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Anti-CCR4 mAb selectively depletes effector-type FoxP3$^+$CD4$^+$ regulatory T cells, evoking antitumor immune responses in humans

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CD4$^+$ Treg cells expressing the transcription factor FOXP3 (forkhead box P3) are abundant in tumor tissues and appear to hinder the induction of effective antitumor immunity. A substantial number of T cells, including Treg cells, in tumor tissues and peripheral blood express C-C chemokine receptor 4 (CCR4). Here we show that CCR4 was specifically expressed by a subset of terminally differentiated and most suppressive CD45RA$^-$FOXP3^{hi}CD4$^+$ Treg cells [designated effector Treg (eTreg) cells], but not by CD45RA$^+$FOXP3^{lo}CD4$^+$ naive Treg cells, in peripheral blood of healthy individuals and cancer patients. In melanoma tissues, CCR4$^+$ eTreg cells were predominant among tumor-infiltrating FOXP3$^+$ T cells and much higher in frequency compared with those in peripheral blood. With peripheral blood lymphocytes from healthy individuals and melanoma patients, ex vivo depletion of CCR4$^+$ T cells and subsequent in vitro stimulation of the depleted cell population with the cancer/testis antigen NY-ESO-1 efficiently induced NY-ESO-1–specific CD4$^+$ T cells. Nondepletion failed in the induction. The magnitude of the responses was comparable with total removal of FOXP3$^+$ Treg cells by CD25$^+$ T-cell depletion. CCR4$^+$ T-cell depletion also augmented in vitro induction of NY-ESO-1–specific CD8$^+$ T cells in melanoma patients. Furthermore, in vivo administration of anti-CCR4 mAb markedly reduced the eTreg-cell fraction and augmented NY-ESO-1–specific CD8$^+$ T-cell responses in an adult T-cell leukemia-lymphoma patient whose leukemic cells expressed NY-ESO-1. Collectively, these findings indicate that anti-CCR4 mAb treatment is instrumental for evoking and augmenting antitumor immunity in cancer patients by selectively depleting eTreg cells.

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Naturally occurring CD25$^+$CD4$^+$ regulatory T (Treg) cells expressing the transcription factor forkhead box P3 (FOXP3) are indispensable for the maintenance of immunological self-tolerance and homeostasis (1, 2). Given that most tumor-associated antigens are antigenically normal self-constituents (3–5), it is likely that natural FOXP3$^+$ Treg cells engaged in self-tolerance concurrently hinder immune surveillance against cancer in healthy individuals and also hamper the development of effective antitumor immunity in tumor-bearing patients. Indeed FOXP3$^+$ CD25$^+$CD4$^+$ Treg cells are abundant in tumor tissues (6–10), and their depletion augments spontaneous and vaccine-induced antitumor immune responses in animal models (10, 11). In humans, increased numbers of FOXP3$^+$CD25$^+$CD4$^+$ Treg cells and, in particular, decreased ratios of CD8$^+$ T cells to FOXP3$^+$ CD25$^+$CD4$^+$ Treg cells among tumor-infiltrating lymphocytes (TIL) are well correlated with poor prognosis in various types of cancers (6, 7, 10). Some clinical studies have shown the potential of depleting CD25-expressing lymphocytes to augment antitumor immune responses (12, 13); yet other similar studies failed to support the effects (10, 14, 15). Because activated effector T cells also express CD25, and their production of IL-2 is required for the expansion of CD8$^+$ cytotoxic lymphocytes, CD25-based cell depletion may reduce activated effector T cells as well, cancelling the effect of Treg-cell depletion to augment antitumor immunity (10). In addition, it has been demonstrated in animal models that depletion of Treg cells as a whole can trigger autoimmunity (1, 16, 17). Therefore, a current key issue is to determine how Treg cells can be controlled to evoke and enhance antitumor immunity without affecting effector T cells or eliciting deleterious autoimmunity.

Human FOXP3$^+$CD4$^+$ T cells are heterogenous in phenotype and function (2). These cells can be dissected into three subpopulations by the expression levels of FOXP3 and the cell-surface molecules CD45RA and CD25: (*i*) FOXP3^{hi}CD45RA$^-$CD25^{hi} cells, designated effector Treg (eTreg) cells, which are terminally differentiating and highly suppressive; (*ii*) FOXP3^{lo}CD45RA$^+$ CD25^{lo} cells, designated naive Treg cells, which differentiate into eTreg cells upon antigenic stimulation; and (*iii*) FOXP3^{lo} CD45RA$^-$CD25^{lo} non-Treg cells, which do not possess suppressive activity but secrete proinflammatory cytokines (18). In principle, these distinct properties of FOXP3$^+$ T-cell subpopulations can be exploited to augment antitumor immunity without inducing autoimmunity, for example, by depleting a particular Treg-cell subpopulation rather than whole Foxp3$^+$-cell population. One of

Significance

Regulatory T (Treg) cells expressing the transcription factor FOXP3 play a critical role in suppressing antitumor immune responses. Here we found that, compared with peripheral blood T cells, tumor-infiltrating T cells contained a higher frequency of effector Tregs, which are defined as FOXP3^{hi} and CD45RA$^-$, terminally differentiated, and most suppressive. Effector Treg cells, but not FOXP3^{lo} and CD45RA$^+$ naïve Treg cells, predominantly expressed C-C chemokine receptor 4 (CCR4) in both cancer tissues and peripheral blood. In vivo or in vitro anti-CCR4 mAb treatment selectively depleted effector Treg cells and efficiently induced tumor-antigen-specific CD4$^+$ and CD8$^+$ T cells. Thus, cell-depleting anti-CCR4 mAb therapy is instrumental for evoking and enhancing tumor immunity in humans via selectively removing effector-type FOXP3$^+$ Treg cells.

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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

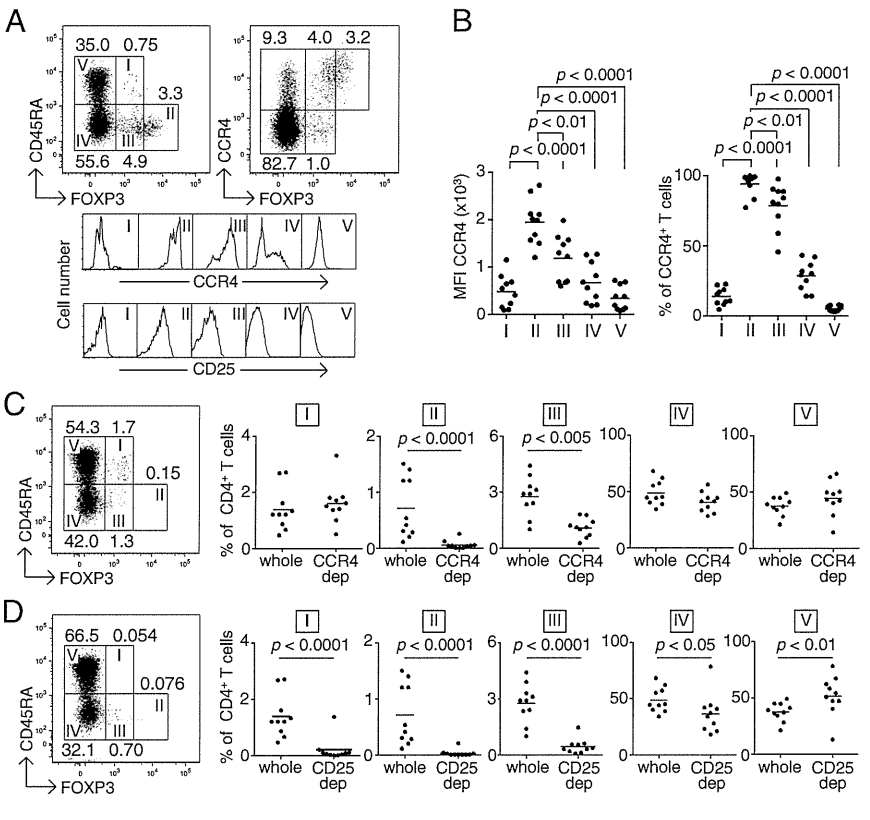

Fig. 1. Reduction of eTreg cells by in vitro depletion of CCR4-expressing T cells. (*A*) CCR4 and CD25 expression by subpopulations of FOXP3$^+$ Treg cells in PBMCs from healthy donors. CCR4 and CD25 expression levels were evaluated for each fraction. Representative data from 10 healthy donors are shown. (*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*.

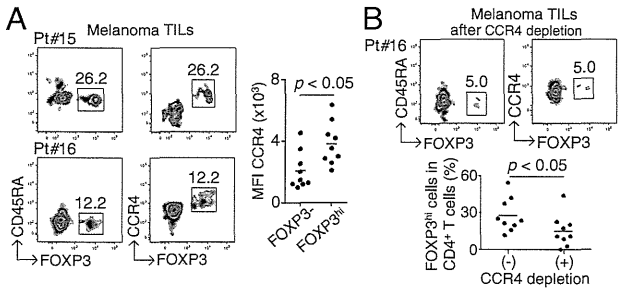

Fig. 2. Predominant infiltration of CCR4$^+$ eTreg cells into melanoma tissues. (*A*) CCR4 expression by melanoma-infiltrating T cells. CD4$^+$ T cells from melanoma sites were fractionated into subpopulations based on the expression of CCR4, CD45RA, and FOXP3; CCR4 expression by each fraction was analyzed. 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).

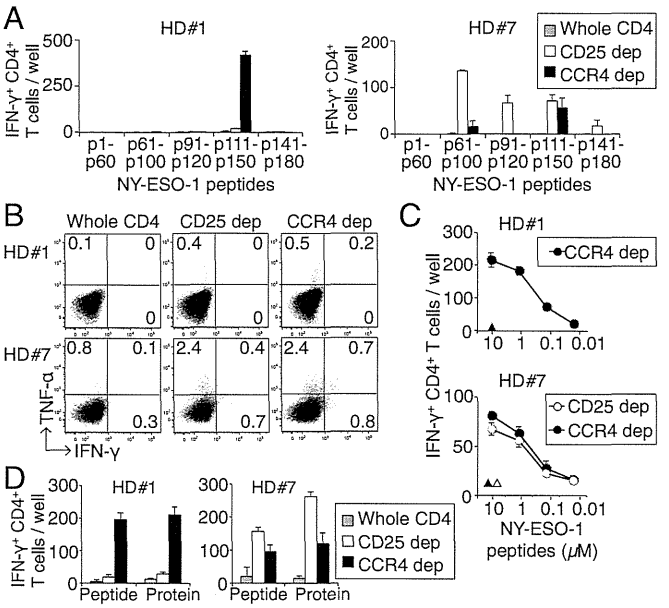

Fig. 3. Induction of cancer/testes antigen-specific CD4$^+$ T cells by depletion of CCR4- or CD25-expressing T cells in healthy donors. (*A*) CD4$^+$ T-cell responses to NY-ESO-1 peptides after depletion of CCR4$^+$ or CD25$^+$ T cells. CD4$^+$ T cells prepared from PBMCs of healthy donors were presensitized 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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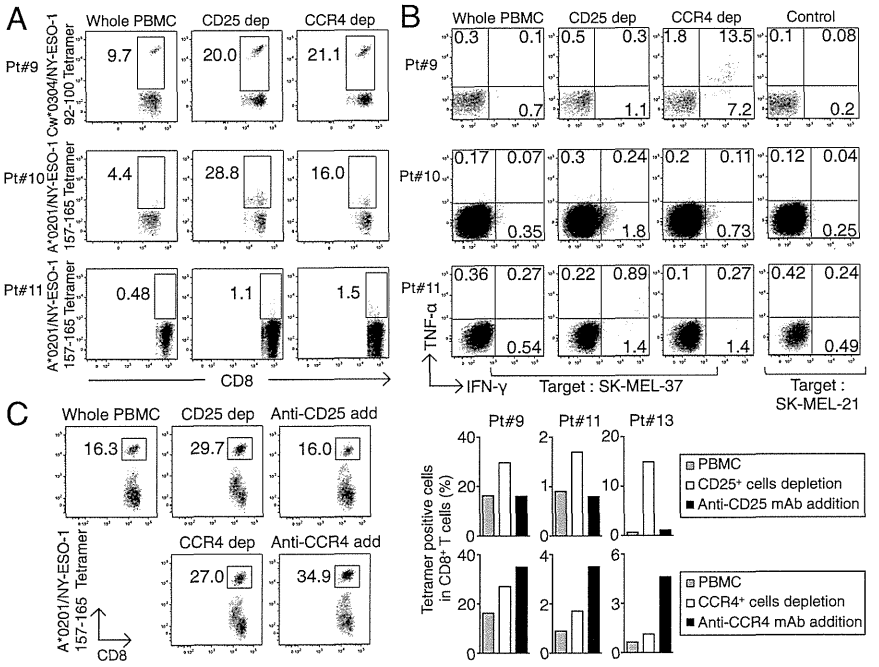

Fig. 4. Augmentation of NY-ESO-1–specific $CD8^+$ T-cell induction in melanoma patients by in vitro $CCR4^+$ T-cell depletion. (*A*) Induction of NY-ESO-1–specific $CD8^+$ T cells. Unfractionated PBMCs, or PBMCs depleted of $CD25^+$ or $CCR4^+$ cells, were prepared from melanoma patients (n = 6), and 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

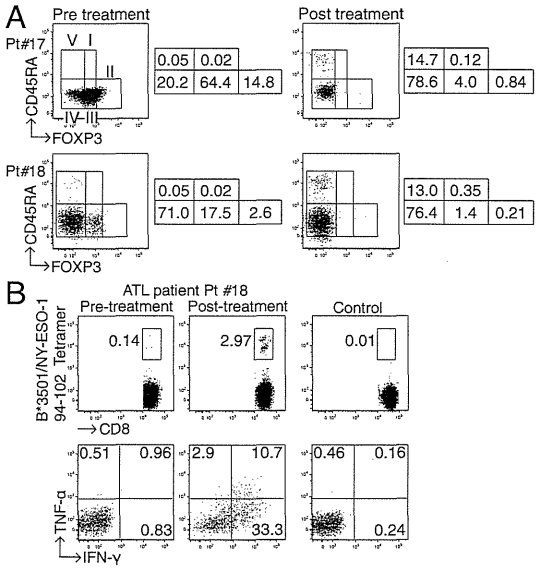

Fig. 5. Reduction of $CD4^+FOXP3^{hi}CD45RA^-$ T cells and augmentation of NY-ESO-1–specific $CD8^+$ T-cell responses in ATL patients after anti-CCR4 mAb (Mogamulizumab) therapy. (*A*) $FOXP3^+$ Treg-cell subpopulations in PBMCs from two ATL patients (Pt. #17: acute type, HLA-A*2402/-, B*3901/5401, C*0102/0702 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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CASE REPORT

Use of anti-tumor necrosis factor biologics in the treatment of rheumatoid arthritis does not change human T-lymphotropic virus type 1 markers: a case series

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Abstract

Anti-tumor necrosis factor (anti-TNF) biologics are effective in the treatment of rheumatoid arthritis (RA); however, it is still not clear whether this treatment promotes the development of malignancies such as lymphoma. Human T-lymphotropic virus type 1 (HTLV-1), which is a causative agent of adult T-cell lymphoma (ATL), is prevalent in Japan. Many HTLV-1-positive patients with RA are assumed to exist; however, there have thus far been no reports on the effect of anti-TNF biologics on HTLV-1-positive patients. We analyzed the response to treatment with anti-TNF biologics and change of HTLV-1 markers in two cases of RA. The two cases showed no response based on the European League Against of Rheumatism response criteria 60–96 weeks after administration of anti-TNF biologics (infliximab and etanercept). No signs of ATL were observed and HTLV-1 markers, such as proviral load and clonality of HTLV-1-infected cells, showed no significant change in either of two cases. Therefore, treatment with anti-TNF biologics did not induce activation of HTLV-1, although the effect on RA was not as effective as in HTLV-1-negative patients in this limited study. Further long-term study with a greater number of patients is necessary to clarify the safety and efficacy of anti-TNF biologics in HTLV-1-positive patients with RA.

Keywords

Anti-TNF biologics, Human T-lymphotropic virus type 1, Lymphoma, Rheumatoid arthritis, Viral infection

History

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Introduction

The effectiveness of biologics, which target inflammatory cytokines, has revolutionized the treatment of rheumatoid arthritis (RA); however, there are many concerns regarding potential adverse effects to be resolved. It is still not clear whether this treatment promotes the development of malignancies such as lymphoma, and epidemiological studies on this matter are ongoing [1]. RA has been considered a risk factor for the development of lymphoma. The most common lymphoma associated with RA is diffuse large B-cell type non-Hodgkin lymphoma [1, 2].

In Japan, human T-lymphotropic virus type 1 (HTLV-1), which is a causative agent of adult T-cell lymphoma (ATL), is prevalent, with the number of HTLV-1 carriers estimated to be 1.08 million individuals [3]. Therefore, the number of patients in Japan with RA who are also infected with HTLV-1 can be estimated at approximately 10,000. Moreover, a cohort study in Nagasaki prefecture, one of the highest areas of HTLV-1-prevalence in Japan, showed the rate of HTLV-1 infection in patients with RA to be higher than that of healthy blood donors [4]. HTLV-1 has been reported to be associated not only with ATL, but also with chronic inflammatory diseases, such as HTLV-1-associated myelopathy (HAM), uveitis, arthropathy, Sjogren syndrome (SS), and myositis [5, 6]. These chronic inflammatory diseases are often treated with corticosteroids and immunosuppressive agents. An important question is whether these treatments adversely affect HTLV-1 infection. In fact, progression to ATL in HTLV-1 carriers treated with the immunosuppressive agent tacrolimus after liver transplant has been reported [7]. Therefore, it is important to clarify whether treatment with biologics increases the risk of ATL in HTLV-1-positive patients. Higher proviral load (PVL), advanced age, family history of ATL, and first opportunity for HTLV-1 testing during treatment for other diseases have been reported as risk factors in the progression of ATL [8]. The clonal evolution of HTLV-1-infected cells has also been reported to occur before the onset of ATL [9]. In this study, we investigated the change of HTLV-1 markers (HTLV-1 proviral load and clonality of HTLV-1-infected cells) in two patients with RA before and after treatment with anti-TNF biologics. In addition, we evaluated RA disease activity to clarify the response to anti-TNF biologics for 60–96 weeks.

Patients and methods

Patients

Case 1

A 52-year-old woman with polyarthralgia, rheumatoid factor and anti-cyclic citrullinated peptide antibody (ACPA) was diagnosed with RA based on the 1987 American College of Rheumatology (ACR) classification criteria for RA 3 years prior to the present study [10]. She had pneumonitis and was treated with bucillamine

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and salazosulfapyridine, but not with methotrexate (MTX). As these medicines proved inefficacious, we recommended biologics. She had dry mouth and tested positive for anti-Ro/SSA antibody. She was diagnosed with SS based on the American-European consensus criteria for SS [11]. She tested positive for HTLV-1 antibody. Her disease activity score in 28 joints (DAS28) based on the European League Against Rheumatism (EULAR) response criteria was 4.05. After obtaining informed consent, she was started on treatment with etanercept (ETN). Her RA seemed to respond at 12 weeks after treatment with ETN. However, after this time point, DAS28, the levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) gradually increased despite treatment with ETN (Figure 1-A). The number of swollen and tender joints and her modified health assessment questionnaire (mHAQ) did not improve significantly (Figure 1-A). As a result, she was judged to have had no response by EULAR response criteria [12].

Case 2

A 32-year-old woman with polyarthralgia, rheumatoid factor, and ACPA was diagnosed with RA based on the 1987 ACR classification criteria for RA [10] when she was pregnant 4 years prior to the present study. She tested positive for HTLV-1 antibody. After giving birth, treatment with MTX was started; however, she complained of worsening arthralgia. As MTX dosage could not be increased because of the adverse effect, she accepted our recommendation to use biologics. She also had dry eyes and dry mouth. She tested positive for anti-Ro/SSA antibody and was diagnosed with SS based on the American-European consensus criteria for SS [11]. Her DAS28 was 5.17. After obtaining informed consent, she was started on treatment with infliximab (IFX) in addition to MTX. Her DAS28 remained unchanged and high disease activity continued. For this reason, we changed IFX to ETN and the dosage of MTX was escalated; however, DAS28 and mHAQ at 60 weeks after the beginning of biologics remained high. Therefore, she was judged to be non-responsive to anti-TNF agents (Figure 2-A) [12]. The levels of serum CRP gradually decreased; however, the levels of CRP and ESR remained high, in this case. DAS28 after the IFX and ETN treatments was judged not to have changed in this case based on the absence of significant improvement in the number of swollen joints, the number of tender joints, the levels of inflammatory markers and her mHAQ.

Change of HTLV-1 PVLs and clonality of infected cells in peripheral blood

HTLV-1 PVLs and clonality of HTLV-1-infected cells in peripheral blood in these two cases were analyzed. Written informed consent was obtained, and the study protocol was approved by the institutional review board of University of Miyazaki.

The methods for measuring HTLV-1 PVL and clonality of HTLV-1-infected cells are described in detail elsewhere [13, 14]. In brief, peripheral blood mononuclear cells (PBMCs) were obtained from both cases and genomic DNA was isolated. Real-time polymerase chain reaction (PCR) using primers and probe for HTLV-1 *pX* regions and human RNase P gene were performed to evaluate PVL (HTLV-1 copies per 100 PBMCs).

Inverse-long PCR (IL-PCR) was performed with slight modification to determine the clonality of HTLV-1-infected cells in each case [14]. In brief, the genomic DNA was digested with *EcoR* I, and then self-ligated by T4 ligase following digestion with *Mlu* I. The resultant DNA was amplified using the LA Taq Hot start version (Takara Bio, Shiga, Japan) in triplicate. PCR products were analyzed using 0.6% agarose gel, and each band represented the individual HTLV-1-infected clone.

The PVL of Case 1 before ETN therapy was low at 0.2 copies per 100 PBMCs and continued at the same level until 96 weeks after the beginning of treatment (Figure 1-B). Analysis using IL-PCR in this case showed many bands with different sizes, suggesting oligoclonal expansion of HTLV-1-infected cells before ETN therapy, which did not change thereafter (Figure 1-C). The PVL of Case 2 before anti-TNF biologics was also low at 0.3 copies per 100 PBMCs. It increased slightly after the beginning of IFX; however, it had returned to nearly the same level by 60 weeks after the beginning of treatment (Figure 2-B). Analysis using IL-PCR also showed oligoclonal expansion of HTLV-1-infected cells before anti-TNF therapy, which remained unchanged to the end of observation (Figure 2-C). In addition, there were no signs, symptoms, or laboratory abnormalities, suggesting ATL or HAM during treatment with anti-TNF agents.

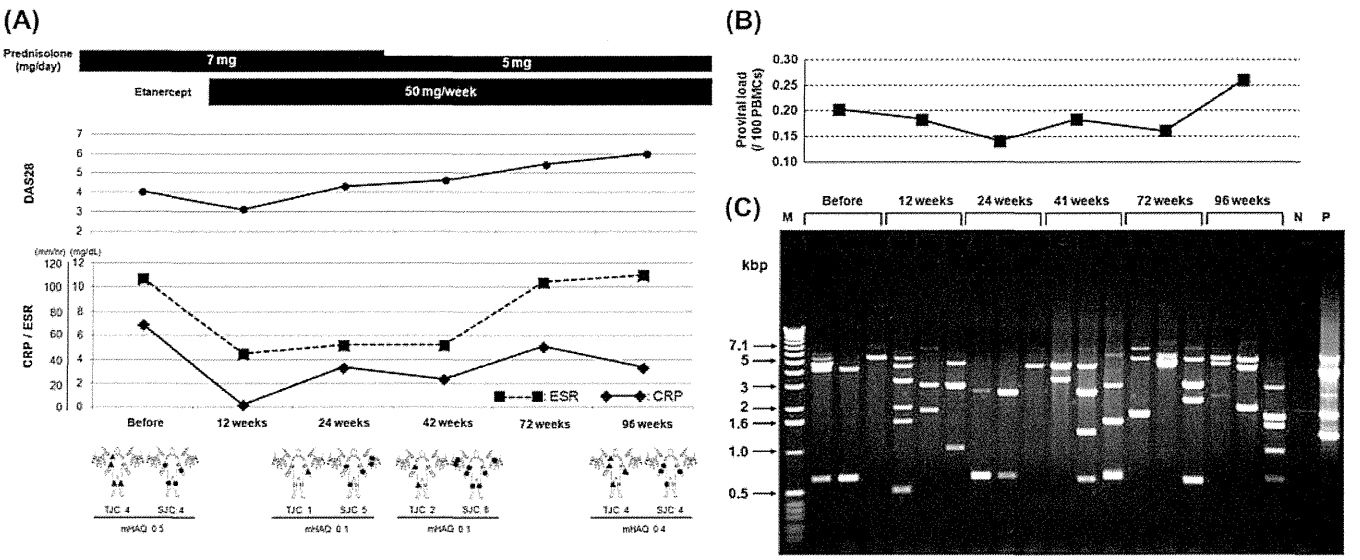

Figure 1. Case 1. Clinical course (A); time sequential analysis of HTLV-1 proviral loads (B) and detection of clonalities of HTLV-1-infected cells in Case 1 by IL-PCR (C). IL-PCR assays were performed in triplicate. ESR: erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; TJC, tender joint counts (▲); SJC, swollen joint counts (●); and mHAQ, modified health assessment questionnaire. M, molecular weight marker; N, PBMCs from HTLV-1-negative subject as negative control; P, HTLV-1-infected cell line; and HUT102, as positive control.

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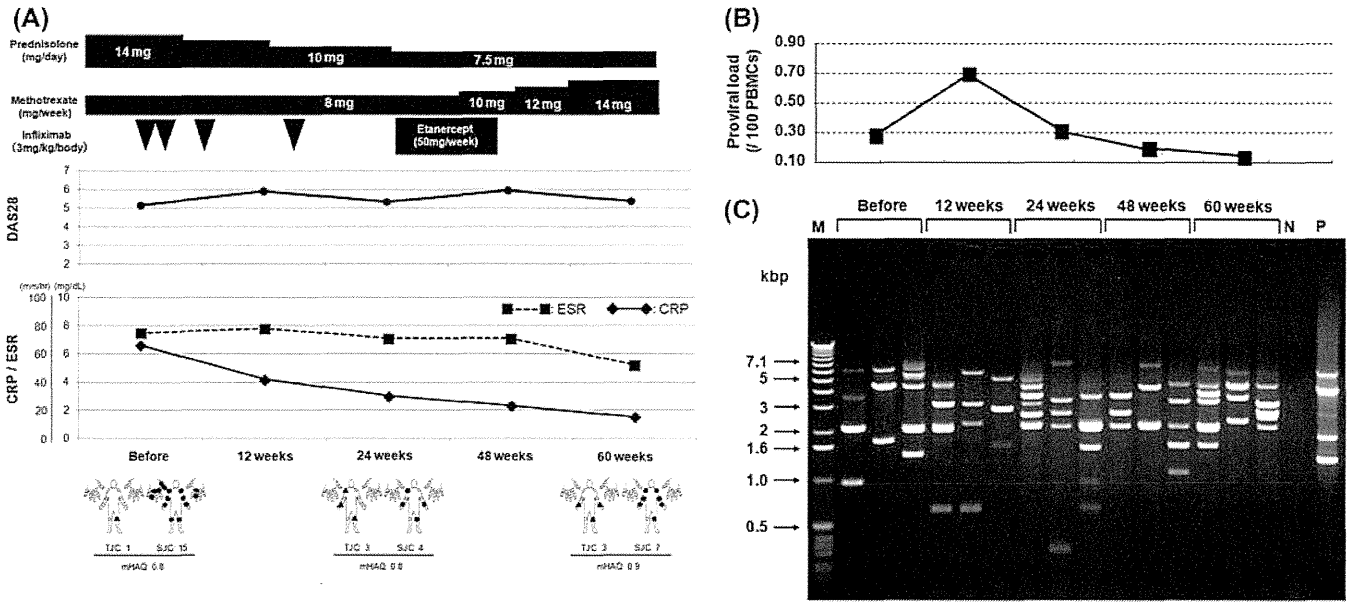

Figure 2. Case 2. Clinical course (A); time sequential analysis of HTLV-1 proviral loads (B) and detection of clonalities of HTLV-1-infected cells in Case 2 by IL-PCR (C). IL-PCR assays were performed in triplicate. The abbreviations used in this figure were same as those in Figure 1.

Discussion

We experienced two cases of RA associated with SS in HTLV-1 carriers. A high incidence of arthritis and SS in HTLV-1carriers has been reported in Japan [4, 15]. Sato et al. reported oligo-arthritis in the shoulders, wrists, and knees in HTLV-1 carriers in Japan [16]. The reported patients tended to have high inflammation and extra-joint symptoms such as SS. Both cases in the present study had features similar to the patients reported by Sato et al. At the same time, the present cases were ACPA positive and fulfilled both the definition of ACR (1987) and ACR/EULAR criteria for RA in 2010 [17].

As conventional disease-modifying anti-rheumatic drugs proved inefficacious, these two HTLV-positive cases were treated with anti-TNF therapy. According to the RECONFIRM study, 84.5% of Japanese patients with RA showed good or moderate response to treatment with IFX by EULAR response criteria [18]. On the basis of post-marketing surveillance, approximately 80% of Japanese patients with RA showed good or moderate response to treatment with ETN [19]. However, the two HTLV-1-positive cases of RA in the present study showed no response to anti-TNF agents.

It has been reported that patients with advanced RA and long disease histories showed low response rates to anti-TNF treatments; however, the duration of RA in the present study was only 3–4 years, and neither case was advanced (Steinbrocker's Classification stage II, data not shown). Both of the cases in the present study had SS in addition to RA; however, association of SS was not always a factor in RA resistance to anti-TNF therapy [20, 21].

Thus far, there have been no reports on the effectiveness of anti-TNF agents or other biologics in HTLV-1 carriers with RA. In an animal model experiment, transgenic mice carrying the HTLV-I genome showed strong expression of mRNA of IL-1 and IL-6, but not TNF [22]. There is a possibility that cytokines such as IL-1 and IL-6, but not TNF, are more important to RA activity in HTLV-1-positive patients than in HTLV-1-negative patients. In fact, one of the two patients in the present study was treated with IL-6 inhibitor (tocilizumab), thereafter, and showed a better response, although the period of observation was not sufficient for a definite conclusion to be reached (data not shown).

There have been no studies on the risk of progression to ATL in HTLV-1 carriers receiving biologics for the treatment of RA. Patients with RA are considered to be at high risk of lymphoma, mainly B-cell type [2]. Thus far, anti-TNF therapy has not been reported to be associated with lymphoma [23, 24]. However, re-activation of Epstein–Barr virus has been reported to be associated with MTX-related lymphoproliferative diseases in RA [2]. In addition, progression to ATL in HTLV-1 carriers who received the immunosuppressive agent tacrolimus after liver transplant has been reported [7]. Therefore, it is important to clarify whether treatment of RA with the biologics increases the risk of ATL.

High HTLV-1 PVL has also been reported in patients with various connective tissue diseases [25]. High PVL, greater than 4–5 copies per 100 PBMCs, has been reported to be associated with progression to ATL in carriers [8, 9]. Therefore, HTLV-1 PVL was monitored during treatment with anti-TNF reagents in the present study. HTLV-1 PVL was low at less than 0.5 copies per 100 PBMCs before treatment with anti-TNF agents in both the present cases. In fact, we thought that the two cases in the present study were not in the high-risk group for the development of ATL and could choose anti-TNF agents for their treatment. Fortunately, even after the beginning of treatment, the levels of PVL showed no significant increase.

In addition, the clonal evolution of HTLV-1-infected cells has also been reported to occur before the onset of ATL [9]. We also monitored the clonality of HTLV-1-infected cells, and neither case showed significant change. In addition, there were no signs (lymphadenopathy, eruption), symptoms or laboratory abnormalities (abnormal lymphocytes on blood smear) related to the progression of ATL. Therefore, no data suggesting the progression of HTLV-1-related diseases such as ATL were observed for 60–96 weeks after anti-TNF therapy in the present cases.

This study has a number of limitations. The number of patients was small, and the observation period was short. Generally, expansion into ATL from HTLV-1 exposure requires a period of 50–60 years. Therefore, we have to follow patients over a longer period of time to see the actual incidence of ATL among them. Because RA patients with high PVL were not included in this study, we could not say whether such patients would show the same course. From this point of view, future study should include RA patients with various levels of PVL ranging from low to high.

In conclusion, two HTLV-1-positive patients with RA were treated with anti-TNF agents. They had high RA disease activity and did not exhibit a good response to anti-TNF agents. Virological study on HTLV-1 infection showed no data suggesting that progression of ATL was promoted by these treatments. Further study including a greater number of patients is necessary to clarify whether these results can be generalized or whether HTLV-1 screening is necessary for RA patients before treatment with biologics.

Conflict of interest

None.

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Adult T-cell leukemia cells are characterized by abnormalities of *Helios* expression that promote T cell growth

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Molecular abnormalities involved in the multistep leukemogenesis of adult T-cell leukemia (ATL) remain to be clarified. Based on our integrated database, we focused on the expression patterns and levels of Ikaros family genes, *Ikaros*, *Helios*, and *Aiolos*, in ATL patients and HTLV-1 carriers. The results revealed profound deregulation of Helios expression, a pivotal regulator in the control of T-cell differentiation and activation. The majority of ATL samples (32/37 cases) showed abnormal splicing of Helios expression, and four cases did not express Helios. In addition, novel genomic loss in *Helios* locus was observed in 17/168 cases. We identified four ATL-specific short Helios isoforms and revealed their dominant-negative function. Ectopic expression of ATL-type Helios isoform as well as knockdown of normal Helios or Ikaros promoted T-cell growth. Global mRNA profiling and pathway analysis showed activation of several signaling pathways important for lymphocyte proliferation and survival. These data provide new insights into the molecular involvement of Helios function in the leukemogenesis and phenotype of ATL cells, indicating that Helios deregulation is one of the novel molecular hallmarks of ATL. (*Cancer Sci* 2013; 104: 1097–1106)

Adult T-cell leukemia (ATL) is a highly aggressive malignancy of mature CD4+ T cells and is caused by HTLV-1. After HTLV-1 infection, ATL is thought to develop following a multitude of events, including both genetic and epigenetic changes in the cells. Although many aspects of HTLV-1 biology have been elucidated, the detailed molecular mechanism of ATL leukemogenesis remains largely unknown.[1,2] Therefore, to precisely define the comprehensive abnormalities associated with ATL leukemogenesis, we previously carried out global mRNA and miRNA profiling of ATL cells derived from a large number of patients.[3,4] In this study, we focused on Ikaros family genes, especially Helios, on the basis of our integrated profiling of expression and gene copy number in ATL cells, which revealed the deregulated expression of this family of genes and genomic loss of *Helios* locus.

Ikaros family genes are specifically expressed in the hematopoietic system and play a vital role in regulation of lymphoid development and differentiation.[5–11] In addition, they are known to function as tumor suppressors during leukemogenesis according to several genetic studies carried out in mouse models.[12–15] Recently, many studies reported the deregulated splicing of Ikaros and the deletion of *Ikaros* locus in several human leukemias.[16–23] These abnormalities are associated with poor prognoses.[24–27] Helios is mainly expressed in the T-cell lineage.[10,11] Genomic changes and abnormal expression of Helios are also observed in some patients with T-cell malignancies.[18,28–31] However, in contrast to Ikaros, the substantial impact of aberrant Helios expression remains to be elucidated because of the absence of functional information, including the target genes of Helios.

In this study, we carried out a detailed expression analysis of Ikaros family genes in a large panel of clinical samples from ATL patients and HTLV-1 carriers and consequently identified a novel molecular characteristic, that is, abnormal splicing of Helios and loss of expression, which seems to be a significant key factor in leukemogenesis affecting the regulation of T-cell proliferation.

Materials and Methods

Cell lines and clinical samples. HeLa and 293T cells were cultivated in DMEM supplemented with 10% FCS. Human leukemic T cells, Jurkat, Molt-4, and CEM, ATL-derived, MT-1 and TL-Om1, and HTLV-1-infected MT-2 and Hut-102 cell lines were all maintained in RPMI-1640 with 10% FCS. The PBMCs from ATL patients of four clinical subtypes[32] and healthy volunteers were a part of those collected with informed consent as a collaborative project of the Joint Study on Prognostic Factors of ATL Development. The project was approved by the Institute of Medical Sciences, University of Tokyo Human Genome Research Ethics Committee (Tokyo, Japan). Clinical information of ATL individuals is provided in Table S1.

RNA isolation and RT-PCR analysis. The preparation of total RNA and synthesis of the first strand of cDNA were described previously.[3] The mRNAs of Ikaros family genes were examined by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). The PCR products were sequenced by automated DNA sequencer. Nested PCR amplification was carried out with diluted full-length PCR products by Accuprime Taq DNA polymerase High Fidelity (Invitrogen). Quantitative PCR was carried out as previously described.[3] The specific primer sets for each PCR are described in Table S2.

Immunoblot analysis. Cells were collected, washed with PBS, and lysed with RIPA buffer. For immunoprecipitation, cells were lysed with TNE buffer and incubated with specific antibody. Proteins samples were then analyzed by immunoblots with specific antibodies: anti-tubulin, anti-Ikaros, and anti-Helios antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-FLAG antibody (M2) was from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-HA

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antibody was from MBL (Nagoya, Japan). Anti-mouse, rabbit, and goat secondary antibodies were from Promega (Fitchburg, WI, USA).

Immunostaining. HeLa cells were cultured on coverslip slides and transfected with the indicated expression vectors by Lipofectamine LTX (Invitrogen). At 24 h post transfection, cells were washed three times with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Then, cells were stained with primary antibodies (diluted 1:500 to 1:2000). Alexa-488 or 546-conjugated secondary antibodies (Molecular Probes, Life Technologies, Carlsbad, CA, USA) were used for detection of specific targets, and DAPI was used for nuclear staining. Images were acquired by using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan).

Electrophoretic mobility-shift assay. Experimental conditions and detail methods were previously reported.[3] For evaluation of DNA binding activity, 3–5 μg nuclear extracts from each transfectant were used per each lane of electrophoresis. The oligonucleotide sequences used as a probe are provided in Table S2.

Luciferase assay. The pGL4.10-firefly vector (Promega) containing *Hes1* promoter was used as a reporter vector and RSV-renilla vector was used as a control vector. HeLa cells were transiently transfected with these reporters and each Ikaros or/and Helios expression vector by Lipofectamine 2000 reagent (Invitrogen). The luciferase activities were quantified by the Dual-Luciferase Reporter Assay System (Promega) at 24 h post-transfection.

Retroviral construction and transduction. The FLAG-Hel-5 cDNA sequence was subcloned into retrovirus vector pRx-puro. Stable cell populations expressing Hel-5 were selected by puromycin. The shRNA-expressing retroviral vectors and virus production procedures have been established.[3] The shRNA sequences are listed in Table S2. Stable cell populations were obtained by puromycin or G418 selection.

Proliferation assays. Cells (0.5 or 1.0×10^4) were plated in 96-well plates with media supplemented with 10% or 0.2% FCS. The cell numbers were evaluated for 4 days by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The averages of at least three independent experiments are shown.

Gene expression microarray analyses. Gene expression microarray used the 4 × 44K Whole Human Genome Oligo Microarray (Agilent Technologies, Santa Clara, CA, USA); detailed methods were previously reported.[3] Coordinates have been deposited in the Gene Expression Omnibus database with accession numbers GSE33615 (gene expression microarray), GSE33602 (copy number analyses), and GSE41796 (Jurkat models).

Results

Abnormal expression of short Helios transcripts in primary ATL cells. To characterize the gene expression signature in primary ATL cells, we previously carried out mRNA microarray analyses on a large number of samples. The comprehensive survey unveiled deregulated expression of Ikaros family genes; transcription levels of Ikaros and Aiolos were downregulated in ATL samples, whereas Helios was upregulated (Fig. S1). Thus, we examined the detailed expression patterns and levels of Ikaros family members in PBMCs derived from a panel of ATL patients and HTLV-1 carriers (Fig. 1a). Compared with control PBMCs from normal volunteers (Fig. 1b), the expression levels of Ikaros and Aiolos seemed to be downregulated in ATL samples, consistent with our microarray results. However, there were obvious abnormalities in the expression patterns of Helios. The main isoform of Helios was changed from full-length Hel-1 to Hel-2, which lacks exon 3 that contains the first N-terminal zinc finger in the DNA-binding domain. In addition, four ATL-specific Helios short transcripts were identified (Fig. 1c). Among them, Hel-5 and Hel-6 have been reported to be expressed in ATL.[29] We also identified two novel variants, Hel-v1 that lacks exons 3 and 4 and Hel-v2 that lacks exons 2, 3, and 6. These abnormal Helios variants were also expressed in the samples of high-risk HTLV-1 carriers, who subsequently developed ATL in the next few years. Furthermore, nested PCR revealed that Hel-5 or Hel-6 were expressed in a majority of ATL samples (17/22 acute cases, 10/10 chronic cases, and 5/5 smoldering cases; total, 32/37 cases) (Fig. 1d, upper panels), whereas Hel-v1 was expressed only in limited cases of ATL (Fig. 1d, lower panels). In four cases, Helios was not expressed. Collectively, our mRNA analysis showed that Helios expression was generally deregulated in ATL cells.

Genomic abnormalities at the *Helios* locus in primary ATL cells. To investigate the *Helios* locus in ATL, we retrieved data from our gene copy number analysis[3] and found that specific genomic deletion was accumulated at the *Helios* locus in ATL samples (17/168 cases, Fig. 2). All 17 cases were aggressive-type ATL (12/17 lymphoma types and 5/17 acute types). Furthermore, we found that two acute ATL cases in Figure 1(a) (#9 and #14), which showed severely deregulated or lost Helios expression, had a genomic deletion of the *Helios* locus.

Dimerization ability of ATL-type Helios isoforms with wild-type Helios or Ikaros. Consistent with a previously published report,[33] co-immunoprecipitation analyses confirmed that wild-type Hel-1 formed homodimers with themselves and heterodimers with wild-type Ikaros (Ik-1) protein (Fig. 3a, top panel, lane 1 and lane 4). In contrast, the dimerization activity of another artificial Helios mutant (Hel-ΔC), which lacks the dimerization domain at the C-terminal region, was dramatically declined (Fig. 3b, top panel, lane 1 and lane 4). We confirmed that all ATL-type Helios proteins could interact with Hel-1 and Ik-1, despite the fact that all of them lack various sets of the N-terminal exons (Fig. 3c–f).

Cytoplasmic localization of ATL-type Helios isoforms lacking exon 6. Ectopically expressed Hel-1 and Ik-1 were localized in the nucleus (Fig. 4a, top two panels). Regarding the ATL-type Helios isoforms, we found that Hel-5 and Hel-v1 were localized in the nucleus, whereas Hel-6 and Hel-v2, both of which lack exon 6, were substantially localized in the cytoplasm (Fig. 4a, middle four panels). We also confirmed the cytoplasmic localization of Hel-Δexon 6, which is an artificial Helios mutant lacking only exon 6 (Fig. 4a, bottom panel). Thus, exon 6 appears to be critical for nuclear localization of Helios proteins. Furthermore, defect of exon 6 led to disruption of the

Fig. 1. (On the next page) Abnormal expression of Helios mRNA in primary adult T-cell leukemia (ATL) cells. (a) Expression analysis of Ikaros family genes in PBMCs by full-length RT-PCR (Acute, $n = 22$; Chronic, $n = 10$; Smoldering, $n = 5$; HTLV-1 carriers, $n = 5$; High-risk carriers, $n = 4$). To detect and distinguish alternative splicing variants, PCR analyses were carried out with the sense and antisense primer sets designed in the first and final exons of each full-length transcript of Ikaros family genes. Obtained cDNAs were cloned and their sequences were analyzed. The samples acute #4, 4′, and 4″ were derived from the same patient, but were studied independently. (b) Expression of Ikaros family genes in PBMCs from normal volunteers ($n = 10$). (c) Schematic representation of Hel-1, Hel-2, and ATL-type Helios isoforms identified in this study. Hel-variant 1 (Hel-v1) and Hel-variant 2 (Hel-v2) are novel isoforms in ATL. Arrows indicate primer locations of full-length PCR for Helios. Ex, exon; F1–F6, functional zinc-finger domains. (d) Nested PCR with specific primer sets, which were designed at exon junction of exon 1–5 or exon 2–5 for detection of Hel-5 and Hel-6 (upper panel), or detection of Hel-v1 (lower panel), respectively. Arrows indicate primer locations.

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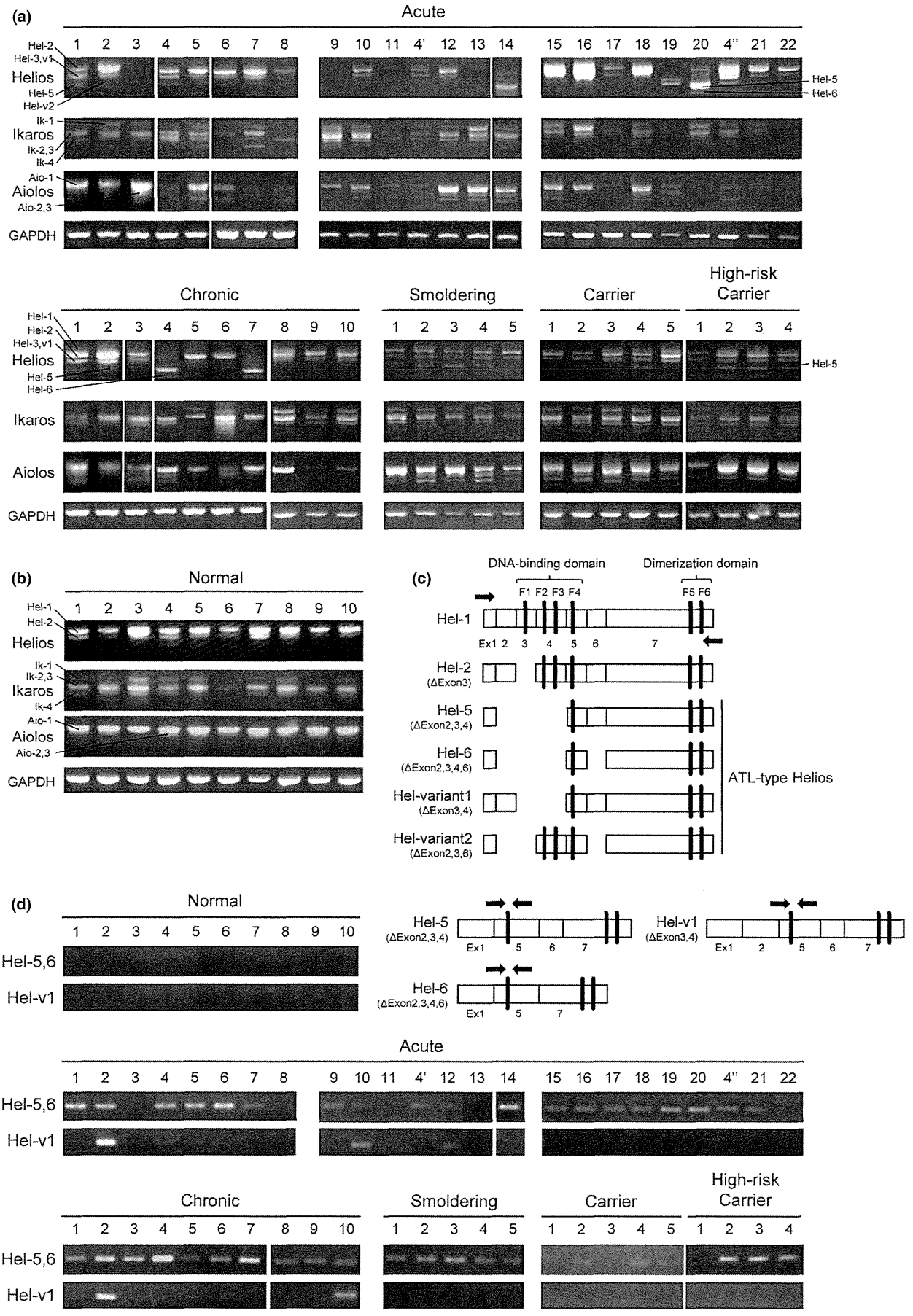

cellular localization of binding partners. When Hel-6 or Hel-v2 were co-expressed with Hel-1 or Ik-1, they were co-localized in the cytoplasm (Fig. 4b, Fig. S2).

Dominant-negative function of ATL-type Helios isoforms against wild-type Helios and Ikaros. We next examined the functional aspects of these ATL-type Helios isoforms by evaluating their DNA-binding capacities. For EMSA, we used an oligonucleotide probe derived from the promoter region of human *Hes1*, which was a direct target of Ikaros.[34,35] Ectopically expressed Hel-1 or Ik-1 could bind human *Hes1* promoter DNA (Fig. 5a). Supershift assays confirmed the binding specificity (Fig. 5b). In contrast, all ATL-type Helios isoforms did not show any specific binding to the *Hes1* promoter (Fig. 5a). This impossibility of specific DNA binding of ATL-type Helios was confirmed with another independent DNA probe, IkBS4[33,36] (data not shown). In addition, it was found in co-expression experiments that Hel-5 had antagonistic effects on the DNA binding capacity of Ik-1 in a dose-dependent manner (Fig. 5c). Reporter assays showed that Hel-1 and Ik-1 suppressed *Hes1* promoter activity. However, ATL-type Helios isoforms did not show any suppressive activity, and actually slightly activated the promoter (Fig. 5d). Furthermore, they also inhibited the suppressive function of Hel-1 and Ik-1 in a dose-dependent manner (Fig. 5e, Fig. S3). These data clearly indicate that ATL-type Helios isoforms are functionally defective because of a DNA binding deficiency and act dominant-negatively in transcriptional suppression induced by Hel-1 or Ik-1. We also confirmed that Hel-2, which lacks only exon 3 and is a major isoform in ATL cells, did not possess suppressive activity against *Hes1* promoter in spite of having binding activity (Fig. 5a,d).

Major ATL-type Helios variant, Hel-5, promotes T cell growth. Given the tumor-suppressive roles of Ikaros family members,[12–15] it was expected that abnormal splicing of Helios could contribute to T cell leukemogenesis. The mRNA level of Helios was significantly downregulated in ATL-related cell lines compared with that in T-cell lines without HTLV-1 (Fig. 6a, Fig. S4). Moreover, Helios protein was not detected in any ATL-derived or HTLV-1-infected cell lines used in this study (Fig. 6b). In contrast, the expression levels of Ikaros mRNA did not show major differences between HTLV-1-infected and uninfected T-cell lines. Those of Aiolos were low in most cell lines irrespective of HTLV-1 infection (Fig. 6a, Fig. S4). Ikaros protein was detected in all T-cell lines used in this study (Fig. 6b). To elucidate the cellular effects of the expression of dominant-negative ATL-type Helios isoforms in T cells, we established stable Jurkat cells expressing Hel-5 (Fig. 6c). A cell proliferation assay confirmed that Hel-5 expression significantly promoted Jurkat cell proliferation

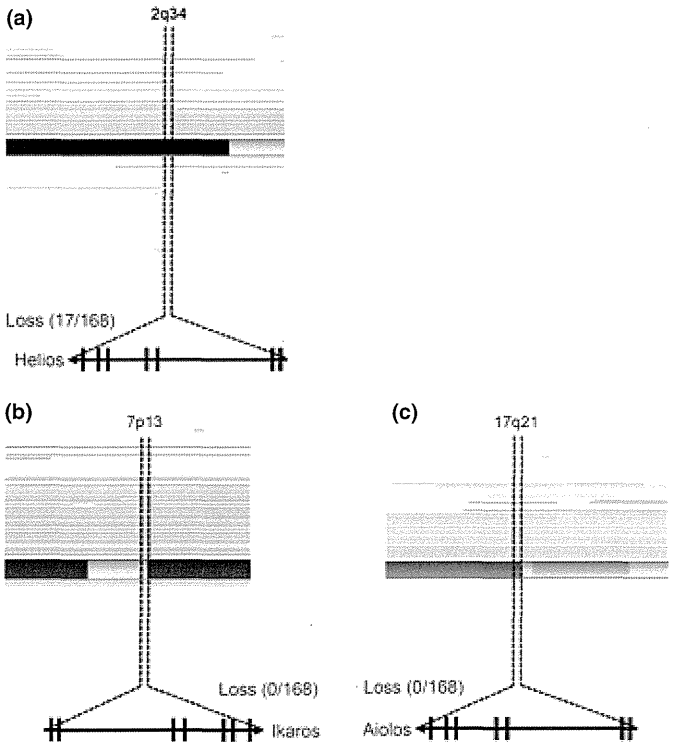

Fig. 2. Genetic abnormalities in *Helios* locus in primary adult T-cell leukemia cells. The results of our copy number analyses[3] (total number, $n = 168$; acute type, $n = 35$; chronic type, $n = 41$; lymphoma type, $n = 44$; smoldering type, $n = 10$; intermediate, $n = 1$; unknown diagnosis, $n = 37$). Tumor-associated deletion of *Helios* region (17/168) was detected (a). No specific genomic losses were observed in *Ikaros* (b) or *Aiolos* loci (c). Recurrent genetic changes are depicted by horizontal lines based on Copy Number Analyser for GeneChip output of the single nucleotide polymorphism array analysis.

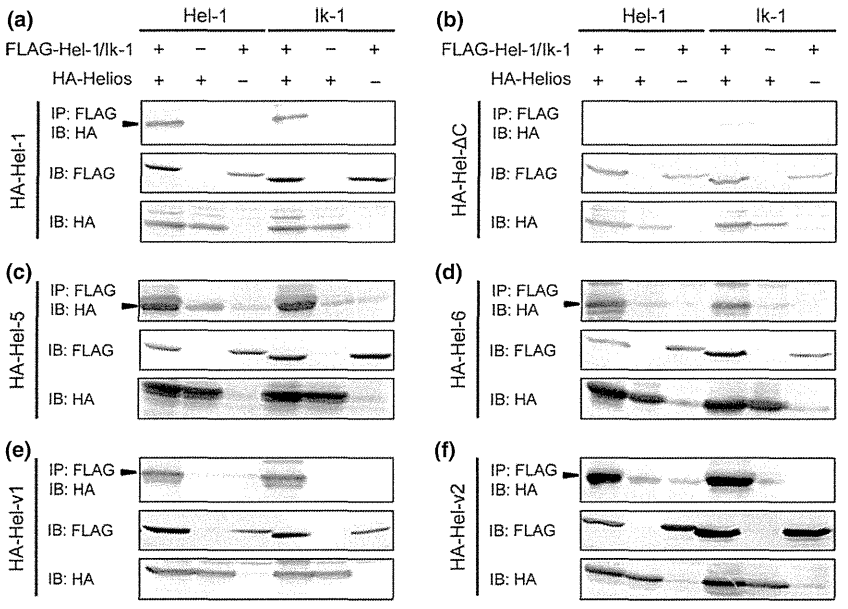

Fig. 3. Dimerization ability of adult T-cell leukemia (ATL)-type Helios isoforms. *In vitro* dimerization assays by co-immunoprecipitation between ATL-type Helios and wild-type Helios or Ikaros proteins. 293T cells were transfected with the indicated combination of expression vectors and subjected to co-immunoprecipitation analyses (top panels). Arrowheads indicate the complex of FLAG and HA-tagged proteins. Middle and bottom panels show the input samples. Hel-1 (a) and Hel-ΔC (b) included as positive and negative controls, respectively. ATL-specific isoforms, Hel-5 (c), Hel-6 (d), Hel-v1 (e), and Hel-v2 (f) were tested. IB, immunoblot; IP, immunoprecipitant.

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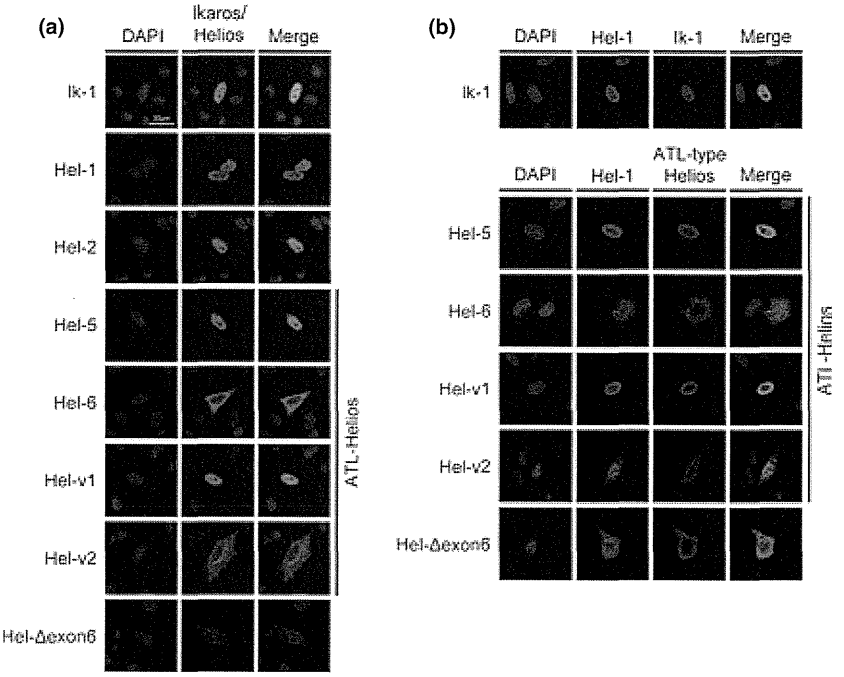

Fig. 4. Subcellular localization of adult T-cell leukemia (ATL)-type Helios isoforms. Immunostaining analyses of Helios and Ikaros proteins. HeLa cells were transfected with each individual expression vector (a) or the indicated combination of expression vectors (b). Each protein was visualized with anti-FLAG (green) or anti-HA antibodies (red). Nuclei were detected by DAPI staining (blue). Colocalization between Ik-1 and ATL-type Helios was shown in Fig. S2. Hel-v1, Hel-variant 1; Hel-v2, Hel-variant 2.

(Fig. 6d). To examine whether the cellular effect of Hel-5 was due to its dominant-negative function against Hel-1 and Ik-1, we carried out further knockdown analyses with specific shRNAs (Fig. 6e). The results showed that knockdown of wild-type Helios or Ikaros led to enhanced cell growth (Fig. 6f), which was consistent with the results of enforced Hel-5 expression. These results collectively suggested that counteraction of Ikaros or Helios by dominant-negative isoforms contributed to T cell growth.

Helios deficiency causes expression of various genes in T cells. We globally searched mRNA expression changes using microarray analysis of Jurkat cells expressing Hel-5 and those of knocked-down Helios or Ikaros (Fig. 7a,b). The results clearly showed differentially expressed gene sets between the transformants and control cells (Fig. 7c). Furthermore, pathway analysis[37] of each upregulated gene set identified activation of several signaling cascades. In particular, we focused on six common pathways identified in both Hel-5 transduced and Helios or Ikaros knocked-down Jurkat cells (Fig. 7d). These pathways are important for various T cell regulations, for example, cell growth, apoptosis resistance, and migration activity. Among these pathways, it has not been reported that the shingosine-1-phosphate (S1P) pathway is regulated by the Ikaros family. We confirmed overexpressed *S1PR1* and *S1PR3*, which are critical receptors for the activation of the S1P pathway, in manipulated Jurkat samples (Fig. 7e).

Discussion

In the present study, on the basis of the integrated analysis of ATL cells using our biomaterial bank in Japan, we revealed a novel molecular characteristic of ATL cells, which is a profound abnormality in the expression of Helios. The abnormal alternative splicing and, in some cases, loss of Helios expression appear to be a part of the basis for advantageous cell growth and survival in ATL cells. We also showed the tumor-suppressive function and target genes, as well as pathways of Helios, in mature human T cells.

Characterization of Ikaros family members revealed profound abnormalities in Helios expression in ATL cells: (i) biased and increased expression of alternatively spliced variants; (ii) suppression of Hel-1 expression; (iii) lack of Helios expression in some cases; and (iv) frequent genomic defects of the *Helios* locus. Our results also revealed that alternatively spliced Helios variants are expressed in PBMCs of HTLV-1 carriers, suggesting that the abnormal splicing of Helios may occur in HTLV-1-infected cells at the carrier state until progression to leukemia development. However, the genomic deletions appear to be one of the important genetic events during the latter stages of leukemia development, as they were observed only in aggressive subtypes of ATL.

The structural characteristics of the ATL-type Helios variants involve a selective lack of one or more zinc fingers in the N-terminal domain. The results of this study indicated that these variant proteins lost DNA binding activity, whereas the capacity of dimerization was preserved. Therefore, these variant proteins hindered transcriptional activities of Ikaros family proteins, showing dominant-negative effects. In addition, a part of ATL-type Helios isoform, which lacks exon 6, is linked to abnormal localization of wild-type Helios and Ikaros. We confirmed that Helios isoforms lacking exon 6 were overexpressed in primary ATL cells (Fig. S5). Interestingly, Hel-2 has reduced transcriptional suppressive activity compared with Hel-1, although it can bind to the target sequence as well as Hel-1. This is similar to a previous report,[36] which noted that the activity of mouse Ik-2 protein for the reporter gene was remarkably lower than that of Ik-1, whereas the binding affinities of Ik-1 and Ik-2 were similar. The exon 3 skip occurred more frequently in ATL cells, compared to PBMCs from normal volunteers (Fig. S6). These results collectively indicate that all abnormalities of Helios expression, including loss of or decreased Hel-1 expression and upregulated Hel-2 and ATL-type Helios, result in abrogation of Ikaros family functions in ATL cells.

We also confirmed that *Hes1*, a target gene of the Notch pathway, is one of the targets of Helios as well as Ikaros.[34,35] A recent study reported that activated Notch signaling may be important to ATL pathogenesis and that Hes1 is upregulated in ATL cells.[38] Thus, we examined expression levels of Hes1 mRNA by quantitative RT-PCR and confirmed the

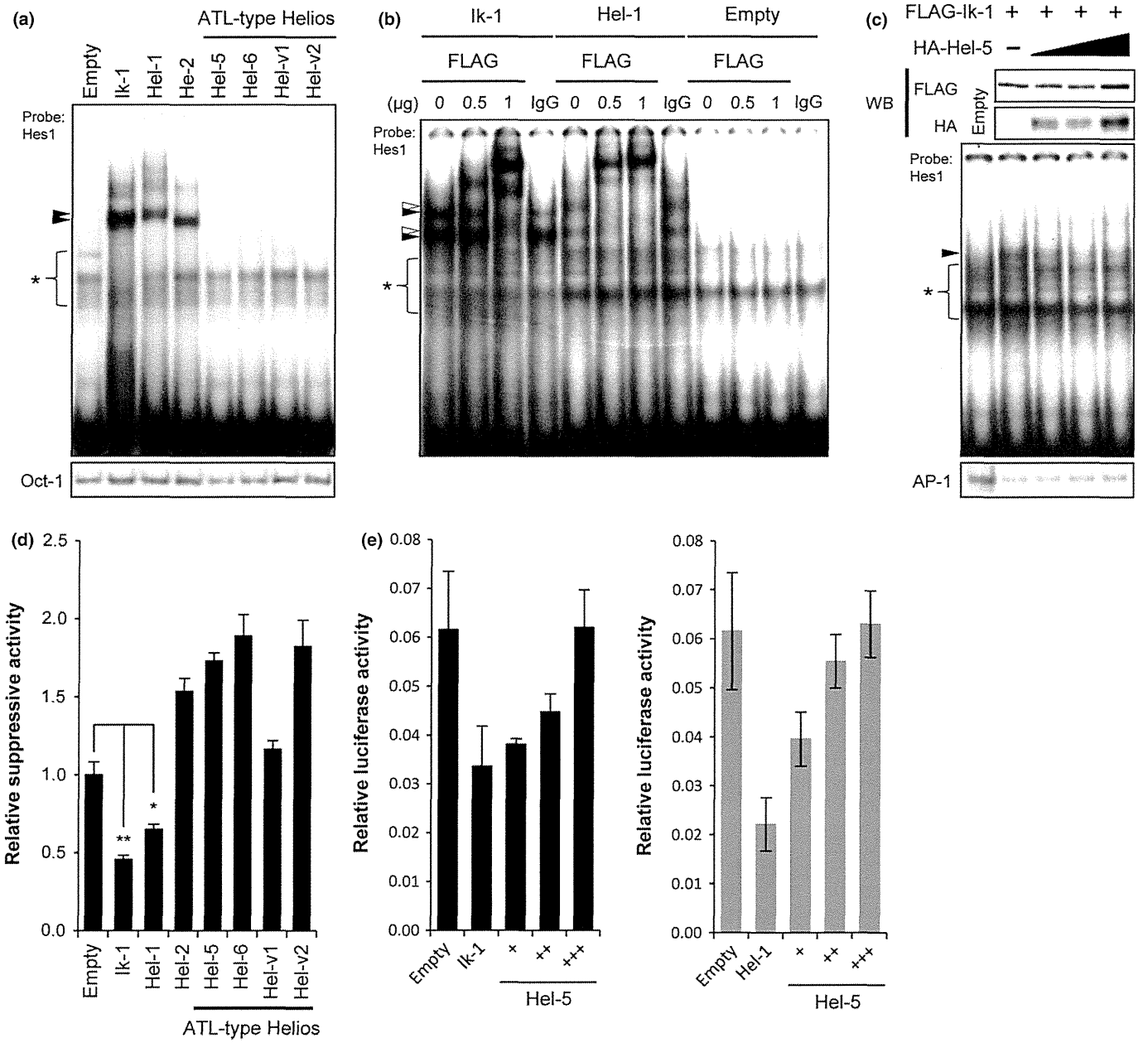

Fig. 5. Dominant-negative function of adult T-cell leukemia (ATL)-type Helios isoforms. (a) DNA-binding activities of wild-type Helios or Ikaros and ATL-type Helios proteins. Each FLAG-tagged Helios or Ikaros isoforms were ectopically expressed in 293T cells and their nuclear extracts were subjected to EMSA with a [γ-^{32}P]-labeled *Hes1* promoter probe. Oct-1 probe was used as an internal control. Arrowheads indicate Helios or Ikaros complexes. *Non-specific bands. Hel-v1, Hel-variant 1; Hel-v2, Hel-variant 2. (b) Results of supershift assays. Anti-FLAG (0, 0.5, 1 μg) or control IgG (1 μg) antibodies were added to each nuclear extract prior to electrophoresis. The black and white arrowheads indicate the supershifted bands of Ik-1 and Hel-1, respectively. (c) Antagonistic effects of Hel-5 on DNA-binding of Ik-1 tested by EMSA. The molar ratios of Ik-1 to Hel-5 plasmids are 1:1, 1:4, and 1:8. Expression levels of FLAG-Ik-1 and HA-Hel-5 were assessed by immunoblotting. The arrowheads indicate the Ik-1 specific band. AP-1 probe was used as an internal control. WB, western blot. (d) Transcriptional suppression activities of various Helios or Ikaros isoforms tested by *Hes1* promoter-luciferase reporter systems (n = 3, mean ± SD). Basal *Hes1* promoter activity was defined as firefly/renilla ratio, and suppression activities of Helios or Ikaros are relatively presented. Statistical significance was evaluated by unpaired Student's t-test (*P < 0.05; **P < 0.01). (e) Inhibitory function of Hel-5 against Ik-1 and Hel-1 tested by *Hes1* promoter assay (n = 3, mean ± SD). The molar ratios of Ik-1 or Hel-1 to Hel-5 plasmids are 1:1, 1:2, and 1:3. Relative luciferase activities were defined as firefly/renilla ratio.

upregulation in our ATL samples (Fig. S7). Hes1 has been reported to directly promote cell proliferation through the transcriptional repression of p27kip1.[39] Taken together, our results suggest a possibility that abnormalities in Helios expression are one of the causes of Hes1 activation, which may be one of the genetic events involved in ATL leukemogenesis.

Our results show that the Hel-5 variant may have an oncogenic role, whereas the wild-type Helios, Hel-1, shows tumor suppressor-like activity. These findings are consistent with previous findings in mice.[15] Furthermore, our description of expression profiles of stable cells followed by pathway analyses showed activation of several important pathways in lymphocytes for the regulation of proliferation, survival, and others. In particular, we discovered novel molecular cross-talk between the Ikaros family and the S1P pathway. The S1P–S1PR1 axis is known to play important

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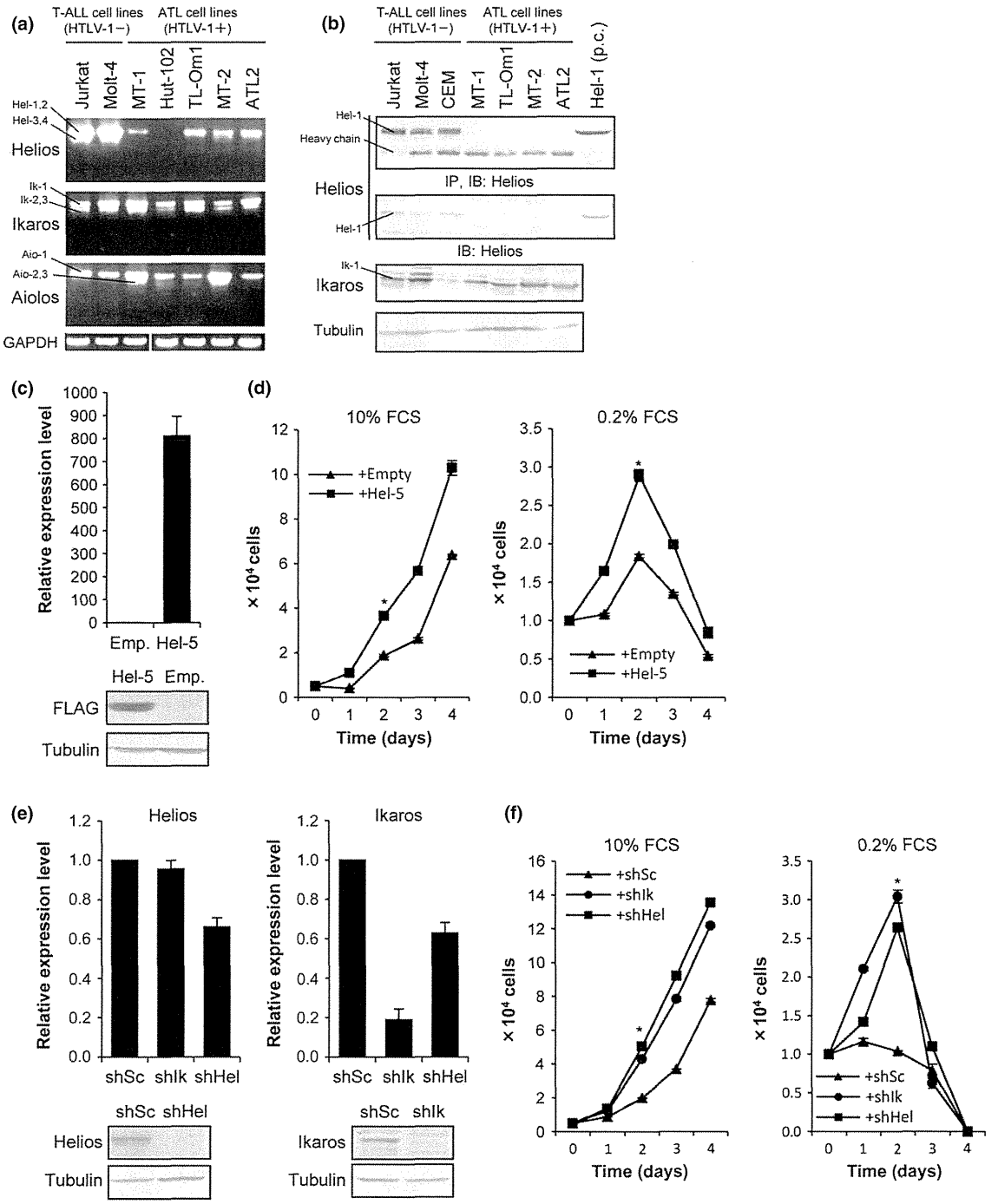

Fig. 6. Hel-5 functions in T cell growth and survival. (a) Expression patterns and levels of Ikaros family genes in various cell lines examined by RT-PCR. ATL, adult T-cell leukemia; T-ALL, acute T lymphoblastic leukemia. (b) Results of immunoblotting analyses of the immunoprecipitants (top panel) and cell lysates (lower panels). Positive control (p.c.), Hel-1 transfectant. IB, immunoblot; IP, immunoprecipitant. (c) Establishment of Jurkat cells stably expressing Hel-5. The Hel-5 level was quantified by quantitative RT-PCR (top, $n = 3$, mean ± SD) and immunoblotting (bottom). (d) Cell proliferation analysis of control cells (▲) and Hel-5-expressing Jurkat cells (■) under two FCS conditions ($n = 3$, mean ± SD). Statistical significance was observed (*P <0.01, Student's t-test). (e) Knockdown analyses of Helios or Ikaros in Jurkat cells. The Helios and Ikaros levels were evaluated by quantitative RT-PCR (top, $n = 3$, mean ± SD) and immunoblotting (bottom), respectively. (f) Cell proliferation curves of scrambled shRNA (shSc) cells (▲), shIkaros (shIk) cells (●), and shHelios (shHel) cells (■) were examined in two FBS conditions ($n = 3$, mean ± SD; *P < 0.01).

roles in regulation of the immune system, apoptosis, cell cycle, and migration of lymphocytes.[40–42] Recently, activation of the S1P pathway in various diseases, including leukemia, has been reported, and the therapeutic potential of S1PR1 inhibitors was suggested.[42] Studies of functional roles of S1P pathway activation in ATL cells are now underway in our laboratory.

In conclusion, our present study revealed a novel aspect of molecular abnormalities in ATL cells: a profound deregulation in Helios expression, which appears to play an important role in T-cell proliferation. Our experimental approaches also imply that, in addition to genetic and epigenetic abnormalities, ATL shows abnormal splicing, which has been observed in various human diseases including cancers.[43–45]

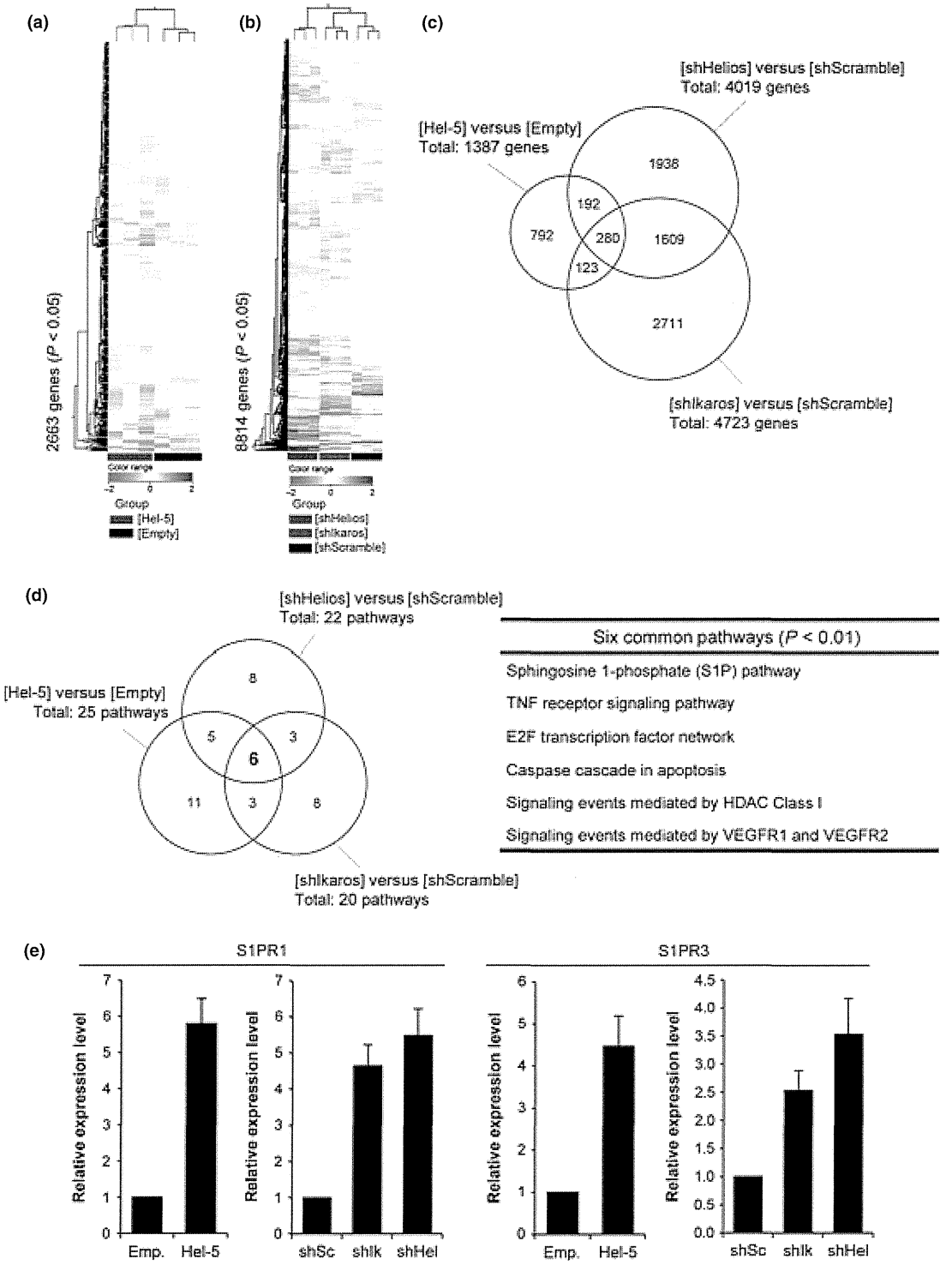

Fig. 7. Comprehensive search for Helios target genes by microarray analysis. (a,b) Gene expression analysis of Jurkat stable cells. The gene expression patterns of Jurkat cells expressing Hel-5 (n = 3), shIkaros (n = 3), and shHelios (n = 3) were comprehensively analyzed by microarray technique. The obtained 2D hierarchical clusters and Pearson's correlation between the cells expressing Hel-5 or not (a) and the cells introducing shHel, shIk, or shSc (b). (c) Venn diagram of differential gene expression pattern in the Jurkat sublines. The each differential expression gene set (5-fold changes, $P < 1 \times 10^{-5}$) was compared. (d) Venn diagram depicting the overlap between the outputs of pathway analysis in Jurkat sublines. The analysis was based on the NCI-Nature Pathway Interaction Database.[37] Each differential pathway set (t-test, $P < 0.01$) was compared and the common pathways listed. (e) Results of quantitative RT-PCR of shingosine-1-phosphate receptor 1 (S1PR1) and receptor 3 (S1PR3) in Jurkat sublines (n = 3, mean ± SD). HDAC, histone deacetylase; VEGFR, vascular endothelial growth factor receptor.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Deregulated expression of Ikaros family genes in primary adult T-cell leukemia cells.