the candidate molecules for such differential control of Treg-cell subpopulations is chemokine receptors, which allow Treg cells to migrate to a specific inflammation site via sensing specific chemokine milieu (19).

It has been shown that tumor-infiltrating macrophages and tumor cells produce the chemokine (C-C motif) ligand 22 (CCL22), which chemoattracts Treg cells as well as effector T cells expressing C-C chemokine receptor type 4 (CCR4) (6, 10, 20). In this report, we have addressed whether CCR4-targeting treatment is able to selectively reduce a particular Treg-cell subpopulation, rather than whole Treg population, and thereby elicit or augment in vitro and in vivo antitumor immune responses in humans.

Results

Depletion of CCR4$^+$ T Cells Predominantly Depletes eTreg Cells. In peripheral blood mononuclear cells (PBMCs) of healthy individuals, CCR4$^+$ T cells were present in both FOXP3$^+$ and FOXP3$^-$ T-cell fractions, and FOXP3^{hi} cells in particular were CCR4$^+$ (Fig. 1*A*). When FOXP3$^+$ T cells were classified into three populations by the levels of FOXP3 and CD45RA expression (18), FOXP3^{hi}CD45RA$^-$ eTreg cells (Fr. II) predominantly expressed CCR4 at the protein and mRNA level (Fig. 1*A*, and Figs. S1 and S2*A*). In contrast, FOXP3^{lo}CD45RA$^+$ naive Treg cells (Fr. I) scarcely expressed the molecule, whereas FOXP3^{lo}CD45RA$^-$ non-Treg cells (Fr. III) exhibited a moderate expression. Among FOXP3$^-$ cells, some CD45RA$^-$CD4$^+$ memory or activated T cells expressed CCR4, whereas CD45RA$^+$ CD4$^+$ naive T cells did not. CD25 expression was well correlated with CCR4 expression with the highest CD25 expression by eTreg cells (Fr. II). Analyses of multiple samples of PBMCs from healthy individuals showed similar patterns of CCR4 expression by FOXP3 subsets (Fig. 1*B*). CD8$^+$ T cells, natural killer (NK) cells, CD14$^+$ monocytes/macrophages, dendritic cells, and B cells hardly expressed CCR4 at the protein and mRNA level (Fig. S2). In vitro depletion of CCR4$^+$ cells from PBMCs by magnet-bead sorting with anti-CCR4 mAb predominantly decreased CD4$^+$FOXP3^{hi} CD45RA$^-$ eTreg cells (Fr. II) and, to a lesser extent, CD4$^+$ FOXP3^{lo}CD45RA$^-$ non-Treg cells (Fr. III), but spared CD4$^+$ FOXP3^{lo}CD45RA$^+$ naive Treg cells (Fr. I) and FOXP3$^-$ cells (Fr. IV and V) (Fig. 1*C*). In contrast with anti-CCR4 mAb treatment, similar in vitro cell depletion with anti-CD25 mAb significantly reduced all of the FOXP3$^+$ subpopulations (Fr I, II, and III) and, to a lesser extent, FOXP3$^-$CD45RA$^-$CD4$^+$ activated or memory T cells (Fr. IV), with a relative increase in FOXP3$^-$ CD45RA$^+$CD4$^+$ naive T cells (Fr. V) (Fig. 1*D*). PBMCs of melanoma patients showed similar patterns of CCR4 expression by FOXP3$^+$ subpopulations and similar changes in the composition of FOXP3$^+$ T-cell subsets after in vitro CCR4$^+$ T-cell depletion (Fig. S3).

Taking these data together, we find that CCR4 is predominantly expressed by eTreg cells and depletion of CCR4$^+$ cells results in selective reduction of eTreg cells, while preserving naive Treg cells and the majority of FOXP3$^-$CD4$^+$ T cells.

Tumor-Infiltrating Treg Cells Exhibit the eTreg-Cell Phenotype and Can Be Depleted in Vitro by Anti-CCR4 mAb. Although there is accumulating data that FOXP3$^+$ T cells predominantly infiltrate into tumor tissues (6, 7, 10, 21), their detailed phenotypes remain to be determined. Our analysis of TILs in nine melanoma samples revealed infiltration of a high percentage of CCR4$^+$ T cells, the majority of which were CD4$^+$FOXP3^{hi}CD45RA$^-$ eTreg cells (Fr. II), with only a small number of CD4$^+$FOXP3^{lo}CD45RA$^+$ naive Treg cells (Fr. I) (Fig. 2*A*). In vitro depletion of CCR4$^+$ T cells indeed dramatically reduced these tumor-infiltrating eTreg cells (Fig. 2*B*), indicating that anti-CCR4 mAb treatment is able to selectively deplete eTreg cells abundantly infiltrating into tumors.

In Vitro Induction of NY-ESO-1–Specific CD4$^+$ T Cells After CCR4$^+$ T-Cell Depletion from PBMCs of Healthy Donors and Melanoma Patients. With the efficient depletion of the eTreg-cell population by in vitro anti-CCR4 mAb treatment, we next examined

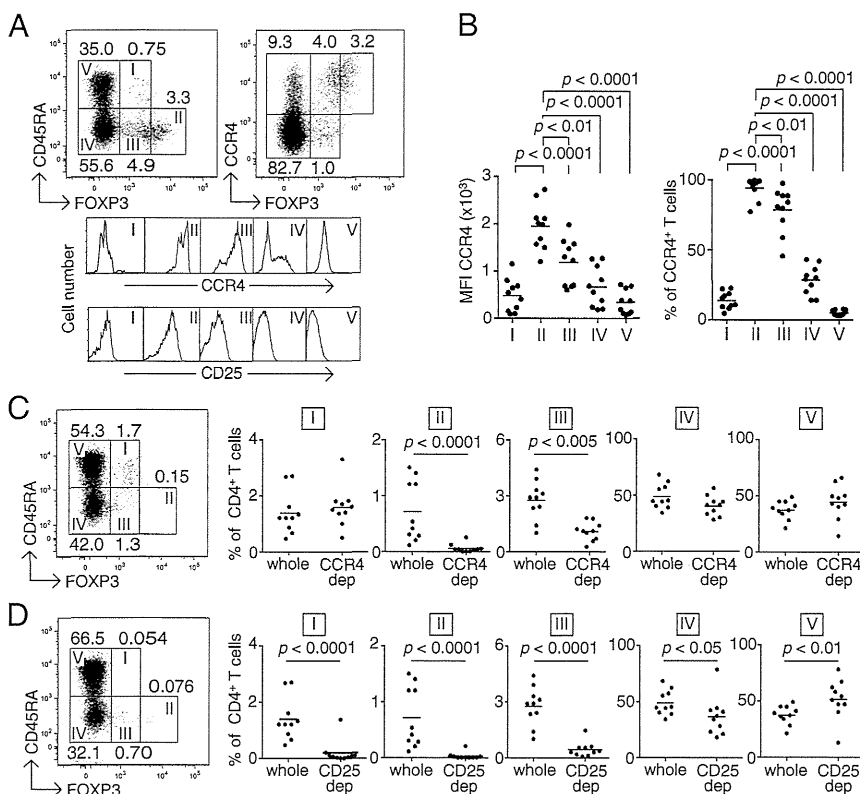

Fig. 1. Reduction of eTreg cells by in vitro depletion of CCR4-expressing T cells. (*A*) CCR4 and CD25 expression by subpopulations of FOXP3$^+$ Treg cells in PBMCs from healthy donors. CCR4 and CD25 expression levels were evaluated for each fraction. Representative data from 10 healthy donors are shown. (*B*) Median fluorescence intensity (MFI, *Left*) and frequency (*Right*) of CCR4 expression by each fraction of T cells in PBMCs of healthy donors (n =10). (*C*) Changes in the proportion of T-cell subpopulations after CCR4$^+$ T-cell depletion (CCR4 dep) (n = 10). (*D*) Changes in the proportion of T-cell subpopulations after CD25$^+$ T-cell depletion (CD25 dep) (n = 10). The numbers in *A*, *C*, and *D* indicate the percentage of gated CD4$^+$ T cells. Representative staining profiles in *A*, *C*, and *D* are from the same donor, and the same PBMC samples were analyzed in *B*–*D*.

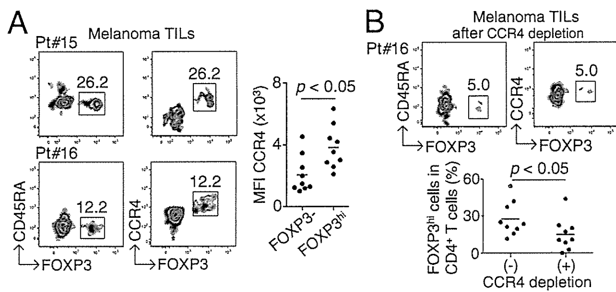

Fig. 2. Predominant infiltration of CCR4$^+$ eTreg cells into melanoma tissues. (*A*) CCR4 expression by melanoma-infiltrating T cells. CD4$^+$ T cells from melanoma sites were fractionated into subpopulations based on the expression of CCR4, CD45RA, and FOXP3; CCR4 expression by each fraction was analyzed. Data from two representative patients are shown. (*Right*) Summary of MFI of CCR4 expression by FOXP3$^-$ or FOXP3$^+$ cells ($n = 9$). (*B*) CCR4$^+$ CD4$^+$ T cells from melanoma tissues (Pt #16) were depleted of CCR4$^+$ T cells and then analyzed for the proportion of FOXP3^{hi} eTreg cells. (*Lower*) Percentages of FOXP3^{hi} cells among CD4$^+$ T cells after CCR4$^+$ cell depletion or nondepletion ($n = 9$). The numbers in *A* and *B* indicate the percentage of gated CD4$^+$ T cells.

whether CCR4$^+$ T-cell depletion from PBMCs of healthy donors was able to induce tumor antigen-specific CD4$^+$ T cells. We assessed specific T-cell responses to NY-ESO-1, a cancer/testis antigen, which is normally expressed by human germ-line cells and also by various types of cancer cells (4, 22). CCR4$^-$CD4$^+$ T cells or CD25$^-$CD4$^+$ T cells were cultured with CD4$^-$CD8$^-$ PBMCs as antigen-presenting cells (APCs), which were pulsed overnight with series of overlapping peptides covering the entire sequence of the NY-ESO-1 protein and X-irradiated (35 Gy) before use, as previously described (23, 24). Fifteen to 20 d later, NY-ESO-1–specific CD4$^+$ T cells secreting IFN-γ were enumerated by enzyme-linked immunospot (ELISpot) assay. Significant numbers of IFN-γ–secreting NY-ESO-1–specific CD4$^+$ T cells were induced in 7 of 16 healthy donors (43.8%), but only in the cultures with CCR4$^+$ or CD25$^+$ T-cell–depleted T cells (Fig. 3*A*, and summarized in Table S1). Furthermore, the frequencies of IFN-γ–secreting NY-ESO-1–specific CD4$^+$ T cells were higher after CCR4$^+$ T-cell depletion compared with CD25$^+$ T-cell depletion in five of seven healthy donors (71.4%) (Table S1). This result could be attributed in part to possible depletion of NY-ESO-1–specific CD25$^+$ activated T cells by anti-CD25 mAb treatment. The NY-ESO-1–specific CD4$^+$ T cells produced IFN-γ and TNF-α (Fig. 3*B*). Those cells induced in vitro after CCR4$^+$ T-cell depletion recognized NY-ESO-1 peptides at the concentration as low as 0.1 μM (Fig. 3*C*), and also NY-ESO-1 peptides produced by natural processing of the NY-ESO-1 protein by APCs, as previously shown with CD25$^+$ T-cell depletion (22, 24) (Fig. 3*D*).

We also attempted to determine whether Treg-cell depletion would evoke anti–NY-ESO-1 responses in apparently nonresponsive melanoma patients. With PBMCs from patients bearing NY-ESO-1–expressing melanomas, but without detectable NY-ESO-1–specific Ab in the sera, in vitro depletion of CCR4$^+$ or CD25$^+$ T cells and subsequent in vitro peptide stimulation induced IFN-γ– and TNF-α–secreting NY-ESO-1–specific CD4$^+$ T cells in three of eight patients (37.5%) (Fig. S4 *A* and *B* and Table S2). These NY-ESO-1–specific CD4$^+$ T cells appeared to express high-avidity T-cell receptors that recognized NY-ESO-1 peptides at a concentration as low as 0.1 μM, as seen with healthy donor T cells (Fig. S4*C*).

Thus, in healthy individuals as well as melanoma patients who had not raised spontaneous NY-ESO-1 immune responses, removal of eTreg cells by CCR4$^+$ T-cell depletion is able to efficiently induce high-avidity NY-ESO-1–specific CD4$^+$ T cells secreting effector cytokines.

CCR4$^+$ T-Cell Depletion Augments in Vitro Induction of NY-ESO-1–Specific CD8$^+$ T Cells from PBMCs of Melanoma Patients. PBMCs from melanoma patients were subjected to in vitro depletion with anti-CCR4 mAb or anti-CD25 mAb, and cultured with NY-ESO-1 peptide capable of binding to HLA class I of each patient. Seven to 10 d later, NY-ESO-1–specific CD8$^+$ T cells were detected by NY-ESO-1/HLA tetramers and analyzed for intracellular cytokine production. NY-ESO-1–specific CD8$^+$ T cells were induced in four of six patients (66.7%), and the responses were markedly augmented after depletion of CCR4$^+$ or CD25$^+$ cells (Fig. 4*A*). In addition, these NY-ESO-1–specific CD8$^+$ T cells recognized an HLA-matched malignant melanoma cell line and secreted IFN-γ and TNF-α (Fig. 4*B*). For example, Pt. #9 (HLA-A*02/29, B*44/27, C*03/04) harbored not only HLA-C*03–restricted NY-ESO-1–specific CD8$^+$ T-cells detected by HLA Cw*0304/NY-ESO-1 tetramers, but also those NY-ESO-1–specific CD8$^+$ T cells that recognized the SK-MEL 37 melanoma line (A*0201$^+$, NY-ESO-1$^+$) in an HLA-A2–restricted manner.

We also examined whether NY-ESO-1–specific CD8$^+$ T cells could be induced by directly adding mAb into cell cultures. Addition of anti-CD25 mAb or anti-CCR4 mAb reduced the frequency of CD4$^+$FOXP3^{hi}CD45RA$^-$ eTreg cells (Fr. II) (Fig. S5).

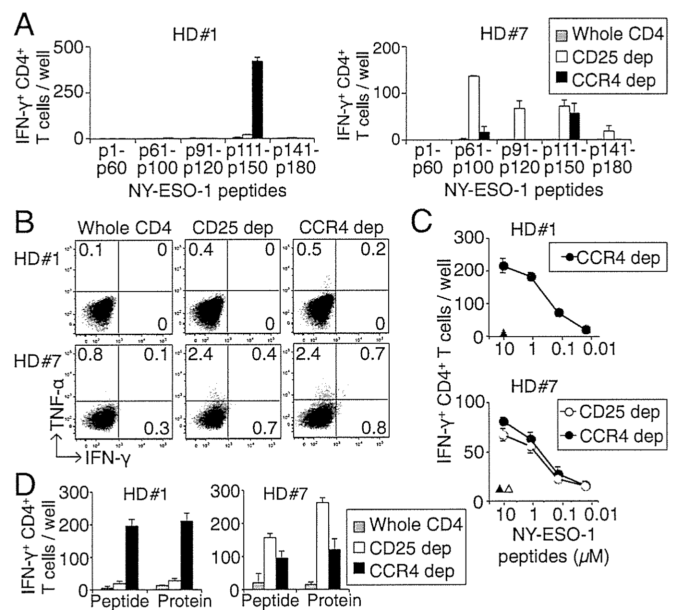

Fig. 3. Induction of cancer/testes antigen-specific CD4$^+$ T cells by depletion of CCR4- or CD25-expressing T cells in healthy donors. (*A*) CD4$^+$ T-cell responses to NY-ESO-1 peptides after depletion of CCR4$^+$ or CD25$^+$ T cells. CD4$^+$ T cells prepared from PBMCs of healthy donors were presensitized with APCs pulsed with NY-ESO-1 peptide covering the entire sequence of NY-ESO-1. Results of 2 (HD#1 and HD#7) among 16 healthy donors are shown. The numbers of IFN-γ–secreting CD4$^+$ T cells were assessed by ELISpot assay. (*B*) Intracellular cytokine secretion of CD4$^+$ T cells shown in *A*. The numbers in figures indicate the percentage of gated CD4$^+$ T cells. (*C*) Peptide dose-dependent recognition of NY-ESO-1–specific IFN-γ–secreting CD4$^+$ T cells. NY-ESO-1–specific CD4$^+$ T cells derived from CCR4$^+$ or CD25$^+$ T-cell–depleted cells (CCR4 dep and CD25 dep, respectively) were cultured with autologous activated T-cell APCs pulsed with graded amounts of NY-ESO-1 peptides and assessed for the number of IFN-γ–secreting cells as in *A*. Triangles indicate responses to control peptide at 10 μM. (*D*) Recognition of naturally processed NY-ESO-1 protein antigen by NY-ESO-1–specific CD4$^+$ T cells derived from whole CD4$^+$, CCR4$^+$ cell-depleted, or CD25$^+$ cell-depleted cells. NY-ESO-1–specific CD4$^+$ T cells from two healthy donors were cultured with autologous dendritic cells pulsed with NY-ESO-1 or control protein, or with NY-ESO-1 or control peptide. The experiments were independently performed twice with similar results.

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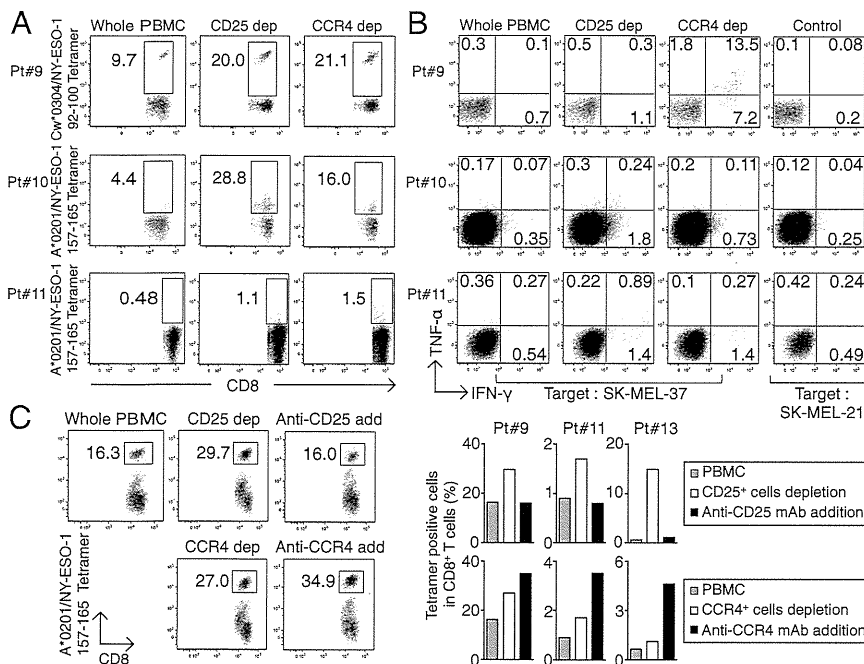

Fig. 4. Augmentation of NY-ESO-1–specific CD8$^+$ T-cell induction in melanoma patients by in vitro CCR4$^+$ T-cell depletion. (*A*) Induction of NY-ESO-1–specific CD8$^+$ T cells. Unfractionated PBMCs, or PBMCs depleted of CD25$^+$ or CCR4$^+$ cells, were prepared from melanoma patients (n = 6), and presensitized in peptides capable of binding to patients' HLA. NY-ESO-1–specific CD8$^+$ T cells were analyzed with NY-ESO-1/HLA tetramers (Pt. #9: A*02/29, B*44/27, C*03/04, Pt. #10: A*02/11, B*35/44, C*04/05, and Pt. #11: A*02/-, B*13/18, C*06/07). (*B*) Cytokine secretion of NY-ESO-1–specific CD8$^+$ T cells upon recognition of the HLA-A*0201$^+$ melanoma cell line SK-MEL 37 (NY-ESO-1$^+$), or SK-MEL-21 (NY-ESO-1$^-$) analyzed by intracellular cytokine staining. Data from three representative patients are shown. (*C*) Induction of antigen-specific CD8$^+$ T cells by addition (add) of anti-CD25 or anti-CCR4 mAb (KM2160) to cell cultures, or by CCR4$^+$ or CD25$^+$ cell depletion or nondepletion, as shown in *A* (Pt. #13 A02/03, B07/41, C07/17). A representative result (*Left*) and summary of three melanoma patients (*Right*) are shown. The numbers in the panels indicate the percentage of gated CD8$^+$ T cells. These experiments were performed independently at least twice with similar results.

Interestingly, although NY-ESO-1–specific CD8$^+$ T-cell induction was augmented in the cell culture containing anti-CCR4 mAb, the addition of anti-CD25 mAb reduced the frequency of NY-ESO-1–specific CD8$^+$ T cells (Fig. 4*C*), indicating that it might have killed some CD25$^+$CD8$^+$ activated effector T cells in addition to CD25$^+$CD4$^+$ Treg cells.

These results indicate that depletion of CCR4$^+$ T cells before in vitro induction or even simple incubation with anti-CCR4 mAb during the induction effectively augments NY-ESO-1–specific CD8$^+$ T-cell responses by selectively reducing eTreg cells.

Anti-CCR4 mAb Administration into Adult T-Cell Leukemia-Lymphoma Patients Reduces CD4$^+$FOXP3^{hi}CD45RA$^-$ eTreg Cells and Augments NY-ESO-1–Specific CD8$^+$ T-Cell Responses. In adult T-cell leukemia-lymphoma (ATL), which is caused by human T-lymphotropic virus 1 infection, ATL cells are CD4$^+$ and the majority—if not all—of them express FOXP3, CD25, CTLA-4, and CCR4, thus resembling naturally occurring FOXP3$^+$ Treg cells (25–28). Although it is currently difficult to discriminate whether anti-CCR4 mAb reduces ATL cells or normal FOXP3$^+$ Treg cells (29), we examined whether in vivo administration of anti-CCR4 mAb (Mogamulizumab), which has a cell-depleting effect by antibody-dependent cellular cytotoxicity, was able to reduce FOXP3$^+$ cells or a subpopulation thereof. Analysis of PBMCs from ATL patients collected before and after anti-CCR4 mAb therapy revealed that CD4$^+$FOXP3^{hi}CD45RA$^-$ cells including both ATL cells and eTreg cells were markedly reduced after the therapy (Fig. 5*A*). In addition, in a patient whose ATL cells expressed NY-ESO-1, NY-ESO-1–specific CD8$^+$ T cells producing IFN-γ and TNF-α were induced after several rounds of anti-CCR4 mAb administration (Fig. 5*B*). NY-ESO-1–specific CD8$^+$ T cells producing these cytokines were much higher in frequency than NY-ESO-1–specific CD8$^+$ T cells detected by NY-ESO-1/HLA-B*3501 tetramers, suggesting that this patient additionally possessed CD8$^+$ T cells recognizing other epitopes of NY-ESO-1. These results collectively indicate that anti-CCR4 mAb therapy for ATL is able to selectively deplete eTreg cells as well as ATL cells in vivo, and induce/augment tumor antigen-specific T-cell responses, although it is possible that anti-CCR4 mAb-induced reduction of FOXP3$^+$ ATL cells, which reportedly exhibit a Treg-cell–like in vitro suppressive activity (27, 28), might also contribute to the augmentation of immune responses.

Discussion

Accumulating evidence indicates that effective cancer immunotherapy needs to control FOXP3$^+$ Treg cells naturally present in the immune system and abundantly infiltrating into tumor tissues (10, 11, 30). Here, we have shown that CD4$^+$FOXP3^{hi}CD45RA$^-$ eTreg cells, which are terminally differentiated and most suppressive, highly express CCR4, that they are predominant among FOXP3$^+$ T cells infiltrating into tumor tissues (e.g., melanoma), and that specific depletion of eTreg cells in vivo or in vitro by anti-CCR4 mAb evoked tumor antigen-specific immune responses mediated by CD4$^+$ and CD8$^+$ T cells in healthy individuals and cancer patients.

Besides high expression of CCR4 in eTreg cells, CCR4 is expressed, although to a lesser extent, in non-Treg CD4$^+$ T-cell fractions [i.e., the FOXP3^{lo}CD45RA$^-$ cells (Fr. III) and FOXP3$^-$CD45RA$^-$ cells (Fr. IV)]. The former are capable of secreting cytokines, such as IL-4 and IL-17, as previously reported with PBMCs of healthy individuals (18). It has also been shown that Th2 cells and a fraction of central memory CD8$^+$ T cells express CCR4 (31–33). It is thus likely that tumor-infiltrating activated macrophages, and presumably some tumor cells produce CCL22, which predominantly chemoattracts and recruits from peripheral blood both CCR4$^+$ eTreg and CCR4$^+$ effector T cells that recognize tumor-associated antigens (such as cancer/testis antigen) and presumably self-antigens released from tumor cells (6, 10, 21, 34). However, the frequency of IL-4– or IL-17–secreting CD4$^+$ T cells were much lower than eTreg cells among CCR4$^+$CD4$^+$ T cells in PBMCs and TILs in melanoma tissues of nontreated patients; and CCR4 expression by CD8$^+$ TILs were limited. Moreover, addition of anti-CCR4 mAb into in vitro peptide stimulation more effectively induced antigen-specific CD8$^+$ T cells than CCR4$^+$ T-cell depletion, indicating that anti-CCR4 mAb had reduced eTreg cells but spared CD8$^+$ effector T cells. The result contrasted with the addition of anti-CD25 mAb, which appeared to deplete CD25$^+$CD8$^+$ T cells and cancel the enhancing effect of Treg-cell depletion. These results taken together indicate that anti-CCR4 mAb treatment to augment antitumor immunity mainly target CCR4$^+$ eTreg cells

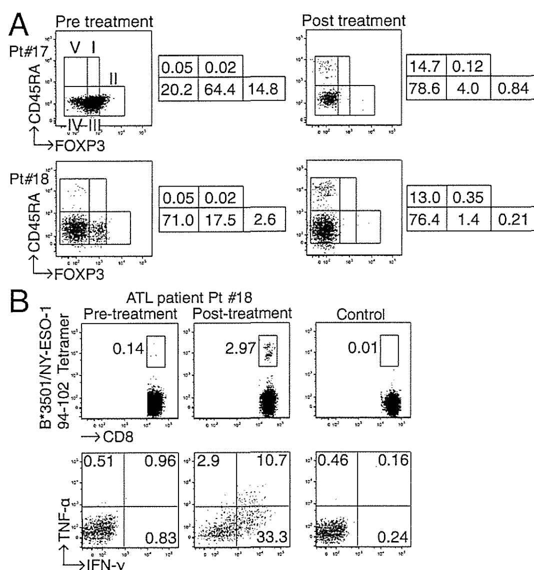

Fig. 5. Reduction of CD4$^+$FOXP3^{hi}CD45RA$^-$ T cells and augmentation of NY-ESO-1–specific CD8$^+$ T-cell responses in ATL patients after anti-CCR4 mAb (Mogamulizumab) therapy. (*A*) FOXP3$^+$ Treg-cell subpopulations in PBMCs from two ATL patients (Pt. #17: acute type, HLA-A*2402/-, B*3901/5401, C*0102/0702 and Pt. #18: lymphoma type, HLA-A*0201/3101, B*3501/4002, C*0303/0401) before and after anti-CCR4 mAb therapy. These experiments were performed at least twice with similar results. The numbers indicate the percentage of gated CD4$^+$ T cells. (*B*) Analysis of NY-ESO-1–specific CD8$^+$ T-cell induction before and after anti-CCR4 mAb therapy. PBMCs from Pt. #18 were presensitized in the presence of APCs pulsed with NY-ESO-$1_{91–110}$ peptide corresponding to the patient's HLA. NY-ESO-1–specific CD8$^+$ T cells were detected with NY-ESO-1/HLA tetramers, and cytokine secretion of these NY-ESO-1–specific CD8$^+$ T cells upon recognition of autologous activated T-cell APCs pulsed with NY-ESO-$1_{91–110}$ or control peptide was analyzed by intracellular cytokine staining. The numbers in figures indicate the percentage of gated CD8$^+$ T cells. The result was derived from a single assay because of limited availability of the patient's samples.

in tumor tissues and the regional lymph nodes, as well as peripheral blood, which would otherwise be a reservoir of fresh tumor-infiltrating Treg cells. Further study is warranted to determine whether depletion of CCR4$^+$CD4$^+$ and CD8$^+$ effector T cells in vivo affects antitumor immunity to a clinically significant extent.

Both NY-ESO-1–specific CD4$^+$ and CD8$^+$ T cells induced by in vitro anti-CCR4 mAb treatment possessed high-avidity T-cell receptors, and responded to dendritic cells processing tumor antigens and histocompatible tumor cell lines, respectively. This finding raises the issue of whether Treg depletion by anti-CCR4 mAb activates and expands already present antigen-primed effector T cells or newly induces effector T cells from a naive T-cell pool. We previously showed that in vitro NY-ESO-1-peptide stimulation following CD25$^+$CD4$^+$ T-cell depletion could activate NY-ESO-1–specific naive CD4$^+$ T-cell precursors in healthy individuals and in melanoma patients who possessed NY-ESO-1–expressing tumors but failed to develop anti-NY-ESO-1 Ab (23). In contrast, most NY-ESO-1–specific CD4$^+$ T cells in melanoma patients who had spontaneously developed anti–NY-ESO-1 Ab were derived from a memory population and could be activated even in the presence of CD25$^+$CD4$^+$ Treg cells (23). In addition, following vaccination of ovarian cancer patients with a HLA-DP–restricted NY-ESO-1 peptide, development of NY-ESO-1–specific high-avidity effector T cells from naive T cells was hampered by the presence of CD25$^+$CD4$^+$ Treg cells, although the vaccination could expand low-avidity NY-ESO-1–specific CD4$^+$ T cells that were apparently present in an effector/memory fraction before the vaccination (24). These results collectively indicate that elimination of eTreg cells by CCR4$^+$ T-cell depletion abrogates Treg cell-mediated suppression on NY-ESO-1–specific high-avidity naive T-cell precursors, allowing their activation and differentiation into high-avidity effector T cells capable of mediating strong antitumor immune responses. This successful induction of tumor antigen-specific CD4$^+$ and CD8$^+$ T cells indicates that the combination of anti-CCR4 mAb administration and vaccination with tumor antigens, such as NY-ESO-1, could be an ideal strategy for immunotherapy of a variety of cancers including ATL, which express NY-ESO-1 (35).

On the other hand, it was noted that not all healthy individuals or melanoma patients developed NY-ESO-1–specific T cells in vitro after Treg depletion for several possible reasons. For example, individuals who do not have a proper HLA haplotype may fail to select NY-ESO-1–reactive T cells thymically (22), hence possessing few NY-ESO-1–specific T-cell precursors. Other types of suppressor cells (such as myeloid-derived suppressor cells, immunosuppressive macrophages, and Foxp3$^-$ Treg cells) might contribute to inhibiting the induction of the responses (30). Alternatively, T cells specific for NY-ESO-1, a cancer/testis antigen, may also be subjected to other mechanisms of immunological self-tolerance—for example, anergy—hence being hyporesponsive to the antigen (36). These possibilities are under investigation to make anti-CCR4 mAb therapy more effective.

Would in vivo anti-CCR4 mAb treatment to deplete Treg cells elicit harmful autoimmunity? It has been shown in animal models that a longer period and a more profound degree of Treg-cell depletion is required to elicit clinically and histologically evident autoimmunity than evoking effective antitumor immunity (37, 38). In humans, naive Treg cells are generally well preserved in peripheral blood in cancer patients, even if they are low in frequency in tumor tissues. Furthermore, CCR4$^+$ T-cell depletion selectively eliminates eTreg cells but spares naive Treg cells. Assuming that effective tumor immunity can be evoked without significant autoimmunity via controlling the degree and duration of Treg-cell depletion, it is likely that, although anti-CCR4 mAb administrations reduce eTreg cells in the immune system during the treatment, the residual CCR4$^-$ eTreg cells (as shown in Fig. 2), including those which have newly differentiated from naive Treg cells, are sufficient to prevent deleterious autoimmunity. Supporting this notion, only a minor population of ATL patients treated with anti–CCR4 mAb experienced severe immune-related adverse events, except skin rashes (29). Anti-CCR4 mAb therapy can therefore be a unique cancer immunotherapy aiming at depleting eTreg cells without clinically serious adverse effects that would be incurred by total Treg-cell depletion or functional blockade (39).

The critical roles of CCR4 in Treg-cell recruitment to tumors have been reported with various types of human cancers, such as malignant lymphomas, gastric, ovarian, and breast cancers (10). CCR4$^+$ eTreg cells abundantly and predominantly infiltrated into gastric and esophageal cancers as observed with melanoma. Although it remains to be determined whether every cancer tissue has predominant infiltration of CCR4$^+$ eTreg cells, it is envisaged that possible combination of anti-CCR4 mAb treatment, tumor antigen immunization, and antibody-mediated immune checkpoint blockade will further increase clinical efficacy of cancer immunotherapy.

Materials and Methods

Donor Samples. PBMCs were obtained from healthy donors, malignant melanoma patients with NY-ESO-1 expression, and ATL patients. To collect tumor-infiltrating T cells, melanoma tissues were minced and treated with gentleMACS Dissociator (Miltenyi Biotec). All healthy donors were subjects with no history of autoimmune disease. All donors provided written informed consent before sampling according to the Declaration of Helsinki. The present study was approved by the institutional ethics committees of Osaka University, Osaka, Japan and Landesarztekammer Hessen, Frankfurt, Germany.

Antibodies and Peptides. The information of antibodies and synthetic peptides is provided in *SI Materials and Methods*.

Preparation of CD25$^-$ or CCR4$^-$ Cells. PBMCs or CD4$^+$ T cells were treated with biotin-anti-CD25 mAb (BC96) or biotin-anti-CCR4 (1G1) mAb (0.01 mg/mL), otherwise specified, for 15 min at 4 °C. Subsequently, anti-Biotin MicroBeads (Miltenyi Biotec) were added as described in the manufacturer's protocol, then washed using PBS containing 2% (vol/vol) FCS. CD25$^-$ or CCR4$^-$ cells were separated on autoMACS Pro Separator (Miltenyi Biotec).

In Vitro Sensitization of NY-ESO-1–Specific CD4$^+$ T Cells. NY-ESO-1–specific CD4$^+$ T cells were presensitized as previously described (23, 24) and in *SI Materials and Methods*.

In Vitro Sensitization of NY-ESO-1–Specific CD8$^+$ T Cells. For in vitro sensitization of NY-ESO-1–specific CD8$^+$ T cells, 1.5–2 $\times$ 10^6 cells were cultured with NY-ESO-1 peptides (NY-ESO-1$_{157–165}$ for HLA-A*0201 restricted, NY-ESO-1$_{92–100}$ for HLA-Cw*0304 restricted, NY-ESO-1$_{91–110}$ for HLA-B*3501 restricted, 10 μM) (22, 23) in a 48-well dish or round-bottom 96-well plate. After 8 h, one-half of the medium was replaced by fresh medium containing IL-2 (20 U/mL) and IL-7 (40 ng/mL) and repeated twice per week. In some assays, purified anti-CD25 (M-A251) mAb or anti-CCR4 (KM2160) mAb (1 μg/mL) was included in some wells during the entire period of culture.

ELISpot Assay. The number of IFN-γ–secreting NY-ESO-1–specific CD4$^+$ T cells was assessed by ELISpot assay as previously described (23, 24) and in *SI Materials and Methods*.

Intracellular Cytokine Secretion Assay. The presensitized CD4$^+$ and CD8$^+$ T cells were restimulated with peptide-pulsed autologous activated T-cell APCs, SK-MEL-21 cells (NY-ESO-1$^-$, HLA-A*0201$^+$), or SK-MEL-37 cells (NY-ESO-1$^+$, HLA-A*0201$^+$) for 1 h, after which GolgiStop reagent (BD Biosciences) was added. Subsequently, cells were cultured for another 6–8 h at 37 °C. Cells were stained for cell surface markers and then for intracellular cytokines using BD Cytofix/Cytoperm Buffer and BD Perm/Wash Buffer (BD Biosciences). Results were analyzed by flow cytometry (BD LSRFortessa; BD Biosciences) and FlowJo v9.6.2 software (TreeStar).

Tetramer Assay. Tetramer staining was performed as previously described (35, 40) and in *SI Materials and Methods*.

Preparation of Dendritic Cells. Dendritic cells were prepared as previously described (24) and in *SI Materials and Methods*.

Statistical Analysis. The significance of the difference in each data between two groups was assessed by a Mann–Whitney test using Prism version 6 software (GraphPad). *P* values less than 0.05 were considered significant.

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1. Sakaguchi S (2004) Naturally arising CD4$^+$ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22:531–562.
2. Sakaguchi S, Miyara M, Costantino CM, Hafler DA (2010) FOXP3$^+$ regulatory T cells in the human immune system. *Nat Rev Immunol* 10(7):490–500.
3. Kawakami Y, Rosenberg SA (1997) Human tumor antigens recognized by T-cells. *Immunol Res* 16(4):313–339.
4. Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen YT (2002) Cancer/testis antigens: An expanding family of targets for cancer immunotherapy. *Immunol Rev* 188:22–32.
5. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P (2006) Human T cell responses against melanoma. *Annu Rev Immunol* 24:175–208.
6. Curiel TJ, et al. (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10(9):942–949.
7. Sato E, et al. (2005) Intraepithelial CD8$^+$ tumor-infiltrating lymphocytes and a high CD8$^+$/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA* 102(51):18538–18543.
8. Yamaguchi T, et al. (2007) Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity* 27(1):145–159.
9. Mitsui J, et al. (2010) Two distinct mechanisms of augmented antitumor activity by modulation of immunostimulatory/inhibitory signals. *Clin Cancer Res* 16(10):2781–2791.
10. Nishikawa H, Sakaguchi S (2010) Regulatory T cells in tumor immunity. *Int J Cancer* 127(4):759–767.
11. Dougan M, Dranoff G (2009) Immune therapy for cancer. *Annu Rev Immunol* 27:83–117.
12. Dannull J, et al. (2005) Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* 115(12):3623–3633.
13. Rech AJ, et al. (2012) CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patients. *Sci Transl Med* 4(134):134ra162.
14. Attia P, Maker AV, Haworth LR, Rogers-Freezer L, Rosenberg SA (2005) Inability of a fusion protein of IL-2 and diphtheria toxin (Denileukin Diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma. *J Immunother* 28(6):582–592.
15. Litzinger MT, et al. (2007) IL-2 immunotoxin denileukin diftitox reduces regulatory T cells and enhances vaccine-mediated T-cell immunity. *Blood* 110(9):3192–3201.
16. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155(3):1151–1164.
17. Kim JM, Rasmussen JP, Rudensky AY (2007) Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* 8(2):191–197.
18. Miyara M, et al. (2009) Functional delineation and differentiation dynamics of human CD4$^+$ T cells expressing the FoxP3 transcription factor. *Immunity* 30(6):899–911.
19. Campbell DJ, Koch MA (2011) Phenotypical and functional specialization of FOXP3$^+$ regulatory T cells. *Nat Rev Immunol* 11(2):119–130.
20. Ishida T, Ueda R (2006) CCR4 as a novel molecular target for immunotherapy of cancer. *Cancer Sci* 97(11):1139–1146.
21. Bonertz A, et al. (2009) Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma. *J Clin Invest* 119(11):3311–3321.
22. Gnjatic S, et al. (2006) NY-ESO-1: Review of an immunogenic tumor antigen. *Adv Cancer Res* 95:1–30.
23. Nishikawa H, Jäger E, Ritter G, Old LJ, Gnjatic S (2005) CD4$^+$ CD25$^+$ regulatory T cells control the induction of antigen-specific CD4$^+$ helper T cell responses in cancer patients. *Blood* 106(3):1008–1011.
24. Nishikawa H, et al. (2006) Influence of CD4$^+$CD25$^+$ regulatory T cells on low/high-avidity CD4$^+$ T cells following peptide vaccination. *J Immunol* 176(10):6340–6346.
25. Yoshie O, et al. (2002) Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. *Blood* 99(5):1505–1511.
26. Ishida T, et al. (2003) Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: Its close association with skin involvement and unfavorable outcome. *Clin Cancer Res* 9(10 Pt 1):3625–3634.
27. Matsubara Y, Hori T, Morita R, Sakaguchi S, Uchiyama T (2005) Phenotypic and functional relationship between adult T-cell leukemia cells and regulatory T cells. *Leukemia* 19(3):482–483.
28. Matsuoka M, Jeang KT (2007) Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer* 7(4):270–280.
29. Ishida T, et al. (2012) Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: A multicenter phase II study. *J Clin Oncol* 30(8):837–842.
30. Zou W (2006) Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6(4):295–307.
31. Imai T, et al. (1999) Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 11(1):81–88.
32. Lim HW, Lee J, Hillsamer P, Kim CH (2008) Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3$^+$ regulatory T cells. *J Immunol* 180(1):122–129.
33. Kondo T, Takiguchi M (2009) Human memory CCR4$^+$CD8$^+$ T cell subset has the ability to produce multiple cytokines. *Int Immunol* 21(5):523–532.
34. Nishikawa H, et al. (2005) Definition of target antigens for naturally occurring CD4$^+$CD25$^+$ regulatory T cells. *J Exp Med* 201(5):681–686.
35. Nishikawa H, et al. (2012) Cancer/testis antigens are novel targets of immunotherapy for adult T-cell leukemia/lymphoma. *Blood* 119(13):3097–3104.
36. Chappert P, Schwartz RH (2010) Induction of T cell anergy: Integration of environmental cues and infectious tolerance. *Curr Opin Immunol* 22(5):552–559.
37. Shimizu J, Yamazaki S, Sakaguchi S (1999) Induction of tumor immunity by removing CD25$^+$CD4$^+$ T cells: A common basis between tumor immunity and autoimmunity. *J Immunol* 163(10):5211–5218.
38. Ko K, et al. (2005) Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells. *J Exp Med* 202(7):885–891.
39. Sharma P, Wagner K, Wolchok JD, Allison JP (2011) Novel cancer immunotherapy agents with survival benefit: Recent successes and next steps. *Nat Rev Cancer* 11(11):805–812.
40. Nishikawa H, et al. (2006) In vivo antigen delivery by a *Salmonella typhimurium* type III secretion system for therapeutic cancer vaccines. *J Clin Invest* 116(7):1946–1954.

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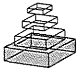

Forefront studies on HTLV-1 oncogenesis

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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 $Sca1^{+}c\text{-}kit^{hi}$ HSC-enriched fraction; they displayed high B and T lymphopoietic potential, but limited myeloerythroid potential and self-renewal ability. In contrast, $Rag1^{-}Sca1^{+}c\text{-}kit^{hi}$ 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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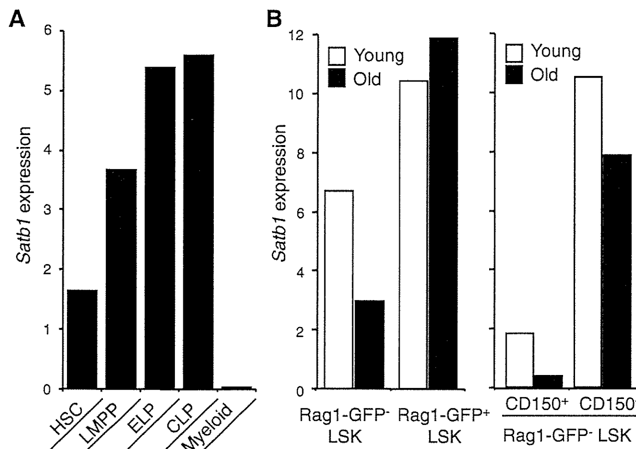

Figure 1. *Satb1* Expression Levels Change with Differentiation and Aging of HSCs

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

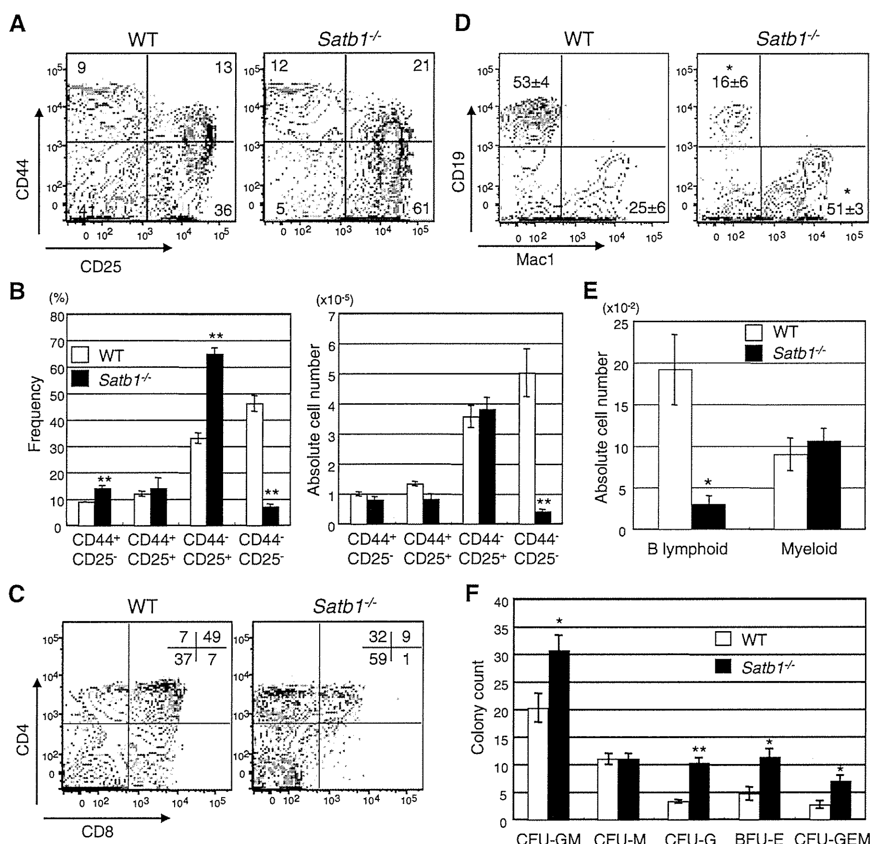

Figure 2. Satb1 Deficiency Alters Lymphoid and Myeloid Activities of Hematopoietic Stem/Progenitor Cells in Culture

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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Satb1 Promotes Lymphoid Priming in HSC

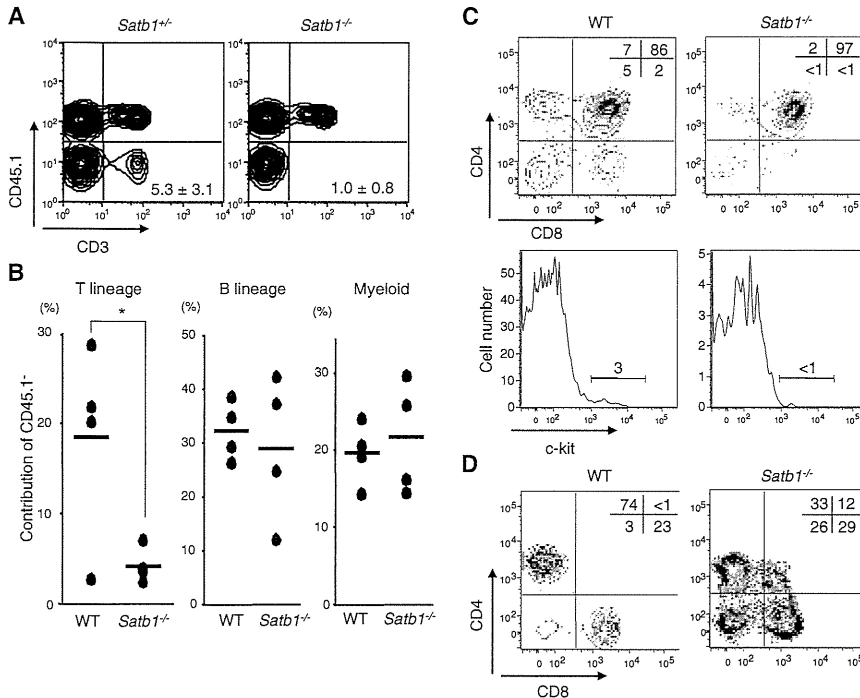


Figure 3. Defective T Lymphopoiesis from Transplanted *Satb1*$^{-/-}$ HSC

(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

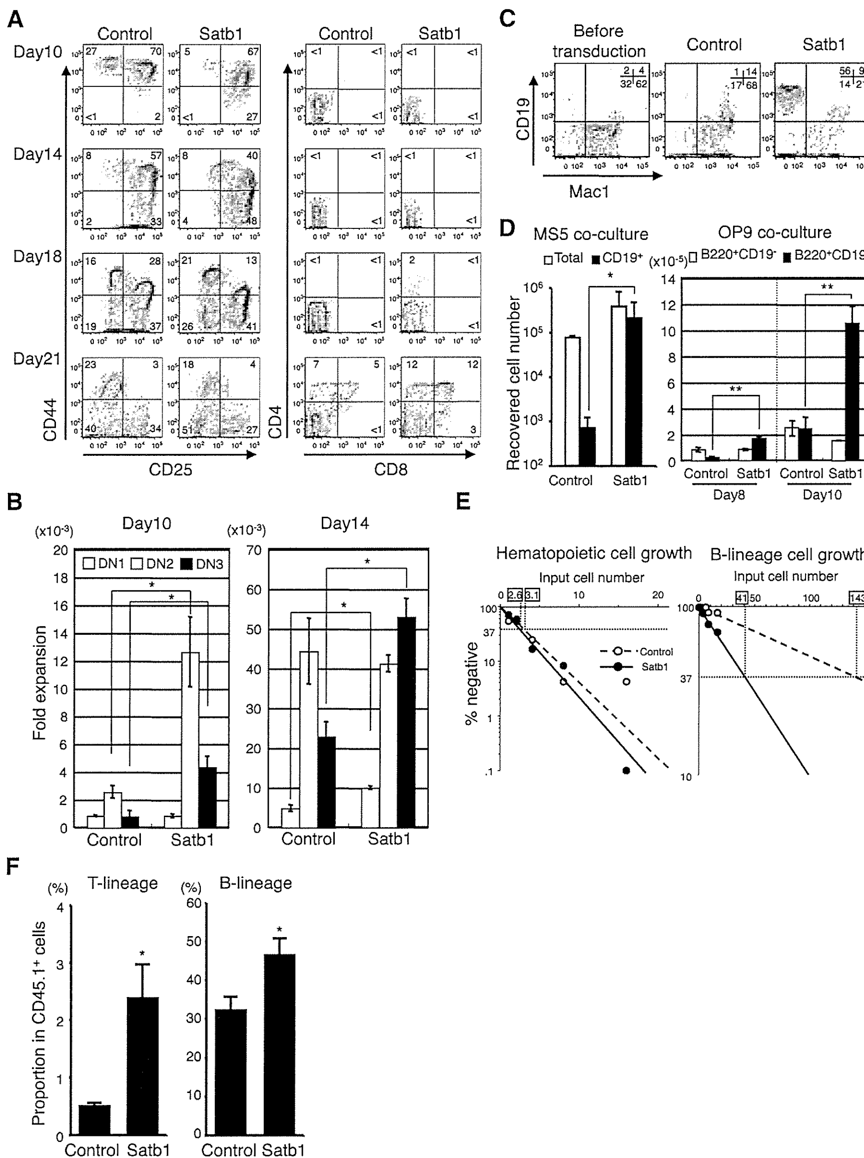


Figure 4. Satb1 Overexpression Promotes Lymphopoiesis

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

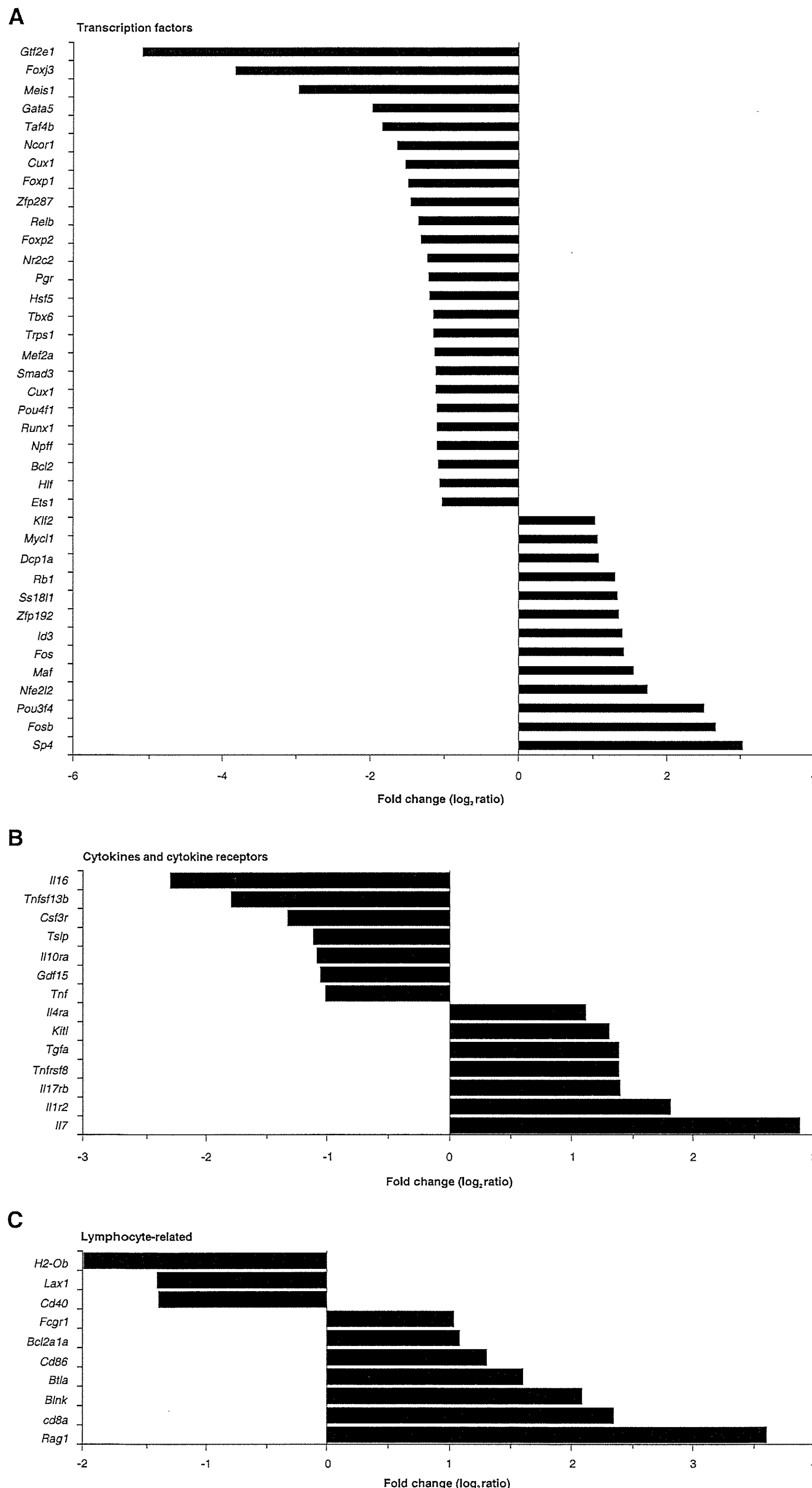


Figure 5. Genes Affected by Satb1 Expression

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.

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-$\gamma\delta$ 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;

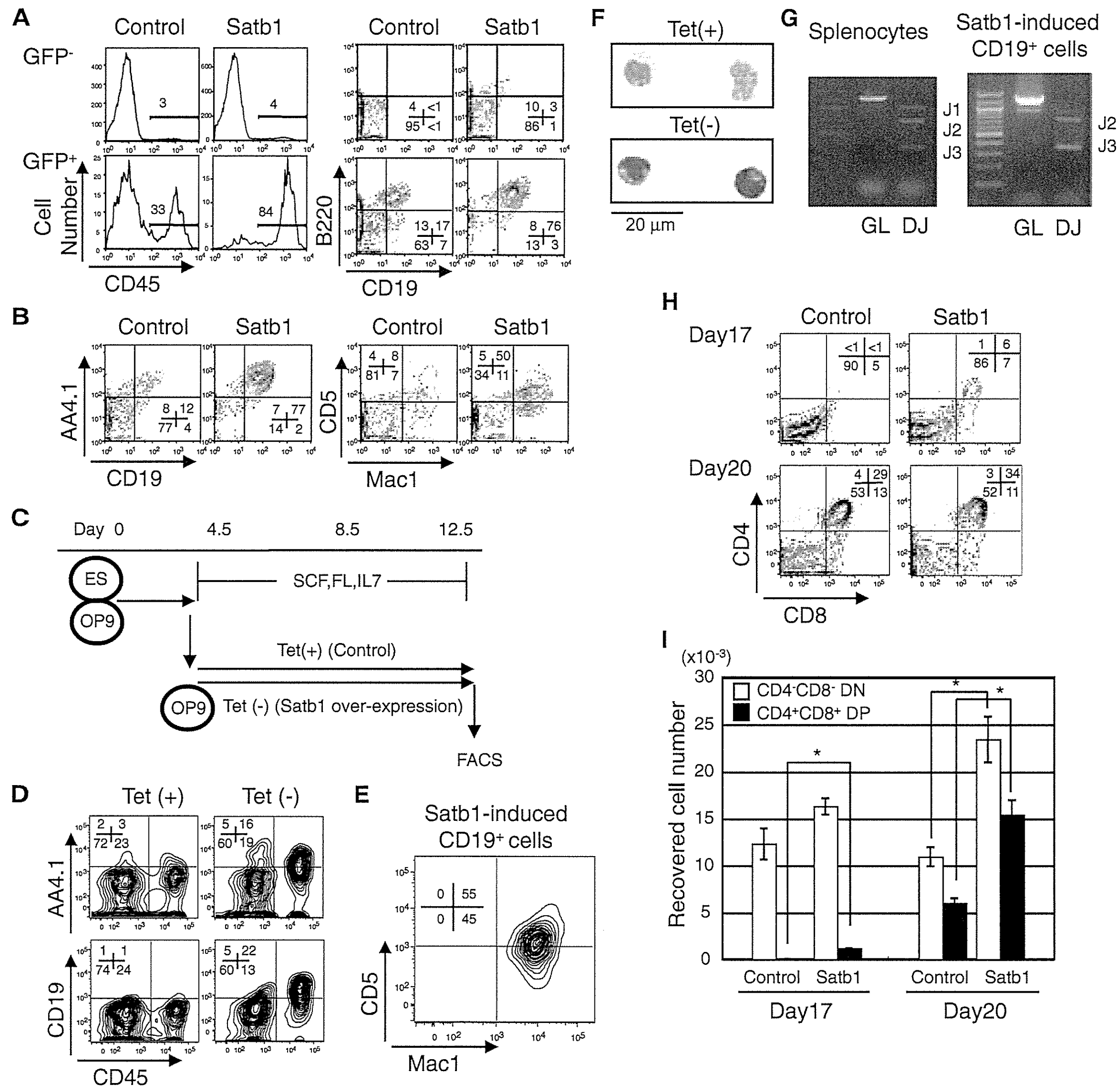


Figure 6. Satb1 Promotes Lymphoid Differentiation from ES-Derived Cells

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

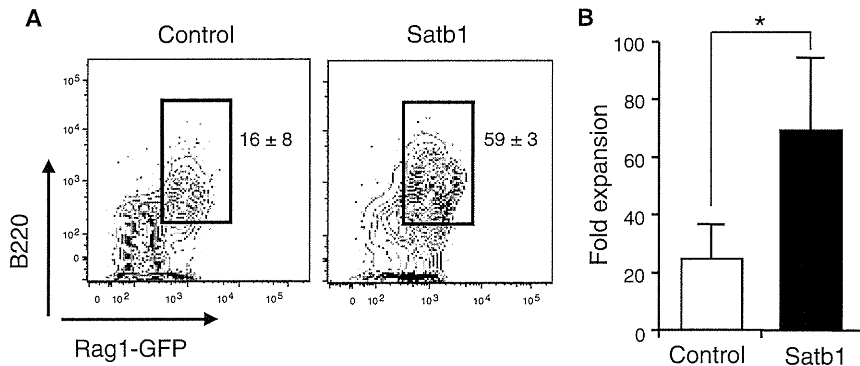


Figure 7. Satb1 Overexpression Restores Lymphopoietic Activity of Aged HSC
(A) Rag1/GFP$^-$ LSK cells were sorted from 2-year-old mice and retrovirally transduced with control or Satb1-DsRed vectors. Successfully transfected cells were cultured on OP9 cells. Cultures were established in triplicate. Numbers 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

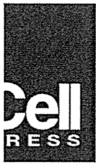
Animal studies were performed with the approval of the Institutional Review Board of Osaka University. Rag1-GFP knockin mice were previously described (Kuwata et al., 1999). *Satb1*$^{-/-}$ mice were also previously established (Alvarez et al., 2000). WT C57BL/6 mice and the congenic C57BL/6SJL strain (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^{hi} 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



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)AAGGGATCTACTA CTGTG; 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.

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-Lla, 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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GUIDELINE

Guidelines for the management of cutaneous lymphomas (2011): A consensus statement by the Japanese Skin Cancer Society – Lymphoma Study Group

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ABSTRACT

In 2010, the first Japanese edition of guidelines for the management of cutaneous lymphoma was published jointly by the Japanese Dermatological Association (JDA) and the Japanese Skin Cancer Society (JSCS) – Lymphoma Study Group. Because the guidelines were revised in 2011 based on the most recent data, we summarized the revised guidelines in English for two reasons: (i) to inform overseas clinicians about our way of managing common types of cutaneous lymphomas such as mycosis fungoides/Sézary syndrome; and (ii) to introduce Japanese guidelines for lymphomas peculiar to Asia, such as adult T-cell leukemia/lymphoma and extranodal natural killer/T-cell lymphoma, nasal type. References that provide scientific evidence for these guidelines have been selected by the JSCS – Lymphoma Study Group. These guidelines, together with the degrees of recommendation, have been made in the context of limited medical treatment resources, and standard medical practice within the framework of the Japanese National Health Insurance system.

Key words: adult T-cell leukemia/lymphoma, cutaneous lymphoma, guideline, mycosis fungoides, Sézary syndrome.

INTRODUCTION

A number of guidelines on the management of cutaneous lymphoma have already been published in Europe and North America. However, the prevalence and clinical types of cutaneous lymphoma vary among different ethnic groups, and medical systems vary from country to country. As a result, the unmodified European/US guidelines may not be well-suited for use in Japan. We wanted to provide a "best treatment" consensus on clinical practice guidelines for cutaneous lymphoma, based on the actual situation in Japan.

In these guidelines, the diagnosis of cutaneous lymphoma is based on classifications from the World Health Organization (WHO) and European Organization for Research and Treatment of Cancer, Cutaneous Lymphomas Task Force (EORTC),[1] and on the 4th edition of the WHO classification published in 2008.[2] The staging and classification of mycosis fungoides (MF)/Sézary syndrome (SS) are based on the tumor

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–node–metastasis (TNM) staging from the International Society for Cutaneous Lymphomas (ISCL) group.[3] For cutaneous lymphomas other than MF/SS, we decided to use the TNM staging system proposed by the ISCL[4] rather than the conventional Ann Arbor classification system.

The British group,[5] EORTC[6] and European Society for Medical Oncology (ESMO)[7] each issued treatment guidelines for MF/SS. In 2009, using published work and overseas guidelines for references, we published the first edition of guidelines based on the actual situation of cutaneous lymphoma in Japan.[8] Because the guidelines were revised in 2011 based on the most recent data, we summarized the revised guidelines in English for two reasons: (i) to inform overseas clinicians about our way of managing common types of cutaneous lymphomas such as MF/SS; and (ii) to introduce Japanese guidelines for lymphomas peculiar to Asia, such as adult T-cell leukemia/lymphoma and extranodal natural killer (NK)/T-cell lymphoma (ENKL), nasal type. References that provide scientific evidence for these guidelines have been selected by the Japanese Skin Cancer Society (JSCS) – Lymphoma Study Group. These guidelines, together with the degrees of recommendation, have been made in the context of limited medical treatment resources, and standard medical practice within the framework of the Japanese National Health Insurance system. The evidence level and degree of recommendation used for the current version are shown in Table 1.

BASIS FOR THE CURRENT GUIDELINES

The cutaneous lymphomas listed in the present guidelines are basically in accordance with the WHO–EORTC classification (2005),[1] but it is difficult to precisely define “primary cutaneous” lymphoma. Ordinarily, a condition is defined as “primary cutaneous” lymphoma if appropriate procedures show no extracutaneous lesions at the time of diagnosis. The present guidelines include lymphomas and hematopoietic malignancies with marked affinity for the skin (Fig. 1, Table 2). The diagnostic nomenclature follows the 4th edition of the WHO classification (2008).[2]

To describe the skin lesions of cutaneous lymphoma, typically MF/SS, uniform terminology is needed. Without consistent terminology, accurate disease staging is impossible, and inconsistencies may develop in prognostic analysis. The ISCL/EORTC group has defined terminology for MF/SS.[9] Those definitions are adopted in the present guidelines (Table S1), and representative clinicopathological findings of various types of cutaneous lymphoma are provided in supporting information (Figs S1–S7).

STAGING

Staging for MF/SS (ISCL/EORTC 2007, modified in 2011)

For the staging of MF/SS, we previously used the categories developed by Bunn *et al.*[10] and Sausville *et al.*[11] In 2007, a new staging system was proposed by the ISCL/EORTC group,[3] which was modified in 2011 (Tables S2 and S3).[12]

In the ISCL/EORTC staging system, peripheral blood findings are classified into three categories: B_0 (atypical lymphocytes accounting for $\leq 5\%$ of peripheral blood lymphocytes), B_1 (atypical lymphocytes accounting for >5% of peripheral blood lymphocytes, but <1000/μL), and B_2 (atypical lymphocyte

Table 1. Standards for the determination of evidence level and degree of recommendation

Classification of evidence level	
I	Systematic review and/or meta-analysis
	Staging/classification proposal and treatment recommendation or consensus paper from WHO, EORTC and ISCL
II	One or more randomized comparative studies
III	Non-randomized comparative studies
IV	Analytical epidemiology studies (cohort research and case–control studies)
	Case series studies ($\geq$5 cases)
V	Descriptive studies (case reports and case series studies [<5 cases])
VI	Opinions of expert committee and individual specialists*
Degree of recommendation classification†	
A	Strongly recommended for implementation (efficacy shown by at least 1 report providing level I or high-quality level II evidence)
B	Recommended for implementation (efficacy shown by $\geq$1 reports providing low-quality level II, high-quality level III, or very high-quality level IV evidence)
B-C1	Recommended for implementation, but less strongly supported than B
C1	Implementation can be considered, but evidence‡ is insufficient (low-quality III–IV, high-quality multiple V, or committee-approved VI evidence)
C2	No evidence‡; cannot be recommended (no evidence of effectiveness, or evidence available of ineffectiveness)
D	Recommended not to implement (high-quality evidence of ineffectiveness or harmfulness)

*Data from basic research and theories derived from such data are placed at this level. †Some of the “degree of recommendation” statements in these guidelines are not in complete agreement with the above table. ‡“Evidence” refers to knowledge from clinical trials and epidemiological research. This is because these “degree of recommendation” grades were based on a consensus among the committee members, taking feasibility into account. This consensus was reached after due consideration of the shortage of evidence internationally on the treatment of skin cancer and the fact that the evidence from overseas is not directly applicable in Japan.

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