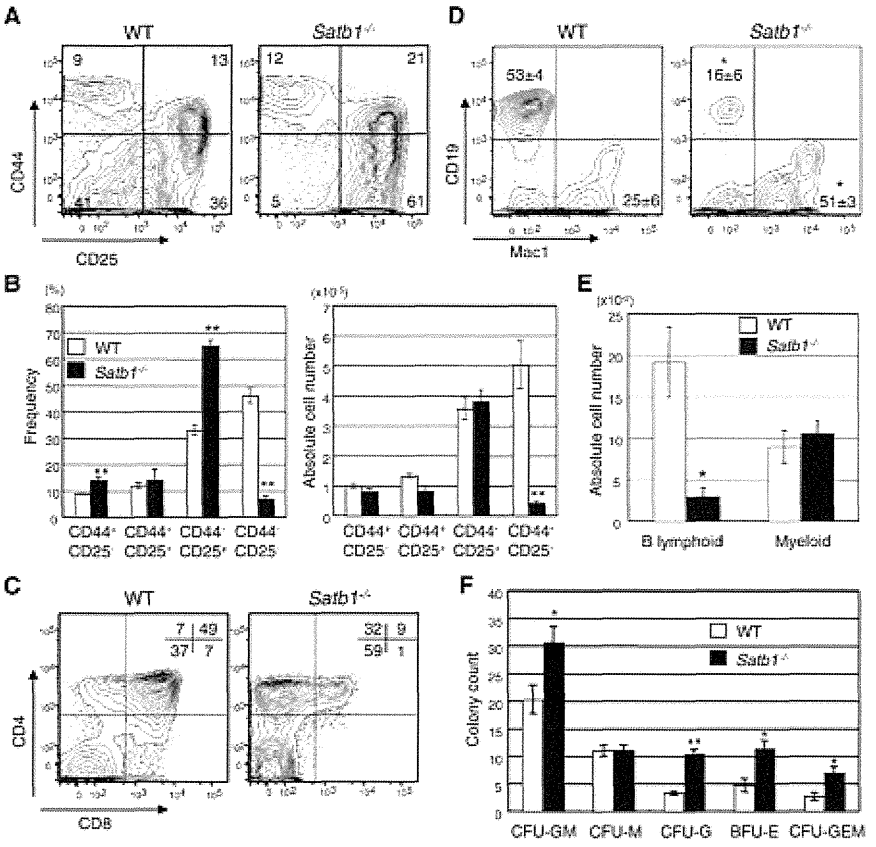

Figure 2. Satb1 Deficiency Alters Lymphoid and Myeloid Activities of Hematopoietic Stem/Progenitor Cells in Culture

Lin^- cells were isolated from FL of E14.5 *Satb1*$^{-/-}$ embryos or their WT littermates.

(A–C) Cells were cocultured with OP9-DL1 stromal cells for evaluation of T-lineage differentiation. (A) Flow cytometry results are shown for cells recovered on day 14 and stained for CD44 and CD25/IL-2Rα. (B) Frequencies and absolute numbers of each phenotype were calculated. (C) A similar analysis was performed for CD4 and CD8α bearing cells recovered cells on day 18.

(D and E) The same cell suspensions were cocultured with MS5 stromal cells to assess B and myeloid lineage potentials and representative data are shown for day 7 of culture.

(F) In parallel, the Lin^- cells were evaluated with methylcellulose colony assays. Each dish contained 1,000 sorted cells and colony counts were performed on day 10. The bars indicate numbers of CFU-GM, CFU-M, CFU-G, BFU-E, or CFU-GEM scored per dish. The results are shown as mean ± SE. Statistically significant differences between WT and *Satb1*$^{-/-}$ cells are marked with asterisks (*$p < 0.05$, **$p < 0.01$) (Figure 2; see also Figure S2).

expressed amounts of *Satb1* comparable to those in ELP from young mice. Recent purification methods for HSC with CD150, a SLAM family receptor that marks HSC even in aged BM (Yilmaz et al., 2006), identified an approximate 80% reduction in *Satb1* transcripts in aged HSC compared with ones from young mice (Figure 1B, right panel). These observations suggest that *Satb1* may be a key molecule related to immunosenescence.

Satb1 Deficiency Reduces the Lymphopoietic Activity of Hematopoietic Stem and Progenitor Cells

T cell development in the thymus is impaired in *Satb1*$^{-/-}$ mice (Alvarez et al., 2000). Although the profile of B220, immunoglobulin M (IgM), and IgD expression appears to be unaffected in the *Satb1*$^{-/-}$ spleen, the total number of B cells is reportedly reduced to approximately 25% of wild-type (WT) at 2 weeks of age (Alvarez et al., 2000). We have determined that the number and frequency of cells that can be recovered from lymphoid organs were reduced in E18.5–19.5 *Satb1*$^{-/-}$ fetuses. Body sizes of *Satb1*$^{-/-}$ fetuses were not different from WT and heterozygous littermates (Figures S2A and S2B).

We then sorted Lin^- cells from FL of *Satb1*$^{-/-}$ mice or their WT littermates and cultured them with stromal cells that support lymphopoiesis. T cell differentiation can be recapitulated in vitro with hematopoietic cells cultured with OP9 expressing the Notch ligand Delta-like 1 (OP9-DL1). Under these coculture conditions, the differentiation patterns of WT and *Satb1*$^{-/-}$ Lin^- cells differed significantly (Figures 2A and 2B). The majority of *Satb1*$^{-/-}$ cells were arrested in the $CD44^-CD25^+$ stage and did not differentiate into $CD44^-CD25^-$ cells. Reduction of IL-7 from culture media normally induces maturation of the $CD4^-CD8^-$ double-negative (DN) into $CD4^+CD8^+$ double-positive (DP) cells and subsequently into either the $CD4^+$ or the $CD8^+$ single-positive cells. However, more than half of the *Satb1*$^{-/-}$ cells were arrested in DN stages even after the IL-7 reduction, and their differentiation to the DP stage was aberrantly skewed toward $CD4^+CD8^-$ (Figure 2C).

Substantial differences were also observed in B-lineage cell production. In coculture with MS5, which supports B and myeloid lineages in the presence of SCF, Flt3-ligand, and IL-7, *Satb1*$^{-/-}$ progenitors exhibited significant reductions in B-lymphopoietic potential (Figures 2D and 2E). Coculture with OP9, which originated with M-CSF-deficient mice and supported the B lineage predominantly, also yielded reduced B/myeloid ratios with *Satb1*$^{-/-}$ progenitors (Figure S2C). Essentially the same results were obtained when cultures were initiated with LSK $Flt3^-$, more stringently purified HSC (Figure S2D, 2E). In addition, B cell lineage output was also reduced when *Satb1*$^{-/-}$ LMPP or CLP were cultured (Figure S2F). In contrast, the myeloid potential was retained in *Satb1*$^{-/-}$ progenitors (Figures 2D and 2E). Indeed, the Lin^- fraction of E14.5 *Satb1*$^{-/-}$ FL contained more myeloid-erythroid progenitors than that of the WT control (Figure 2F).

In transplantation experiments, we observed that $CD45.2^+$ *Satb1*$^{-/-}$ HSC sorted from 2-week-old BM did not effectively reconstitute $CD3^+$ T-lineage cells in lethally irradiated $CD45.1^+$ WT recipients (Figure 3A). Peripheral blood $CD3^+$ T-lineage recoveries from *Satb1*$^{-/-}$ HSC were decreased approximately 90% compared with that from WT HSC (Figure 3B). Conversely, we observed varied amounts of reconstitution of the B lineage and no reduction in reconstitution of the myeloid lineage

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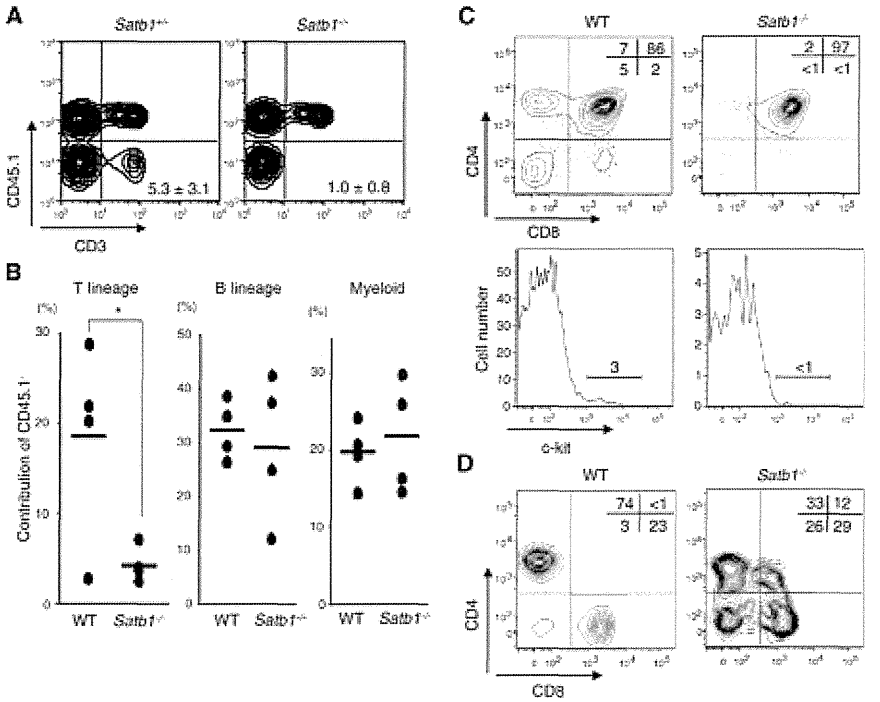

Figure 3. Defective T Lymphopoiesis from Transplanted *Satb1*$^{-/-}$ HSC

(A and B) One thousand stem-cell-enriched Flt3$^-$ LSK cells were sorted from BM of 2-week-old Satb1 deficient or littermate mice (CD45.2). They were then mixed with 4 × 10^5 adult BM cells obtained from WT (CD45.1) mice and were transplanted into lethally irradiated WT CD45.1 mice. At 8 weeks after transplantation, peripheral blood cells of the recipients were identified with anti-CD45.1 and anti-CD3. Numbers in each panel of (A) represent percentages of CD3$^+$ CD45.1$^-$ cells among the total leukocytes and are shown as averages with SD (n = 4 in each). Chimerisms of CD45.1$^-$ cells in the CD3$^+$ T-lineage, the CD45R/B220$^+$ B lineage, or the Gr1$^+$ myeloid lineage were determined. Statistical significance is $^*p < 0.05$.

(C and D) One thousand Flt3$^-$ LSK cells sorted from E14.5 FL of Satb1 homodeficient or their WT littermates (CD45.2) were transplanted into lethally irradiated WT CD45.1 mice. At 8 weeks after transplantation, T-lineage reconstitution in the thymus and the spleen was analyzed. The CD4 and CD8 profiles of CD45.2$^+$ thymocytes (C, upper panels) and the c-kit expression of CD45.2$^+$ CD3$^-$ CD4$^-$ CD8$^-$ CD44$^+$ CD25$^-$ thymocytes (C, lower panels) are shown. (D) Representative CD4 and CD8 profiles are shown for CD45.2$^+$ CD3$^+$ cells in recipient spleens.

resulted from Satb1 ablation (Figure 3B). Compromised T cell lineage contributions of *Satb1*$^{-/-}$ HSC were also evident in the thymus and spleen (Figures 3C and 3D). Although T lymphopoiesis in the thymus was replaced by either WT or *Satb1*$^{-/-}$ donor cells when FL HSCs were transplanted, thymocytes were reduced in the *Satb1*$^{-/-}$ recipients and their differentiation was affected. Besides apparent stagnation at the DP stage and marked reduction of the DN population (Figure 3C, upper panels), c-kit^{hi} cells in the CD44$^+$CD25$^-$ DN1 stage were rare in *Satb1*$^{-/-}$ recipients (Figure 3C, lower panels). The reduced contribution of *Satb1*$^{-/-}$ cells was also evident in CD3$^+$ splenic T lymphocytes. Interestingly, T cells in the spleens of *Satb1*$^{-/-}$ recipients contained substantial percentages of DP and DN cells. Such T cell lineage cells are extremely rare in normal mouse spleens (Figure 3D).

Taken together, these results demonstrate that Satb1 is indispensable for normal T lymphopoiesis, but not for myelopoiesis. The factor may normally have a lesser role in B-lineage differentiation. Furthermore, our data indicate that abnormalities of lymphoid development observed in *Satb1*$^{-/-}$ mice are intrinsic to *Satb1*$^{-/-}$ hematopoietic cells.

Forced Expression of Satb1 in HSC Induces Lymphopoiesis

Next we conducted overexpression experiments to define the role of Satb1 in lineage-fate decisions of HSCs. LSK Flt3$^-$ cells were sorted from BM of adult WT mice and then retrovirally transduced with either a fluorescence-alone expressing control or a native Satb1 construct combined with a GFP-expressing vector. Successfully transduced cells were sorted according to GFP expression. Real-time RT-PCR and immunoblots revealed that Satb1-transduced cells expressed more than 10-fold *Satb1* transcripts and Satb1 proteins compared to control cells (Figure S3A).

The sorted cells were cultured with stromal cells that supported lymphopoieisis. Results from these experiments complemented the observations with *Satb1*$^{-/-}$ cells. Satb1 transduction enhanced T cell lineage growth in OP9-DL1 cocultures (Figures 4A and 4B). By day 10 of the culture, cells had been increased more than 5-fold by Satb1-transduction, and a majority of the recovered cells had progressed to the DN2 and DN3 stages. Differentiation to the DP stage was also advanced by the Satb1-transduction (Figure 4A). The kinetics of cell differentiation and expansion in the B cell lineage showed more changes. Whereas both control and Satb1-transduced cells produced substantial numbers of B-lineage cells, the latter produced B220$^+$CD19$^+$ cells more quickly and efficiently (Figure S3B). Specifically, the Satb1 transduction resulted in approximately 50- to 300-fold and 5-fold greater recovery of B220$^+$CD19$^+$ cells on day 10 in the MS5 and OP9 cocultures, respectively (Figures 4C and 4D). Notably, Satb1 transduction negatively influenced the output of myeloid cells, particularly Mac1^{lo}Gr1$^+$ granulocytes (Figure S3C). In addition, CFU-GM formation of HSC was decreased by Satb1 transduction (Figure S3D).

In stromal-free cultures containing SCF, Flt3-ligand, and IL-7, Satb1 expression strongly induced CD19$^+$ cell production from the LSK fraction (Figure S3E). When calculated on a per-cell basis, one LSK cell with Satb1 overexpression produced approximately 450 CD19$^+$ cells, whereas only 50 cells with this B-lineage marker were produced from control progenitors. As for other hematopoietic lineages, DX5$^+$CD3e$^-$ NK cells emerged when IL-15 was added to the stromal cell-free cultures. Coexpression of NK1.1 and/or CD94 confirmed the NK-lineage, and

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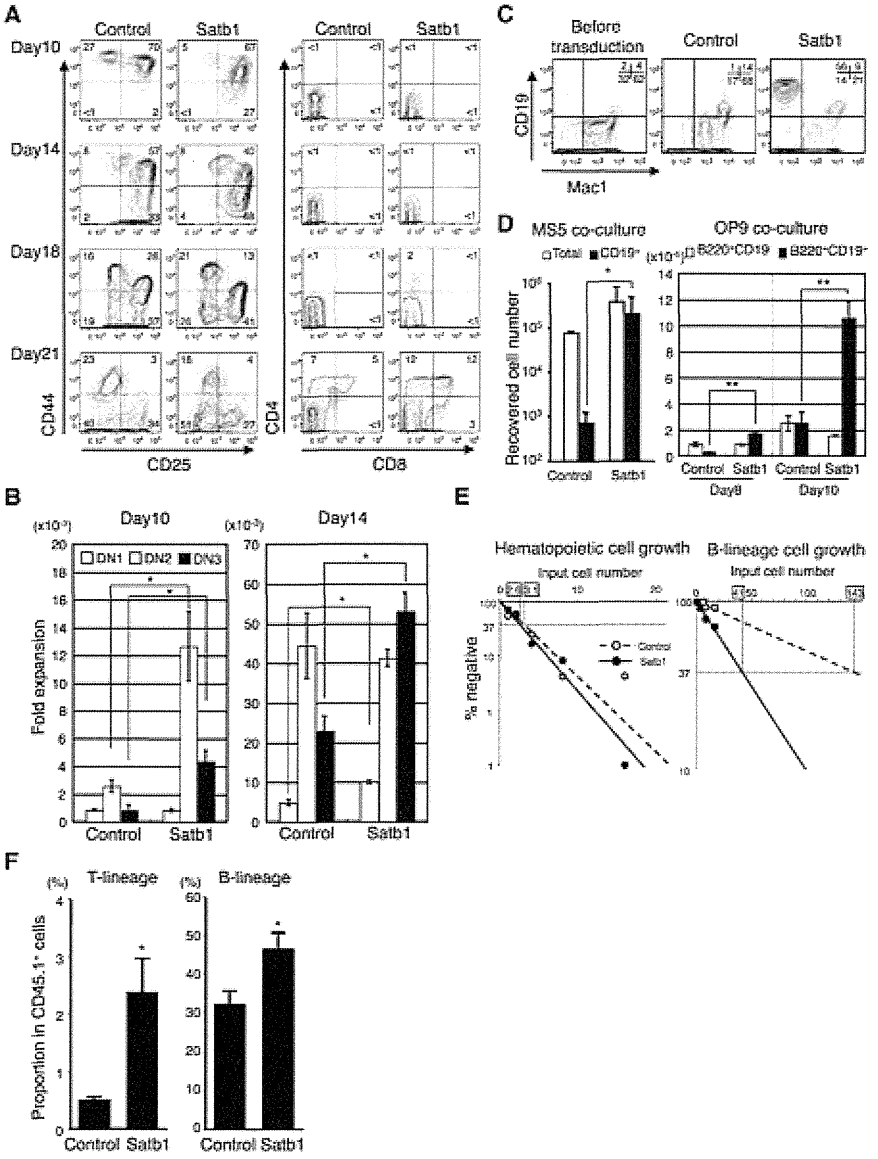

Figure 4. Satb1 Overexpression Promotes Lymphopoiesis

LSK Flt3$^-$ cells obtained from WT BM were retrovirally transduced with either a fluorescence-alone expressing control or a native Satb1 combined with GFP expressing vector. Successfully transduced cells were cultured, and their differentiation and proliferation were analyzed at the indicated period.

(A and B) Time-course analyses were performed for T-lineage cell generation in the OP9-DL1 coculture. Absolute numbers of recovered cells were divided by the numbers of transduced LSK Flt3$^-$ cells used to initiate the cultures to obtain the fold expansion values. Data are shown as mean ± SE.

(C) CD19 and Mac1 profiles are shown for cells recovered from MS5 cocultures on day 10. The left panel shows data obtained from fresh LSK Flt3$^-$ cells that did not undergo the retroviral infection.

(D) The absolute numbers of total recovered cells and B-lymphoid cells in the MS5 coculture (left panel). The output of B220$^+$ CD19$^-$ or B220$^+$ CD19$^+$ B-lineage cells was evaluated in the OP9 coculture (right panel). Cultures were established in triplicate. Data are shown as mean ± SE. Statistical significance is $^*p < 0.05$, $^{**}p < 0.01$.

(E) Limiting-dilution analyses were performed to determine the frequencies of hematopoietic progenitors that could give rise to CD19$^+$ B-lineage cells. Input cell numbers corresponding to each 37% negative value are shown in rectangles.

(F) One thousand LSK Flt3$^-$ cells (CD45.1) transduced with either Satb1-expressing or control vectors were transplanted to lethally irradiated WT mice (CD45.2) with 1×10^5 adult BM cells (CD45.2). Two weeks after transplantation, peripheral blood was collected to determine the proportion of CD4/CD8$^+$ T lineage and CD19$^+$ B lineage in CD45.1$^+$ cells. Data are shown as mean ± SE. Statistical significance is $^*p < 0.05$. (n = 5 in each group) (Figure 4, see also Figure S3).

their numbers were also enhanced by Satb1 overexpression (Figure S3F). Interestingly, the same Satb1-transduced LSKs differentiated to neither conventional nor plasmacytoid dendritic cells (Figure S3G).

The results from in vitro bulk cultures and assessment of lymphoid lineage cell numbers might reflect enhanced survival of lymphoid progenitors rather than priming or expansion of lymphoid potential in individual clones. Notably, no obvious increase in apoptotic cells occurred in any tested cultures with *Satb1*$^{-/-}$ cells or *Satb1*$^{-/-}$ lymphopoietic organs (data not shown and Figure S3H). Additionally, Satb1 overexpression conferred growth advantages to hematopoietic progenitors without influencing their viability in any of the cultures we used (data not shown). To investigate further the mechanisms through which Satb1 exerts its effect on early progenitors, we performed limiting dilution assays. On average, 1 in 3.1 control cells and 1 in 2.6 Satb1-transduced cells gave rise to blood cells, indicating that both are highly potent progenitors for hematopoietic cell growth (Figure 4E, left). Nevertheless, we observed significant differences between them regarding the frequencies of progenitors with lymphopoietic potential. While 1 in 41 Satb1-transduced Flt3$^-$ LSK cells produced B cells, only 1 in 143 control cells were lymphopoietic under these conditions (Figure 4E, right). In the same experiment, fresh Flt3$^-$ LSK cells without retroviral transfection produced hematopoietic cells and B cells at a frequency of 1 in 6.7 cells and 1 in 61 cells, respectively (data not shown).

These results suggest that Satb1 expression affects early lineage decisions in individual HSC and expands the growth and differentiation of lymphoid cells in vitro. To evaluate whether these findings were of practical value, we performed in vivo transplantation experiments with SATB1-transduced LSK Flt3$^-$ cells. We observed enhanced contribution of the SATB1-transduced cells to both T and B lineages in short-term engraftment (Figure 4F). To assess whether the overexpression of SATB1 induces tumors, we evaluated long-term and short-term

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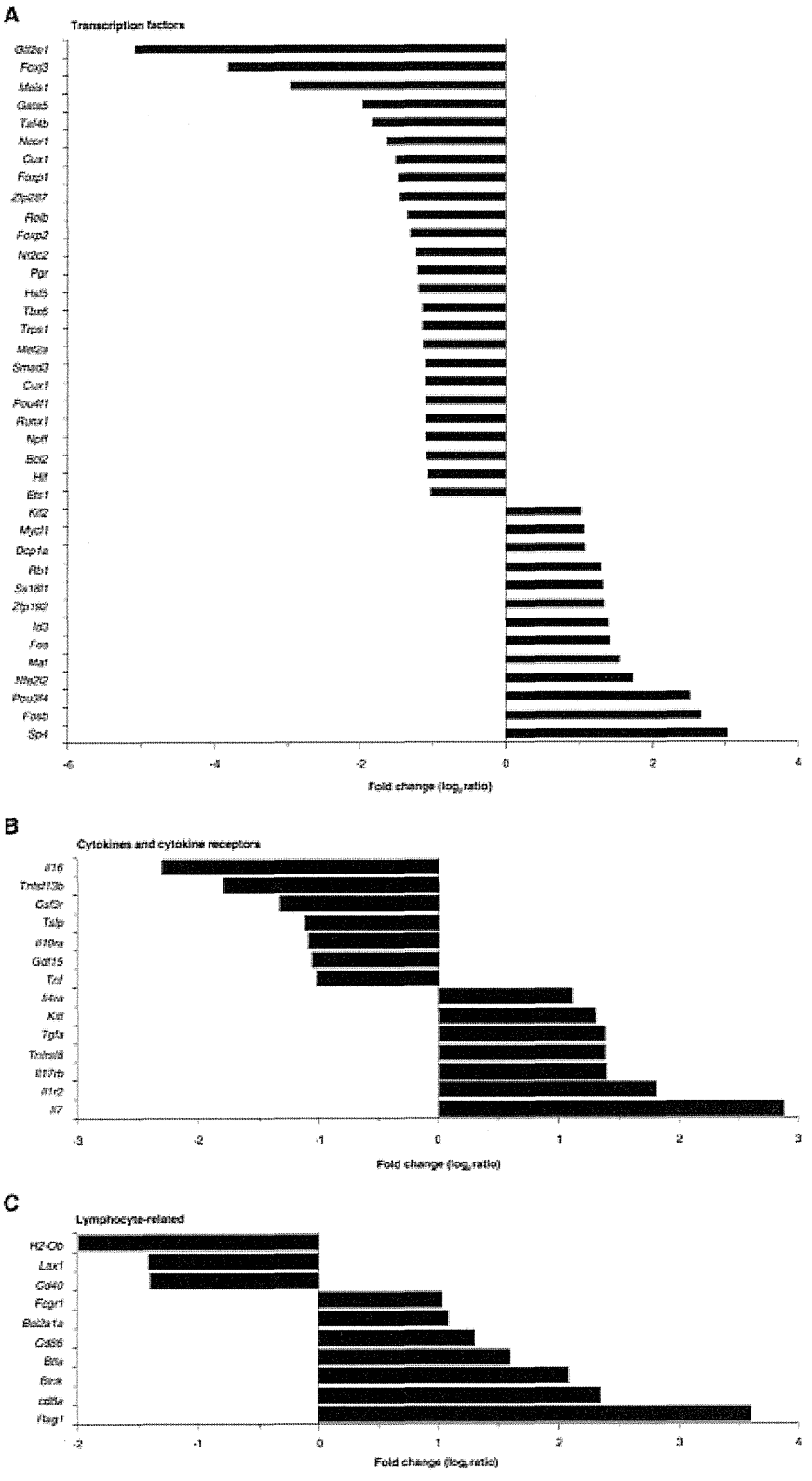

Figure 5. Genes Affected by Satb1 Expression

A microarray experiment was performed to compare gene expression in Satb1 and control-transduced LSK Flt3$^-$ cells. Upregulation in Satb1-transduced cells is shown as positive in each figure. (A) Transcription factors, (B) cytokine and cytokine receptors, and (C) other lymphoid lineage-related genes are summarized (Figure 5; see also Tables S2 and S3).

Satb1 Regulates Lymphoid Lineage-Related Genes in HSC

During early lymphocyte differentiation, several transcription factors have been shown to play roles in a hierarchical manner. To identify the target genes of Satb1, we first examined whether the exogenous expression of Satb1 influences the expression of lineage-relevant transcription factors in LSK Flt3$^-$ cells. Although high Satb1 expression was achieved, no significant upregulation was observed in the expression of *Sfpi1*, *Ikzf1*, *Tcf3*, or *Notch1* (data not shown). The expression of *Cebpa*, which is important for myeloid differentiation, was also not significantly affected (data not shown).

Next, to find candidate genes involved in the Satb1 induction of lymphopoiesis, we performed a microarray comparing gene expression between Satb1- and control-transduced LSK Flt3$^-$ cells (Table S2). In accordance with the results described above, the data showed no significant changes in the expression of *Sfpi1*, *Ikzf1*, *Tcf3*, *Notch1*, or *Cebpa*. However, several transcription factors involved in lymphoid differentiation, *Sp4*, *Maf*, *Fos*, and *Id3*, were upregulated in Satb1-transduced cells (Figure 5A). Cytokines such as *Il7* and *Kitl*, which are critical for lymphocyte differentiation and generally believed to be stromal cell products, were induced in hematopoietic progenitor cells themselves by ectopic expression of Satb1 (Figure 5B). While receptors for IL-4 or IL-17 were induced, *Csf3r*, encoding the G-CSF receptor, was downregulated. Interestingly, among lymphoid-related genes, *Rag1*, which is indispensable for both T and B cell differentiation, was strongly induced by Satb1 (Figure 5C). Expression of the CD86 gene that correlates with lymphoid competency (Shimazu et al., 2012) was also significantly elevated.

lymphohematopoiesis after transplantation. In eight transplanted mice, SATB1-overexpressing cells did not induce tumors, at least during 3 months of observation.

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As a complementary experiment, we performed a set of microarray analyses comparing gene expression signatures between WT and *Satb1*$^{-/-}$ cells (Table S3). We again observed no direct correlations between Satb1 expression and *Ikzf1*, *Tcf3*, or *Notch1*, but confirmed that the expression of numerous lineage-related genes was influenced. The expression of *Il7* and *Kitl* was detectable in WT hematopoietic progenitors, and their levels were significantly lower in the *Satb1*$^{-/-}$ progenitors. Of note, *Satb2*, which is a homolog of *Satb1*, as well as *Bright*, which codes a B cell-specific AT-rich sequence binding protein (Herrscher et al., 1995), were upregulated in *Satb1*$^{-/-}$ HSC. In addition, the *Satb1*$^{-/-}$ HSC aberrantly expressed *Rag1* and *Pax5*, whose levels decreased with differentiation to LMPP. These results indicate that Satb1 expression globally influences many genes involved in lineage-fate decisions during the specification of HSC toward lymphoid lineages.

Satb1 Induces Lymphopoiesis in ESCs

Next, we examined whether the exogenous expression of Satb1 is sufficient to promote lymphopoiesis in ESCs. In the OP9 coculture system (Nakano et al., 1994), ESCs can produce mesoderm cells in 4.5 days, which have potential to become hematopoietic and endothelial cells. After a short period of retroviral transduction with the control-GFP or the Satb1-GFP vector, ES-derived mesoderm cells were cultured with OP9 in the presence of SCF, Flt3-ligand, and IL-7. As shown in Figure 6A, although both control- and Satb1-transfected cells contained substantial numbers of GFP$^+$ cells, the latter produced CD45$^+$ hematopoietic cells efficiently. Further phenotype revealed that most of the CD45$^+$ GFP$^+$ cells produced from the Satb1-transfected cells expressed B220 and CD19 (Figure 6A, right panels). Notably, those cells were also positive for AA4.1, CD11b, and CD5, suggesting that they were likely B1-B-lineage cells (Figure 6B).

Next, we established ESC clones, which can be induced to express Satb1-GFP on removal of tetracycline (Tet) from the culture medium. Eight days after Tet deprivation (day 12.5; Figure 6C), approximately 15% of the recovered cells were GFP$^+$ (data not shown). Thirty-five percent of these cells expressed CD45 and included substantial numbers of AA4.1$^+$ CD19$^+$ B-lineage cells (Figure 6D, right panels). Conversely, in the presence of Tet, the proportions of AA4.1$^+$ and CD19$^+$ cells among the CD45$^+$ faction were very low (Figure 6D, left panels). A majority of the CD19$^+$ cells among the Satb1-GFP$^+$ ES-derived cells were positive for Mac1 or CD5, again indicating a preference for the B1-B lineage (Figure 6E). In cytospin preparations, many of the ES-derived cells cultured with Tet showed myelomonocytic morphology, whereas Satb1/GFP$^+$ cells exhibited lymphocyte-like morphology (Figure 6F). Finally, a PCR-based *Igh* rearrangement assay confirmed D_H-J_H recombination in the Satb1-GFP$^+$ ES-derived cells (Figure 6G).

To test T-lineage potential, we transduced the control-GFP or the Satb1-GFP vector to ES-derived mesoderm cells and cultured them with OP9-DL1 cells. The Satb1-transduced cells effectively produced CD4$^+$ CD8$^+$ DP cells with rapid kinetics (Figures 6H and 6I). Substantial numbers of ES-derived T-lineage cells expressed TCR-γδ or TCR-β, and Satb1-transduced cells were advanced in this regard (data not shown). Taking these results together, we conclude that Satb1 expression directs even ES-derived cells toward lymphoid lineages.

Ectopic Satb1 Expression in Aged HSC Restores Lymphopoietic Potency

As shown in Figure 1B, the *Satb1* expression in HSC declines with age. This decline might be correlated with the age-dependent impairment of lymphopoiesis. Therefore, we examined whether Satb1 expression restores the lymphopoietic activity of progenitors from aged mice. Rag1-GFP$^-$ LSK cells of 2-year-old mice were transduced with control or Satb1-DsRed vectors. After 72 hr of transduction, DsRed$^+$ cells were sorted and cultured on OP9 in the presence of SCF, Flt3-ligand, and IL-7. The Satb1-transduced cells produced a percentage of Rag1-GFP$^+$ B220$^+$ cells that was significantly higher than that of control cells (Figure 7A). Indeed, most of the aged Rag1-GFP$^-$ LSK cells were prone to differentiate into Rag1-GFP$^+$ cells as a result of exogenous Satb1 expression. With respect to the recovered B-lineage cell counts, approximately 3-fold more B220$^+$ Rag1-GFP$^+$ Mac1$^-$ cells were obtained through Satb1 overexpression (Figure 7B).

Conversely, fewer B-lineage cells were generated from aged ELP than from young ELP despite their similar expression of *Satb1* (Figure 1B; Figure S4A). B-lineage differentiation of aged ELP also showed decreased Rag1 expression (Figure S4B). Nonetheless, aged ELP showed substantial lymphopoietic activity in MS5 cocultures, in which aged HSC scarcely produced B-lineage cells (Figure S4A). These results suggest that the downregulation of Satb1 expression is involved in the compromised lymphopoietic potential of aged HSC and that ectopic induction of Satb1 can at least partially restore the activity.

DISCUSSION

Despite accumulating evidence that multiple transcription factors support lymphocyte differentiation, ones that specifically direct HSC to the lymphoid lineage have remained elusive. One aim of this study was to describe molecular signatures of early stages of lymphopoiesis by comparing gene expression patterns between HSC and ELP. While we observed that many genes specific for the lymphoid lineage including *Tcr*, *Igh* and *Il7r* were highly induced at the ELP stage, some lymphoid genes were already expressed at low levels in the HSC-enriched fraction. Among them, we were particularly interested in chromatin modifiers because of their ability to control spatial and temporal expression of essential genes. Our screen identified Satb1, whose expression was previously linked to T lymphocyte differentiation (Alvarez et al., 2000). We show that Satb1 plays a critical role in directing HSC to lymphoid lineages.

Satb1 was originally identified as a protein that binds specifically to genomic DNA in a specialized DNA context with high base-unpairing potential (termed base-unpairing regions; BURs) (Dickinson et al., 1992). Satb1 is predominantly expressed in the thymus and subsequent studies revealed critical roles in thymocyte development (Alvarez et al., 2000), T cell activation (Cai et al., 2006), and Th2 differentiation (Notani et al., 2010). In thymocyte nuclei, Satb1 has a cage-like distribution and tethers BURs onto its regulatory network, thus organizing 3-dimensional chromatin architecture (Cai et al., 2003). By recruiting chromatin modifying and remodeling factors, Satb1 establishes region-specific epigenetic status at its target gene loci and regulates a large number of genes (Yasui et al., 2002;

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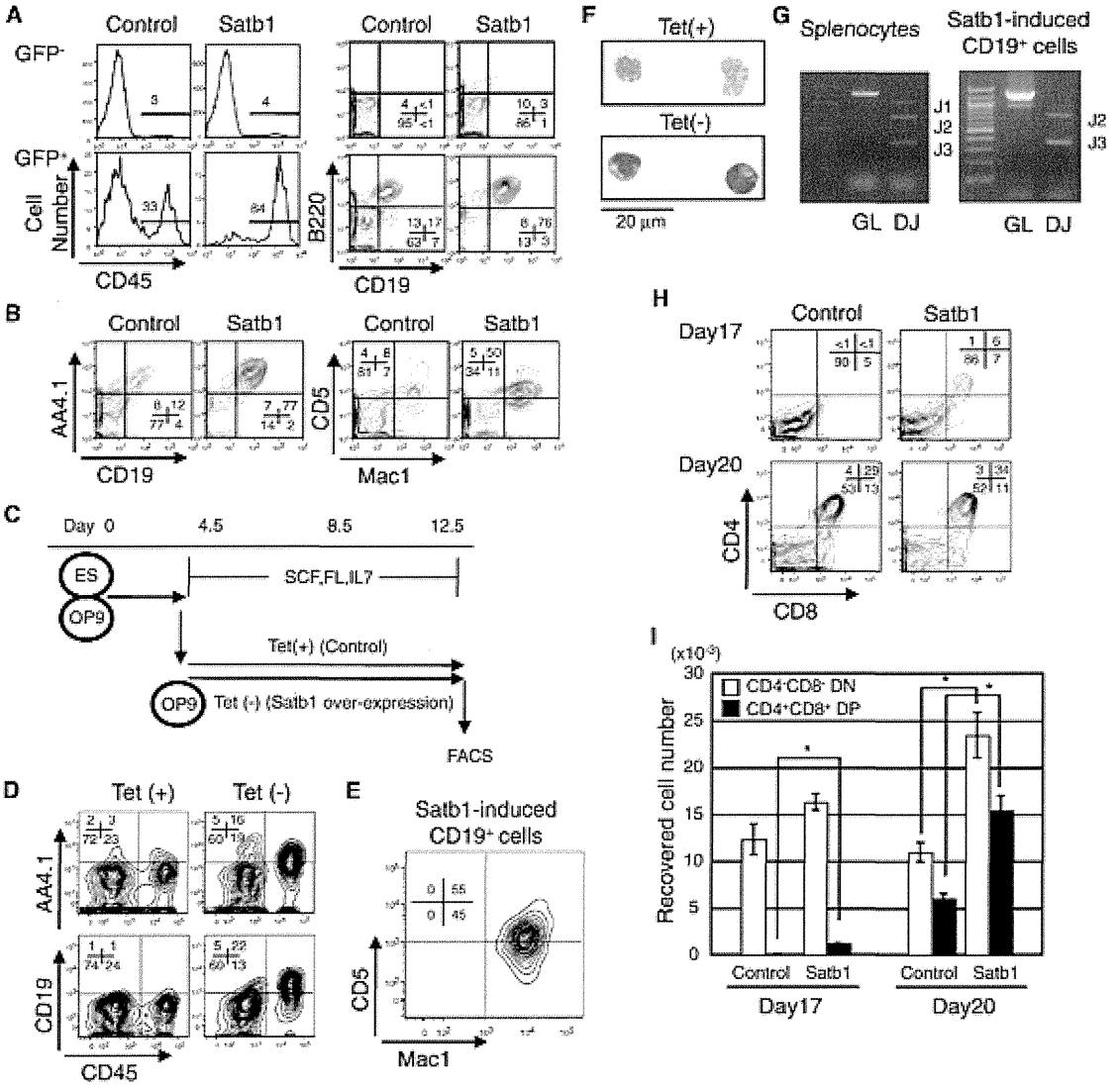

Figure 6. Satb1 Promotes Lymphoid Differentiation from ES-Derived Cells

E14tg2a ESCs were deprived of leukemia inhibitory factor and seeded onto OP9 cells. After 4.5 days, the differentiated mesoderm cells were infected with retroviral supernatants containing control-GFP or Satb1-GFP expressing vectors. Subsequently, the cells were cultured on OP9 for 8 days. At the end of culture, all cells were harvested and stained with the antibodies indicated in each panel.

(A) Total recovered cells were divided according to GFP expression (left panels). The percentages of CD45$^+$ cells in GFP$^-$ (upper panels) and GFP$^+$ populations (lower panels) are shown. CD45R/B220 and CD19 profiles of the CD45$^+$ cells corresponding to the left panels (right panels) are shown.

(B) Representative AA4.1 and CD19 or Mac1 and CD5 profiles of the GFP$^+$ CD45$^+$ cells recovered from control or Satb1-transduced culture.

(C) The experimental design used with a Tet-off system (upper panel). ESCs, which inducibly express Satb1 by Tet deprivation, were established. After 4.5 days of culture without leukemia inhibitory factor in the presence of Tet, the differentiated cells were reseeded onto new OP9 stromal cells with or without Tet. Subsequently, FACS analysis was performed after 8 days of culture (day 12.5).

(D) Tet (+) indicates profiles of GFP$^-$ cells cultured with Tet (left panels). Tet (−) panels show profiles of Satb1/GFP$^+$ cells cultured without Tet (right panels).

(E) Mac1 and CD5 expression on the Satb1/GFP$^+$ CD19$^+$ cells grown without Tet.

(F) Morphology of ES-derived hematopoietic cells on day 12.5.

(G) DNA PCR assays of germline (GL) or D_H-J_H rearranged *Igh* chain (DJ) genes were performed with the Satb1/GFP$^+$ CD19$^+$ cells recovered without Tet (right panel). Splenocytes were used as a positive control for the D_H-J_H recombination (left panel). On each gel, a size marker was loaded in the left lane.

(H and I) E14tg2a ESCs were differentiated to mesoderm cells for 4.5 days and then infected with the retroviral supernatant containing control-GFP or Satb1-GFP expressing vectors for 3 days. Subsequently, the cells were cultured on OP9-DL1 and T-lineage output was evaluated on the indicated days. Data are shown as mean ± SE. Statistical significance is $^*p < 0.05$.

Cai et al., 2003). Increased Satb1 expression in hematopoietic progenitors compared with HSC has been observed by others (Forsberg et al., 2005; Ng et al., 2009); however, no study has been conducted concerning the role of Satb1 in differentiation of HSC to either lymphoid or myeloid progenitors. Our results clearly show a tight association of Satb1 expression with

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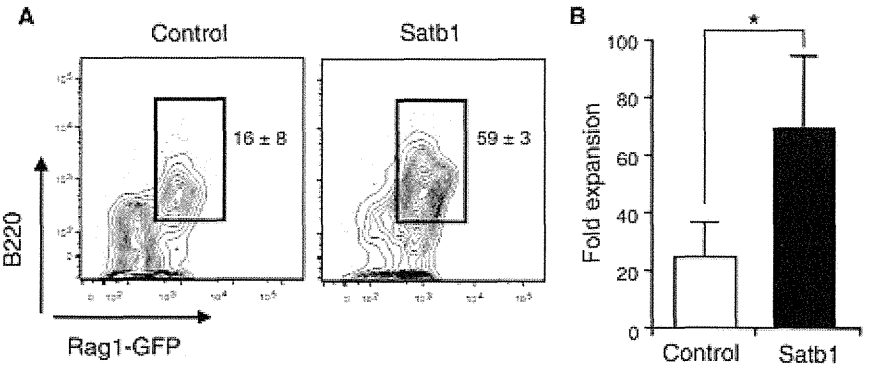

Figure 7. Satb1 Overexpression Restores Lymphopoietic Activity of Aged HSC
(A) Rag1/GFP$^-$ LSK cells were sorted from 2-year-old mice and retrovirally transduced with control or Satb1-DsRed vectors. Successfully transfected cells were cultured on OP9 cells. Cultures were established in triplicate. Numbers in each panel indicate the frequency of Rag1/GFP$^+$ CD45R/B220$^+$ cells.
(B) Yields of CD45R/B220$^+$ Rag1/GFP$^+$ Mac1$^-$ B-lineage cells per 1 input control- or Satb1-transduced Rag1/GFP-LSK cells were calculated and given as averages with SD bars. Statistical significance is $^*p < 0.05$ (Figure 7; see also Figure S4).

lymphoid lineages even at the earliest stages. In addition, *Satb1*$^{-/-}$ HSCs are hindered in producing lymphocytes in vitro and in vivo that are consistent with the phenotypes originally described in *Satb1*$^{-/-}$ mice, suggesting an indispensable role of Satb1 in physiological lymphopoiesis.

Although we have previously identified molecules regulating early lymphoid differentiation, information about ones that initiate the process has been elusive (Oritani et al., 2000; Yokota et al., 2003b, 2008). The present study demonstrates that ectopic expression of Satb1 strongly induces differentiation toward lymphoid lineages and promotes lymphocyte growth from primitive progenitors, even when they are derived from aged BM or ESCs. We believe that these findings are important because they reveal that the earliest step of lymphopoiesis is affected by a global chromatin organizer. In addition, our results suggest that Satb1 expression could be a useful biomarker of aging and be manipulated to reverse immunosenescence.

Lymphoid-fate decisions are not necessarily determined by a few transcription factors or cytokines that positively regulate the differentiation in a hierarchical manner. The process should involve "closed windows" and "open opportunities." Gene array studies comparing HSC and ELP have shown that various lymphoid-related genes appear to be synchronously upregulated in ELP, whereas stem cell-related or myeloid-related ones are downregulated. From these observations, we speculated that a master regulator is present and involved in the synchronicity along with the hierarchical factors; further, we focused on the function of SATB1 in this process. Our results show that once Satb1 is substantially expressed in HSCs, it regulates hundreds of genes, including *Rag1*, *Il7*, *kitl*, and *Csf3r*, which together determine the lymphoid lineage fate. Satb1 itself has the determinant role in regulating a set of genes to exhibit the phenotype that we observed in vitro and in vivo experiments.

Increasing Satb1 beyond physiologic levels in HSCs and ESCs strongly augmented B lymphopoiesis, while depleting Satb1 from HSC dominantly impaired T lymphopoiesis in vivo. Satb1 overexpression in HSCs by itself induces an expression profile that favors B cell production. Conversely, Satb1 deficiency might have disrupted the delicate balance of Satb1 and other BUR-binding proteins such as Satb2 or Bright. We detected minimum levels of *Satb2* and *Bright* expression in WT HSC, and their expression levels significantly increase with B-lineage differentiation (data not shown). Interestingly, both genes were aberrantly induced in Satb1-deficient HSC (Table S2). Satb2 has a binding specificity similar to that of Satb1, and its expression is more predominant in the B lineage than in the T lineage (Dobreva et al., 2003). In ESCs, Satb2 function is antagonistic to Satb1 in regulating some target genes (Savarese et al., 2009). Whether these BUR-binding proteins are antagonistic or sometimes function synergistically, depending on cell differentiation or lineage remains unknown. Further studies of their functional correlation could yield important information about gene regulation in T and B lymphopoiesis.

Although our data provide evidence of a lymphocyte-inductive role of Satb1, an important question remains; that is, what regulates Satb1 expression? Depletion of long-lived mature B cells rejuvenates B-lymphopoiesis in old mice, suggesting that age-associated accumulation of aged B cells seems to be sensed by HSCs or early progenitors in BM (Keren et al., 2011). It will be interesting to learn whether such environmental cues influence Satb1 expression in HSCs. New strategies for boosting lymphocyte regeneration or protecting this capability during aging might emerge from studies of Satb1-related molecular mechanisms.

EXPERIMENTAL PROCEDURES

Animals

Animal studies were performed with the approval of the Institutional Review Board of Osaka University. Rag1-GFP knockin mice were previously described (Kuwata et al., 1999). *Satb1*$^{-/-}$ mice were also previously established (Alvarez et al., 2000). WT C57BL/6 mice and the congenic C57BL/6SJL strain (CD45.1 alloantigen) were obtained from Japan Clea (Shizuoka, Japan) and The Jackson Labs (Bar Harbor, ME), respectively. To obtain mouse fetuses, we considered the morning of the day of vaginal plug observation as E0.5.

Flow Cytometry and Cell Sorting

Cells were stained with Abs indicated in each experiment and analyzed with FACScanto or FACSaria (BD Bioscience). Adult BM cells from Rag1-GFP heterozygotes were used to isolate Lin$^-$ c-kit^{hi} Sca-1$^+$ Flt3$^-$ Rag1-GFP$^-$ IL-7Rα$^-$ (HSC-enriched), Lin$^-$ IL-7Rα$^-$ c-kit^{lo} Sca-1$^+$ Flt3$^+$ Rag1-GFP$^-$ (LMPP-enriched), Lin$^-$ IL-7Rα$^+$ c-kit^{hi} Sca-1$^+$ Flt3$^+$ Rag1-GFP$^+$ (ELP-enriched), Lin$^-$ c-kit^{lo} Sca-1^{lo} Flt3$^+$ Rag1-GFP$^+$ IL-7Rα$^+$ (CLP-enriched), and Lin$^-$ c-kit^{hi} Sca-1$^-$ IL-7Rα$^-$ myeloid progenitors (Adolfsson et al., 2005; Igarashi et al., 2002; Kondo et al., 1997). For culture experiments, we also sorted a HSC-enriched fraction from WT C57BL/6 or *Satb1*$^{-/-}$ mice according to the cell surface phenotype of Lin$^-$ c-kit^{hi} Sca-1$^-$ Flt3$^-$.

Stromal Cell Coculture

Murine stromal cell lines MS5 and OP9 were generous gifts from Dr. Mori (Niigata University) and Dr. Hayashi (Tottori University), respectively. Freshly

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isolated or transduced cells were cocultured with stromal cells in α-MEM supplemented with 10% FCS, rm SCF (10 ng/mL), rm Flt3-ligand (20 ng/mL), and rm IL-7 (1 ng/mL). The cultures were fed twice a week and maintained for the indicated periods in each experiment. OP9-DL1 cells originated by Dr. Kawamoto (Riken, Japan) were obtained from Riken Cell Bank (Tsukuba, Japan) and used to produce T-lineage cells. In this case, cells were cultured in the presence of rm Flt3-ligand (5 ng/mL) and rm IL-7 (1ng/mL) for 14 days, and rm Flt3-ligand (5 ng/mL) alone thereafter. At the end of culture, cells were counted and analyzed by flow cytometry.

Competitive Repopulation Assay

The CD45.1/CD45.2 system was adapted to a competitive repopulation assay. One thousand Flt3$^-$ LSK cells sorted from FL or BM of WT, Satb1 heterodeficient, or Satb1 homozygous-deficient mice (CD45.2) were mixed with 4×10^5 unfractionated adult BM cells obtained from WT C57BL/6-Ly5.1 (CD45.1) mice and were transplanted into C57BL/6-Ly5.1 mice lethally irradiated at a dose of 920 rad. At 8 weeks after transplantation, engraftment of CD45.2 cells was evaluated by flow cytometry.

Retrovirus Transfection

Murine Satb1 expression vector was purchased from OriGene (Rockville, MD). A retrovirus expression vector for Satb1 was generated by subcloning into the pMYs-IRES-GFP or DsRed vector (a gift from Dr. Kitamura, University of Tokoyo). Conditioned medium containing high tighter retrovirus particles was prepared as reported previously (Satoh et al., 2008). Sorted HSC were cultured in D-MEM containing 10% FBS, rm SCF (100 ng/ml), rm TPO (100 ng/ml), and rm Flt3-ligand (100 ng/ml) for 24 hr. Then, the cells were seeded into the culture plates coated with Retronectin (Takara Bio, Shiga, Japan) and cultured with conditioned medium containing retrovirus. After 24 hr, cells were washed and performed second transfection by the same condition. After 48 hr from the second transfection, GFP or DsRed-positive cells were sorted by FACSaria.

Limiting Dilution Assays

The frequencies of lymphohematopoietic progenitors were determined by plating cells in limiting dilution assays by using 96-well flat-bottom plates. Pre-established MS5 layers were plated with 1, 2, 4, 8, or 16 cells each by using the Automated Cell Deposition Unit of the FACSaria. Cells were cultured in α-MEM supplemented with 10% FCS, rm SCF (10 ng/mL), rm Flt3-ligand (20 ng/mL), and rm IL-7 (1 ng/mL). At 10 days of culture, wells were inspected for the presence of hematopoietic clones. Positive wells were harvested and analyzed by flow cytometry for the presence of CD45$^+$ hematopoietic cells and CD45R/B220$^+$ CD19$^+$ Mac1$^-$ B-lineage cells. The frequencies of progenitors were calculated by linear regression analysis on the basis of Poisson distribution as the reciprocal of the concentration of test cells that gave 37% negative cultures.

Lymphocyte Development from Murine ESCs

To induce differentiation toward hematopoietic cells, we deprived E14tg2a ESCs of leukemia inhibitory factor and seeded onto OP9 cells in 6-well plates at a density of 10^4 cells per well in α-MEM supplemented with 20% FBS (Nakano et al., 1994). After 4.5 days, the cells were harvested and whole-cell suspensions were transferred into a new 10 cm dish and incubated in 37°C for 30 min to remove adherent OP9 cells. The collected floating cells were infected with the retroviral supernatant in Retronectin-coated plates by 2 hr spinoculation (1100 *g*) (Kitajima et al., 2006). Subsequently, the cells were cultured on OP9 or OP9-DL1.

Tetracycline-Regulated Inducible Expression of Satb1 in ESCs

To inducibly express Satb1 in ESCs, we utilized a Tet-off system as reported previously (Era and Witte, 2000), in which transcription of the target gene is initiated by the removal of Tet from the culture medium. Briefly, we initially introduced pCAG20-1-tTA and pUHD10-3-puro by electroporation and selected one clone designated E14 by culture with 1 μg/ml of Puro and/or 1 μg/ml of Tet. We further transfected pUHD10-3-Satb1-GFP, which can inducibly express Satb1 and GFP as a single mRNA through the internal ribosome entry site in response to the Tet removal, together with the neomycin-resistant plasmid pcDNA3.1-neo. After the culture with G418, we selected clones that can inducibly express GFP in response to the Tet deprivation.

DNA PCR Assays for *Igh* Rearrangement

DNA PCR assays were performed as reported previously (Schlissel et al., 1991). PCR was performed by using genomic DNA extracted from splenocytes or ES-derived cells as a template. D_H-J_H recombination was detected as amplified fragments of 1,033 bp, 716 bp, and 333 bp by using a primer D_HL(5′) and J3(3′). Germline alleles were detected as an amplified fragment of 1,259 bp by using a primer Mu0(5′) and J3(3′). The sequence of primers are as follows: D_HL(5′), GGAATTCG(AorC)TTTTTGT(CorG)AAGGGATCTACTACTGTG; Mu0(5′), CCGCATGCCAAGGCTAGCCTGAAAGATTACC; and J3(3′), GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG.

Statistical Analyses

Unpaired, two-tailed t test analyses were used for intergroup comparisons, and p values were considered significant if they were less than 0.05.

ACCESSION NUMBERS

The microarray data in Tables S2 and S3 has been deposited in NCBI GEO database under the accession numbers GSE45566 and GSE45299.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.05.014.

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REFERENCES

Adolfsson, J., Månsson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., et al. (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell *121*, 295–306.

Alvarez, J.D., Yasui, D.H., Niida, H., Joh, T., Loh, D.Y., and Kohwi-Shigematsu, T. (2000). The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes during T-cell development. Genes Dev. *14*, 521–535.

Cai, S., Han, H.J., and Kohwi-Shigematsu, T. (2003). Tissue-specific nuclear architecture and gene expression regulated by SATB1. Nat. Genet. *34*, 42–51.

Cai, S., Lee, C.C., and Kohwi-Shigematsu, T. (2006). SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. Nat. Genet. *38*, 1278–1288.

Chambers, S.M., Shaw, C.A., Gatza, C., Fisk, C.J., Donehower, L.A., and Goodell, M.A. (2007). Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. PLoS Biol. *5*, e201.

Dias, S., Månsson, R., Gurbuxani, S., Sigvardsson, M., and Kee, B.L. (2008). E2A proteins promote development of lymphoid-primed multipotent progenitors. Immunity *29*, 217–227.

Dickinson, L.A., Joh, T., Kohwi, Y., and Kohwi-Shigematsu, T. (1992). A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. Cell *70*, 631–645.

Dobreva, G., Dambacher, J., and Grosschedl, R. (2003). SUMO modification of a novel MAR-binding protein, SATB2, modulates immunoglobulin mu gene expression. Genes Dev. *17*, 3048–3061.

Immunity
Satb1 Promotes Lymphoid Priming in HSC

Era, T., and Witte, O.N. (2000). Regulated expression of P210 Bcr-Abl during embryonic stem cell differentiation stimulates multipotential progenitor expansion and myeloid cell fate. Proc. Natl. Acad. Sci. USA *97*, 1737–1742.

Forsberg, E.C., Prohaska, S.S., Katzman, S., Heffner, G.C., Stuart, J.M., and Weissman, I.L. (2005). Differential expression of novel potential regulators in hematopoietic stem cells. PLoS Genet. *1*, e28.

Han, H.J., Russo, J., Kohwi, Y., and Kohwi-Shigematsu, T. (2008). SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. Nature *452*, 187–193.

Herrscher, R.F., Kaplan, M.H., Lelsz, D.L., Das, C., Scheuermann, R., and Tucker, P.W. (1995). The immunoglobulin heavy-chain matrix-associating regions are bound by Bright: a B cell-specific trans-activator that describes a new DNA-binding protein family. Genes Dev. *9*, 3067–3082.

Igarashi, H., Gregory, S.C., Yokota, T., Sakaguchi, N., and Kincade, P.W. (2002). Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity *17*, 117–130.

Ikawa, T., Kawamoto, H., Goldrath, A.W., and Murre, C. (2006). E proteins and Notch signaling cooperate to promote T cell lineage specification and commitment. J. Exp. Med. *203*, 1329–1342.

Keren, Z., Naor, S., Nussbaum, S., Golan, K., Itkin, T., Sasaki, Y., Schmidt-Supprian, M., Lapidot, T., and Melamed, D. (2011). B-cell depletion reactivates B lymphopoiesis in the BM and rejuvenates the B lineage in aging. Blood *117*, 3104–3112.

Kitajima, K., Tanaka, M., Zheng, J., Yen, H., Sato, A., Sugiyama, D., Umehara, H., Sakai, E., and Nakano, T. (2006). Redirecting differentiation of hematopoietic progenitors by a transcription factor, GATA-2. Blood *107*, 1857–1863.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661–672.

Kuwata, N., Igarashi, H., Ohmura, T., Aizawa, S., and Sakaguchi, N. (1999). Cutting edge: absence of expression of RAG1 in peritoneal B-1 cells detected by knocking into RAG1 locus with green fluorescent protein gene. J. Immunol. *163*, 6355–6359.

Lai, A.Y., and Kondo, M. (2008). T and B lymphocyte differentiation from hematopoietic stem cell. Semin. Immunol. *20*, 207–212.

Medina, K.L., Pongubala, J.M., Reddy, K.L., Lancki, D.W., Dekoter, R., Kieslinger, M., Grosschedl, R., and Singh, H. (2004). Assembling a gene regulatory network for specification of the B cell fate. Dev. Cell *7*, 607–617.

Miller, J.P., and Allman, D. (2005). Linking age-related defects in B lymphopoiesis to the aging of hematopoietic stem cells. Semin. Immunol. *17*, 321–329.

Montecino-Rodriguez, E., and Dorshkind, K. (2006). Evolving patterns of lymphopoiesis from embryogenesis through senescence. Immunity *24*, 659–662.

Nakano, T., Kodama, H., and Honjo, T. (1994). Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science *265*, 1098–1101.

Ng, S.Y., Yoshida, T., Zhang, J., and Georgopoulos, K. (2009). Genome-wide lineage-specific transcriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells. Immunity *30*, 493–507.

Notani, D., Gottimukkala, K.P., Jayani, R.S., Limaye, A.S., Damle, M.V., Mehta, S., Purbey, P.K., Joseph, J., and Galande, S. (2010). Global regulator SATB1 recruits beta-catenin and regulates T(H)2 differentiation in Wnt-dependent manner. PLoS Biol. *8*, e1000296.

Oritani, K., Medina, K.L., Tomiyama, Y., Ishikawa, J., Okajima, Y., Ogawa, M., Yokota, T., Aoyama, K., Takahashi, I., Kincade, P.W., and Matsuzawa, Y. (2000). Limitin: An interferon-like cytokine that preferentially influences B-lymphocyte precursors. Nat. Med. *6*, 659–666.

Rossi, D.J., Bryder, D., Zahn, J.M., Ahlenius, H., Sonu, R., Wagers, A.J., and Weissman, I.L. (2005). Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc. Natl. Acad. Sci. USA *102*, 9194–9199.

Satoh, Y., Matsumura, I., Tanaka, H., Ezoe, S., Fukushima, K., Tokunaga, M., Yasumi, M., Shibayama, H., Mizuki, M., Era, T., et al. (2008). AML1/RUNX1 works as a negative regulator of c-Mpl in hematopoietic stem cells. J. Biol. Chem. *283*, 30045–30056.

Savarese, F., Dávila, A., Nechanitzky, R., De La Rosa-Velazquez, I., Pereira, C.F., Engelke, R., Takahashi, K., Jenuwein, T., Kohwi-Shigematsu, T., Fisher, A.G., and Grosschedl, R. (2009). Satb1 and Satb2 regulate embryonic stem cell differentiation and Nanog expression. Genes Dev. *23*, 2625–2638.

Schlissel, M.S., Corcoran, L.M., and Baltimore, D. (1991). Virus-transformed pre-B cells show ordered activation but not inactivation of immunoglobulin gene rearrangement and transcription. J. Exp. Med. *173*, 711–720.

Scott, E.W., Fisher, R.C., Olson, M.C., Kehrli, E.W., Simon, M.C., and Singh, H. (1997). PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. Immunity *6*, 437–447.

Semerad, C.L., Mercer, E.M., Inlay, M.A., Weissman, I.L., and Murre, C. (2009). E2A proteins maintain the hematopoietic stem cell pool and promote the maturation of myelolymphoid and myeloerythroid progenitors. Proc. Natl. Acad. Sci. USA *106*, 1930–1935.

Shimazu, T., Iida, R., Zhang, Q., Welner, R.S., Medina, K.L., Alberola-Ila, J., and Kincade, P.W. (2012). CD86 is expressed on murine hematopoietic stem cells and denotes lymphopoietic potential. Blood *119*, 4889–4897.

Sudo, K., Ema, H., Morita, Y., and Nakauchi, H. (2000). Age-associated characteristics of murine hematopoietic stem cells. J. Exp. Med. *192*, 1273–1280.

Yang, Q., Kardava, L., St Leger, A., Martincic, K., Varnum-Finney, B., Bernstein, I.D., Milcarek, C., and Borghesi, L. (2008). E47 controls the developmental integrity and cell cycle quiescence of multipotential hematopoietic progenitors. J. Immunol. *181*, 5885–5894.

Yasui, D., Miyano, M., Cai, S., Varga-Weisz, P., and Kohwi-Shigematsu, T. (2002). SATB1 targets chromatin remodelling to regulate genes over long distances. Nature *419*, 641–645.

Yilmaz, O.H., Kiel, M.J., and Morrison, S.J. (2006). SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. Blood *107*, 924–930.

Yokota, T., Kouro, T., Hirose, J., Igarashi, H., Garrett, K.P., Gregory, S.C., Sakaguchi, N., Owen, J.J., and Kincade, P.W. (2003a). Unique properties of fetal lymphoid progenitors identified according to RAG1 gene expression. Immunity *19*, 365–375.

Yokota, T., Meka, C.S., Kouro, T., Medina, K.L., Igarashi, H., Takahashi, M., Oritani, K., Funahashi, T., Tomiyama, Y., Matsuzawa, Y., and Kincade, P.W. (2003b). Adiponectin, a fat cell product, influences the earliest lymphocyte precursors in bone marrow cultures by activation of the cyclooxygenase-prostaglandin pathway in stromal cells. J. Immunol. *171*, 5091–5099.

Yokota, T., Oritani, K., Garrett, K.P., Kouro, T., Nishida, M., Takahashi, I., Ichii, M., Satoh, Y., Kincade, P.W., and Kanakura, Y. (2008). Soluble frizzled-related protein 1 is estrogen inducible in bone marrow stromal cells and suppresses the earliest events in lymphopoiesis. J. Immunol. *181*, 6061–6072.

Yoshida, T., Ng, S.Y., Zuniga-Pflucker, J.C., and Georgopoulos, K. (2006). Early hematopoietic lineage restrictions directed by Ikaros. Nat. Immunol. *7*, 382–391.

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Biology and treatment of HTLV-1 associated T-cell lymphomas

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Adult T-cell leukemia-lymphoma (ATL) is a distinct peripheral T-lymphocytic malignancy associated with human T-cell lymphotropic virus type I (HTLV-1) endemics in several regions of the world including the south-west Japan. The three major routes of HTLV-1 transmission are mother-to-child infections via breast milk, sexual intercourse, and blood transfusions. A HTLV-1 infection early in life, presumably from breast feeding, is crucial to the development of ATL. The estimated cumulative risk of developing ATL among HTLV-1-positive individuals is about 3% after transmission from the mother. The diversity in clinical features and prognosis of patients with this disease has led to its subtype-classification into acute, lymphoma, chronic, and smoldering types defined by organ involvement, lactate dehydrogenase (LDH) and calcium values. For the acute, lymphoma and unfavorable chronic subtypes (aggressive ATL), and the favorable chronic and smoldering subtypes (indolent ATL), intensive chemotherapy followed by allogeneic stem cell transplantation and watchful waiting until disease progression has been recommended, respectively, in Japan. A retrospective analysis suggested that the combination of interferon alpha and zidovudine was promising for the treatment of ATL, especially for leukemic subtypes. There are several new trials for ATL, including a defucosylated humanized anti-CC chemokine receptor 4 monoclonal antibody, histone deacetylase inhibitors, a purine nucleoside phosphorylase inhibitor, a proteasome inhibitor and lenalidomide.

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Introduction

Adult T-cell leukemia (ATL) was first described in 1977 by Uchiyama and Takatsuki as a distinct progressive T-cell leukemia of peculiar morphology, so called "flower cells" with a suspected viral etiology because of the clustering of the disease in the southwestern region of Japan [1]. Subsequently, a novel RNA retrovirus, human T-cell leukemia/lymphotropic virus type I (HTLV-1), was isolated from a cell line established from leukemic cells of an ATL patient, and the finding of a clear association with ATL led to its inclusion among human carcinogenic pathogens [2–5]. In the mid-1980s and 1990s, several inflammatory diseases were reported to be associated with HTLV-1 including tropical spastic paraparesis (TSP)/HTLV-1-associated myelopathy (HAM), HTLV-1 uveitis and infective dermatitis [6–9]. At the same time, endemic areas for the virus and diseases have been found such as the Caribbean islands, tropical Africa, South America, Mid East and northern Oceania [10]. Subsequently, diversity in the clinical features of ATL has been recognized including ATL without leukemic manifestation and nomenclature of adult T-cell leukemia/lymphoma (ATLL) and/or adult T cell leukemia-lymphoma (ATL), and a classification of clinical subtypes of the disease was proposed [11]. This chapter will review the current recognition of ATL focusing on the biology and treatment of the disease.

Recent epidemiological findings of HTLV-1 and ATL in Japan

It has been estimated that there are several tens of million HTLV-1-infected individuals reside in the world, with 1.1 million in Japan, and the annual incidence of ATL is approximately 1,000 in Japan. The annual rate of ATL development among HTLV-1 carriers older than 40 years is estimated at 1.5 per 1000 in males and 0.5 per 1000 in females, and the cumulative risk of ATL development among HTLV-1 carriers is estimated to be 2.5%–5% over the course of a 70-year life span [12].

Recently, the prevalence of HTLV-1 in Japan as determined by screening of blood donors was surveyed [13]. The seroprevalence of HTLV-1 among 1,196,321 Japanese first-time blood donors from 2006 to 2007 was investigated. A total of 3787 such donors were confirmed to be positive for the anti-HTLV-1 antibody. This resulted in an estimation of at least 1.08 million current HTLV-1 carriers in Japan, which is 10% lower than that reported in 1988. The adjusted overall prevalence rates were estimated to be 0.66% and 1.02% in men and women, respectively. The peak in carrier numbers was found among individuals in their 70s, which is a shift from the previous peak observed in the 1988 database among individuals in their 50s. As compared to the survey in the 1980s, carriers were distributed throughout the country, particularly in the greater Tokyo metropolitan area.

Factors reportedly associated with the onset of ATL include the following: HTLV-1 infection early in life, increase in age, male sex, family history of ATL, past history of infective dermatitis, smoking of tobacco, serum titers of antibody against HTLV-1, HTLV-1 proviral load and several HLA subtypes [10,14]. However, definitive risk factors for the development of ATL among asymptomatic HTLV-1 carriers have not been elucidated. Recently, Iwanaga and colleagues evaluated 1218 asymptomatic HTLV-1 carriers (426 males and 792 females) who were enrolled during 2002–2008 for a prospective study on the development of ATL [15]. The HTLV-1 proviral load at enrollment was significantly higher in males than females (median, 2.10 vs. 1.39 copies/100 peripheral blood mononuclear cells (PBMC)) ($P < .0001$), in those aged 40 or more years, and in those with a family history of ATL. During the follow-up period, 14 participants developed ATL. Their baseline proviral loads were high (range, 4.17–28.58 copies/100 PBMC). Multivariate Cox regression analyses indicated that not only a higher proviral load but also advanced age, a family history of ATL, and the first opportunity for HTLV-1 testing during treatment for other diseases were independent risk factors for the progression of ATL from a carrier status.

Molecular features of HTLV-1 and ATL

The HTLV-I gene encodes three structural proteins, Gag, Pol and Env, and complex regulatory proteins such as Tax, which not only activates viral replication but also induces the expression of several cellular genes. The expression of the proteins encoded by these cellular genes may enhance the multistep carcinogenesis of ATL. However, the expression including Tax is suppressed in vivo probably

escaping from immune surveillance, and appears just after in vitro culture [10]. A new viral factor, HTLV-1 basic Zip factor (HBZ), encoded by minus strand mRNA was recently discovered and is thought to be involved in viral replication and T-cell proliferation [16]. Several isoforms of HBZ transcripts were reported to be steadily expressed in HTLV-1–infected cells and primary ATL cells in contrast to Tax. The functions of these transcripts and putative proteins in the context of cellular transformation are now under investigation.

Prototypical ATL cells have a mature helper T-cell phenotype (CD3+, CD4+, CD8−). Recent studies have suggested that the cells of some ATL patients may be the equivalent of regulatory T cells because of the high frequency of expression of CD25/CCR4 and about half of that of FoxP3 [17]. By Southern blotting for both HTLV-1 integration and T-cell receptor (TCR) gene rearrangement, about 10–20% of ATL cases showed clonal changes during the transformation from indolent to aggressive disease [18]. Oligoclonal expansion of HTLV-1 infected pre-malignant cells was detected in asymptomatic HTLV-1 carriers by HTLV-1 integrated site-specific PCR [19]. Polycomb-mediated epigenetic silencing of miR-31 is implicated in the aberrant activation of NF-kB signaling in ATL cells [20]. A high rate of chromosomal abnormalities has been detected in HTLV-1-infected T-cell clones derived from HTLV-1 carriers [21]. Abnormalities in tumor suppressors such as p53 and p14/p16 are frequent and rare in acute- and chronic-type ATL, respectively, and both are associated with poor prognosis [22]. Chromosomal abnormalities detected by cytogenetics or comparative genomic hybridization are often more complex and more frequent in acute ATL than in chronic ATL, with aneuploidy and several hot spots such as 14q and 3p [23]. Microarray analyses of the transcriptomes of ATL cells at the chronic and acute stages elucidate the mechanism of stage progression in this disease revealed that several hundred genes were modulated in expression including those for MET, a receptor tyrosine kinase for hepatocyte growth factor and cell adhesion molecule, TSLC1 [24,25].

In summary, ATL is etiologically associated with HTLV-1. However, HTLV-1 does not carry a viral oncogenes, expression of the virus including Tax appears just after in vitro culture. Integration of the provirus into the host genome is random, and chromosomal/genetic abnormalities are complex: therefore, ATL is regarded as a single HTLV-1 disease entity with diverse molecular features resembling the acute-crisis-phase of chronic myeloid leukemia.

Clinical features and prognostic factors of ATL

ATL patients show a variety of clinical manifestations because of various complications of organ involvement by ATL cells, opportunistic infections and/or hypercalcemia [10,11,26]. These three often contribute to the extremely high mortality of the disease. Lymph node, liver, spleen and skin lesions are frequently observed. Although less frequently, digestive tract, lungs, central nervous system, bone and/or other organs may be involved [26]. Large nodules, plaques, ulcers, and erythrodermas are common skin lesions [27–29]. Immune suppression is common. Approximately 26% of 854 patients with ATL had active infections at diagnosis in a prior nationwide study in Japan [14]. The infections were bacterial in 43%, fungal in 31%, protozoal in 18%, and viral in 8% of patients. Individuals with indolent ATL might have no manifestation of the disease and are identified only by health check-ups and laboratory examinations.

ATL cells, so called "flower cells", are usually detected easily in the blood of affected individuals except in smoldering type, which mainly has skin manifestations and lymphoma type [11]. The histological analysis of aberrant cutaneous lesions or lymph nodes is essential for the diagnosis of the smoldering type with mainly skin manifestations and lymphoma type of ATL, respectively. Because ATL cells in the skin and lymph node can vary in size from small to large and in form from pleomorphic to anaplastic and Hodgkin-like cell with no specific histological pattern of involvement, distinguishing the disease from Sezary syndrome, other peripheral T-cell lymphomas and Hodgkin lymphoma can at times be difficult without examinations for HTLV-1 serotype/genotype [26].

Hypercalcemia is the most distinctive laboratory abnormality in ATL as compared to other lymphoid malignancies, and is observed in 31% of patients (50% in acute type, 17% in lymphoma type and 0% in the other two types) at onset [11]. Individuals with hypercalcemia do not usually have osteolytic bone lesions. Parathyroid hormone-related protein or receptor activator of nuclear factor kappa B ligand (RANKL) produced by ATL cells is considered the main factor causing hypercalcemia [30,31].

The diagnosis of typical ATL is not difficult and is based on clinical features, ATL cell morphology, mature helper-T-cell phenotype and anti-HTLV-1 antibody in most cases [11]. Those rare cases which might be difficult to diagnose can be shown to have the monoclonal integration of HTLV-1 proviral DNA in the malignant cells as determined by Southern blotting. However, its sensitivity is around 5% of ATL cells among normal cells. Furthermore, the monoclonal integration of HTLV-1 is also detected in some HAM/TSP patients and HTLV-1 carriers [32]. After the diagnosis of ATL, subtype-classification of the disease, reflecting prognostic factors, clinical features and natural history of the disease are based on the presence of organ involvement, leukemic manifestation and values for LDH and calcium, is necessary for the selection of appropriate treatment (Table 1) [11,33].

Major prognostic indicators for ATL, elucidated among 854 patients with ATL in Japan by multivariate analysis were advanced performance status, high LDH level, age of 40 years or more, more than three involved lesions, and hypercalcemia [34]. Additional factors associated with a poor prognosis include thrombocytopenia, eosinophilia, bone marrow involvement, a high interleukin (IL)-5 serum-level, CC chemokine receptor 4 (CCR4) expression, lung resistance-related protein (LRP), p53 mutation and p16 deletion by multivariate analysis [33]. Specific for the chronic type of ATL, high LDH, high blood urea nitrogen (BUN), and low albumin levels were identified as factors for a poor prognosis by multi-variate analysis [10]. Primary cutaneous tumoral type generally included among smoldering ATL had a poor prognosis in a uni-variate analysis [27].

Recently, a retrospective review of 807 patients in Japan led to a prognostic index for acute- and lymphoma-type ATL based on five prognostic factors; stage, performance status (PS), age, serum albumin and sIL2R. In the validation sample, the index was reproducible with median survival times (MSTs) of 3.6, 7.3, and 16.2 months for patients at high, intermediate, and low risk, respectively [35]. The Japan Clinical Oncology Group (JCOG)-Lymphoma Study Group (LSG) conducted a meta-analysis of three consecutive trials exclusively for aggressive ATL (see below) [36]. OS analysis of a total 276 patients with acute-, lymphoma- or unfavorable chronic-ATL identified two significant prognostic factors, PS and hypercalcemia. In the validation sample, a proposed prognostic index using the two factors into two strata revealed MSTs of 6.3, and 17.8 months for patients at high and low risk, respectively. In both

Table 1
Diagnostic criteria for clinical subtypes of adult T-Cell leukemia-lymphoma.

	Smoldering	Chronic	Lymphoma	Acute
Anti-HTLV-1 antibody	+	+	+	+
Lymphocyte ($\times 10^3/\mu$UL)	<4	≥4	<4	[a]
Abnormal T lymphocytes	≥5%[d]	+[c]	≤1%	+[c]
Flower cells with T-cell marker	[b]	[b]	No	+
LDH	≤1.5 N	≤2 N	[a]	[a]
Corrected Ca^{2+} (mEq/L)	<5.5	<5.5	[a]	[a]
Histology-proven lymphadenopathy	No	[a]	+	[a]
Tumor lesion				
Skin and/or lung	[a]	[a]	[a]	[a]
Lymph node	No	[a]	Yes	[a]
Liver	No	[a]	[a]	[a]
Spleen	No	[a]	[a]	[a]
Central nervous system	No	[a]	[a]	[a]
Bone	No	No	[a]	[a]
Ascites	No	No	[a]	[a]
Pleural effusion	No	No	[a]	[a]
Gastrointestinal tract	No	No	[a]	[a]

HTLV-1, human T-lymphotropic virus type I; LDH, lactate dehydrogenase; N normal upper limit.
With permission from Shimoyama M, Members of the Lymphoma Study Group (1984–1987): Diagnostic criteria and classification of clinical subtypes of adult T-cell leukemia-lymphoma. Br J Haematol 1991; 79:428.

[a] No essential qualification except terms required for other subtype(s).

[b] Typical "flower cells" may be seen occasionally.

[c] If the proportion of abnormal T lymphocytes is less than 5% in peripheral blood, a histologically proven tumor lesion is required.

[d] Histologically proven skin and/or pulmonary lesion(s) is required if there are fewer than 5% abnormal T lymphocytes in peripheral blood.

studies, however, the 5-year OS rate was less than 15% even in the low risk group, indicating that they are not sufficient to properly identify non-candidates for allo-HSCT which can achieve a cure of ATL despite considerable treatment-related mortality.

Treatment of ATL

Current treatment options for ATL include watchful waiting until the disease progresses, interferon alpha (IFN) and zidovudine (AZT) therapy, multi-agent chemotherapy, allogeneic hematopoietic stem cell transplantation (allo-HSCT) and new agents.

Recently, a treatment strategy based on the clinical subtype classification and prognostic factors was suggested as shown in Table 2 [33].

Watchful waiting

At present, no standard management for indolent ATL exists. Therefore, patients with the smoldering or favorable chronic type, may survive one or more years without chemotherapy, excluding topical therapy for cutaneous lesions, are observed and therapy is delayed until disease progression [33]. However, it was recently found that the long-term prognosis of such patients was poorer than expected. In a long-term follow-up study for 78 patients with indolent ATL (favorable chronic- or smoldering-type) with a policy of watchful waiting until disease progression at a single institution in Japan, the MST was 5.3 years with no plateau in the survival curve. Twelve patients remained alive for >10 years, 32 progressed to acute ATL, and 51 died [37].

Chemotherapy

Since 1978, a number of consecutive chemotherapy trials have been conducted for patients newly diagnosed with ATL by the JCOG-Lymphoma Study Group (LSG) (Table 3) [10]. Between 1981 and 1983, JCOG conducted a phase III trial (JCOG8101) to evaluate LSG1-VEPA (vincristine, cyclophosphamide, prednisone, and doxorubicin) vs LSG2-VEPA-M (VEPA plus methotrexate (MTX)) for advanced non-Hodgkin lymphoma (NHL), including ATL [10]. The complete response (CR) rate of LSG2-VEPA-M for ATL (37%) was marginally higher than that of LSG1-VEPA (17%; $P = .09$). However,

Table 2
Strategy for the treatment of adult T-Cell leukemia-lymphoma.

Smoldering-or favorable chronic-type ATL
- Consider inclusion in prospective clinical trials
- Symptomatic patients (skin lesions, opportunistic infections, etc): consider AZT/IFN or watch and wait
- Asymptomatic patients: consider watch and wait

Unfavorable chronic- or acute-type ATL
- If outside clinical trials, check prognostic factors (including clinical and molecular factors if possible):
  - Good prognostic factors: consider chemotherapy (VCAP-AMP-VECP evaluated by a phase III trial against biweekly-CHOP) or AZT/IFN (evaluated by a meta-analysis on retrospective studies)
  - Poor prognostic factors: consider chemotherapy followed by conventional or reduced intensify allo-HSCT (evaluated by retrospective and prospective Japanese analyses, respectively).
  - Poor response to initial therapy: consider conventional or reduced intensity allo-HSCT

Lymphoma-type ATL
- If outside clinical trials, consider chemotherapy (VCAP-AMP-VECP)
- Check prognostic factors (including clinical and molecular factors if possible) and response to chemotherapy:
  - Good prognostic factors and good response to initial therapy: consider chemotherapy followed by observation
  - Poor prognostic factors or poor response to initial therapy: consider chemotherapy followed by conventional or reduced intensity allo-HSCT.

Table 3
Results of sequential chemotherapeutic-trials of untreated patients with ATL (JCOG-LSG).

	J7801	J8101	J8701	J9109	J9303	JCOG9801	
	LSG1	LSG1/LSG2	LSG4	LSG11	LSG15	mLSG15/mLSG19	
Pts. no.	18	54	43	62	96	57	61
CR (%)	16.7	27.8	41.9	28.3	35.5	40.4	24.6
CR + PR (%)				51.6	80.6	72.0	65.6
MST (months)		7.5	8.0	7.4	13.0	12.7	10.9
2 yr. survival (%)				17.0	31.3		
3 yr. survival (%)				10.0	21.9	23.6	12.7
4 yr survival (%)		8.0	11.6				

CR: complete remission, PR: partial remission, MST: median survival time.

the CR rate was significantly lower for ATL than for B-cell NHL and peripheral T-cell lymphoma (PTCL) other than ATL ($P < .001$). The MST of the 54 patients with ATL was 6 months, and the estimated 4-year survival rate was 8%.

In 1987, JCOG initiated a multicenter phase II study (JCOG8701) of a multiagent combination chemotherapy (LSG4) for advanced aggressive NHL (including ATL). LSG4 consisted of three regimens: (1) VEPA-B (VEPA plus bleomycin), (2) M-FEPA (methotrexate, vindesine, cyclophosphamide, prednisone, and doxorubicin), and (3) VEPP-B, (vincristine, etoposide, procarbazine, prednisone, and bleomycin) [10]. The CR rate for ATL patients was improved from 28% (JCOG8101) to 43% (JCOG8701); however, the CR rate was significantly lower in ATL than in B-cell NHL and PTCL ($P < .01$). Patients with ATL still showed a poor prognosis, with an MST of 8 months and a 4-year survival rate of 12%.

The first phase II trial (JCOG9109) with a pentostatin, which was considered to be a promising agent showing responses against relapsed/refractory ATL as a single agent, -containing combination (LSG11) as the initial chemotherapy [38]. A total of 62 untreated patients with aggressive ATL (34 acute, 21 lymphoma, and 7 unfavorable chronic type) were enrolled. Among the 60 eligible patients, there were 17 CRs (28%) and 14 partial responses (PRs) (overall response rate [ORR] = 52%). The MST was 7.4 months, and the estimated 2-year survival rate was 17%. The prognosis of patients with ATL remained poor, even though they were treated with a pentostatin-containing combination chemotherapy.

In 1994, JCOG initiated a phase II trial (JCOG9303) of an eight-drug regimen (LSG15) consisting of vincristine, cyclophosphamide, doxorubicin, prednisone, ranimustine, vindesine, etoposide, and carboplatin for untreated ATL [39]. Dose intensification was attempted with the prophylactic use of granulocyte colony-stimulating factor (G-CSF). In addition, non–cross-resistant agents such as ranimustine and carboplatin, and intrathecal prophylaxis with methotrexate and prednisone were incorporated. Ninety-six previously untreated patients with aggressive ATL were enrolled: 58 acute, 28 lymphoma, and 10 unfavorable chronic types. Approximately 81% of the 93 eligible patients responded (75/93), with 33 patients obtaining a CR (35%). The overall survival rate of the 93 patients at 2 years was estimated to be 31%, with an MST of 13 months. Grade 4 neutropenia and thrombocytopenia were observed in 65% and 53% of the patients, respectively, whereas grade 4 non-hematologic toxicity was observed in only one patient.

To confirm whether the LSG15 regimen would be considered as the new standard for the treatment of aggressive ATL, JCOG conducted a phase III trial comparing modified (m)-LSG15 (Fig. 1) with CHOP-14 (cyclophosphamide, hydroxy-doxorubicin, vincristine [Oncovin], and prednisone), both supported with G-CSF and intrathecal prophylaxis [37]. A total of 118 patients were enrolled. The CR rate was higher in the mLSG15 arm than in the CHOP-14 arm (40% vs. 25%, respectively; $P = .020$). The MST and OS rate at 3 years were 12.7 months and 24% in the mLSG15 arm and 10.9 months and 13% in the CHOP-14 arm {two-sided $P = .169$, and the hazard ratio was 0.75; 95% confidence interval (CI), 0.50 to 1.13}. In mLSG15 vs. CHOP-14, rates of grade 4 neutropenia, grade 4 thrombocytopenia and grade 3/4 infection were 98% vs. 83%, 74% vs. 17% and 32% vs. 15%, respectively. Three treatment-related deaths (TRDs), two from sepsis and one from interstitial pneumonitis related to neutropenia, were reported in the mLSG15 arm. The longer survival at 3 years and higher CR rate with mLSG15 compared with CHOP-14 suggest that mLSG15 is a more effective regimen at the expense of greater toxicity, providing the basis for future investigations in the treatment of ATL [40]. The superiority of VCAP-AMP-VECP in mLSG15 to