were found to be associated with each other (miR-130b versus miR-145, $P = .019$; miR-130b versus miR-223, $P < .0001$; and miR-145 versus miR-223, $P = .043$). To explore which miRNA was the most associated with the patients' prognosis, we performed a multivariate analysis. Only miR-145 lower expression was selected as a prognostic factor ($P = .0005$; hazard ratio, 2.59 [1.52-4.49]), whereas the other 2 miRNAs failed to achieve statistical significance (Table). Fig. 2 shows the overall survival curve of patients with ATL for miR-145 expression. miR-145 expression of patients with ATL relative to that of normal CD4-positive T cells was 0.0495 ± 0.0276 and 3.02 ± 1.03 (mean ± SEM) for lower and higher expressers, respectively. When correlated with clinicopathological factors including age, sex, the presence of B symptoms, performance status, extranodal sites, bone marrow involvement, elevated LDH value, and histologic lymphoma subtype, miR-145 lower expression was not associated with any of these factors.

3.3. Overexpression of miR-145 and cell growth inhibition in ATL cells

Because miR-145 was consistently down-regulated in ATL cell lines and showed the highest prognostic impact on patients' survival, we focused on this miRNA in further analysis. To examine the association between miR-145 expression and the growth of ATL cells, MT-4 cells were transfected with a pre–miR-145 precursor and a pre-miR negative control, and an MTT assay was conducted. Overexpression of mature miR-145 in the transfected cells was confirmed by quantitative RT-PCR (Fig. 3A). Cell growth was significantly inhibited in cells transfected with pre–miR-145 48 hours after the transfection by approximately 40% as compared with those transfected with the pre-miR negative control (Fig. 3B). These results indicated that miR-145 specifically inhibited the cell growth of the MT-4 cell line.

4. Discussion

In this study, we found that 1 miRNA (miR-130b) was consistently up-regulated, and 3 (miR-145, miR-223, and miR-150) were consistently down-regulated in ATL cell lines using an miRNA array and subsequent quantitative RT-PCR. It has been shown that expression of some of these miRNAs is dysregulated in ATL cell lines: miR-130b and miR223 have been reported to be up-regulated [8] and down-regulated [10], respectively. miR155, which was selected in the present miRNA array but was subsequently excluded because of its inconsistent expression in the quantitative RT-PCR, has been frequently up-regulated in ATL [9,10,13]. It is difficult to explain this discrepancy. A similar observation was reported in cutaneous T-cell lymphoma, and it was speculated that cross-hybridization of pre–miR-155 to the hybridization probe might have masked the signal of the mature miR-155 [16]. Recently, Yamagishi et al [11] and Tomita et al [12] determined the miRNA signatures and revealed miR-31 down-regulation and miR-146a up-regulation in primary ATL cells, respectively. However, these 2 miRNAs were not highly dysregulated in our miRNA array. This is partly explained by our use of the ATL tumor samples and control samples. For the ATL tumor samples and controls, we used ATL cell lines and normal CD4-positive lymphocytes while Yamagishi et al used clinical ATL cells as tumor samples [11] and Tomita et al used HTLV-1–uninfected T-cell lines as a control [12].

Of the 4 dysregulated miRNAs we determined (miR-130b, miR-145, miR-150, and miR-223), we searched for miRNAs that had a prognostic impact on clinical ATL cases. For each of the 4 miRNAs, we divided our ATL cases into 2 groups (higher and lower expression) using a respective cutoff value that showed superior segregation into prognostic groups. Univariate prognostic analysis showed that miR-130b, miR-145, and miR-223, but not miR-150, had a significant association with the overall survival of patients with ATL. The reason why miR-150 did not achieve statistical significance is difficult to discern, but may be partly explained as follows. First, some miRNAs are expressed differently in ATL cell lines compared with clinical samples, and miR-150 was reported to be differentially expressed in ATL cell lines and uncultured ATL cells [9]. Second, miR-150 may be more associated with tumor development than tumor progression.

We found that the expressions of 3 selected miRNAs (miR-130b, miR-145, and miR-223) were significantly associated with each other. Multivariate prognostic analysis including these 3 miRNAs revealed that only miR-145 lower expression achieved statistical significance. Interestingly, this miRNA was not correlated with any of the clinicopathological or risk factors examined, suggesting that miR-145 lower expression might be a useful independent prognostic factor. Although down-regulated in all ATL cell lines examined, miR-145 was not always down-regulated in patients with clinical ATL. One possible explanation may be that ATL cell line tumor cells are highly activated and more aggressive than clinical ATL cases.

We then focused on miR-145, and to confirm whether miR-145 expression was inversely associated with ATL cell proliferation, we performed an MTT assay to measure cell proliferation rates before and after enforced miR-145 expression in an ATL cell line, MT-4, and showed that overexpression of miR-145 significantly inhibited tumor cell growth. This inhibition was not complete, suggesting that some other factors or pathways may be involved in tumor cell growth. Down-regulation of miR-145 has been reported in various types of human carcinoma [17-20] and B-cell malignancy [21-23]. These studies suggest that miR-145 plays a role in controlling cell proliferation, thereby serving as a tumor suppressor. The precise upstream mechanism of miR-145 down-regulation in ATL has not been clarified.

Recently, down-regulation of miR-145 by DNA methylation and p53 mutation pathways has been suggested in prostate cancer [24]. We searched a Web site (microRNA.org-Targets and Expression; http://microrna.org/microrna) for specific targets of miR-145 and retrieved more than 20 targets with high matching scores. However, no association has been suggested between these candidate targets and ATL in the literature. Potential targets of miR-145 have been reported including MYC in colon cancer [25], ERG in prostate cancer [26], and connective tissue growth factor in glioblastoma [27].

In conclusion, we investigated ATL cell lines and clinical ATL cases for miRNA expression using miRNA arrays and quantitative RT-PCR, and we found that down-regulation of miR-145 was highly associated with a worsened clinical course in patients. An in vitro functional assay showed that miR-145 expression was inversely associated with tumor cell proliferation. To the best of our knowledge, the involvement of miR-145 in ATL has not been reported. Our findings shed light on the biological and clinical roles of miR-145 in ATL and provide the basis for the development of new miRNA-targeted therapeutic strategies against this tumor.

Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.humpath.2014.01.017.

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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.

cancer immunotherapy | immunomodulation

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

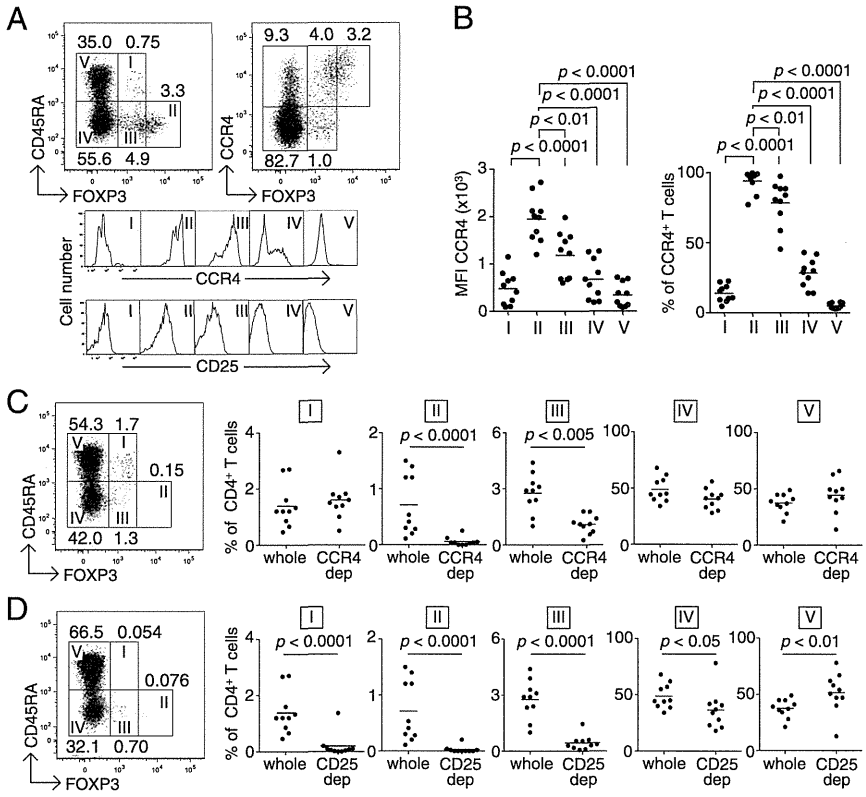

Fig. 1. Reduction of eTreg cells by in vitro depletion of CCR4-expressing T cells. (*A*) CCR4 and CD25 expression by subpopulations of FOXP3$^+$ Treg cells in PBMCs from healthy donors. CCR4 and CD25 expression levels were evaluated for each fraction. Representative data from 10 healthy donors are shown.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

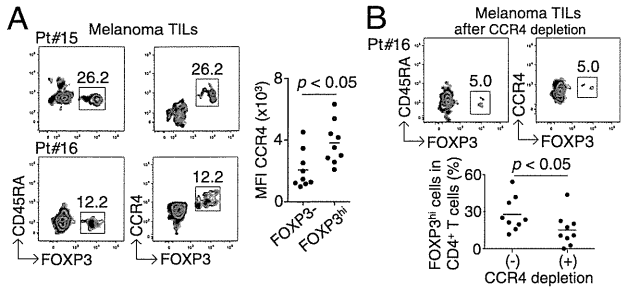

Fig. 2. Predominant infiltration of CCR4$^+$ eTreg cells into melanoma tissues. (*A*) CCR4 expression by melanoma-infiltrating T cells. CD4$^+$ T cells from melanoma sites were fractionated into subpopulations based on the expression of CCR4, CD45RA, and FOXP3; CCR4 expression by each fraction was analyzed.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

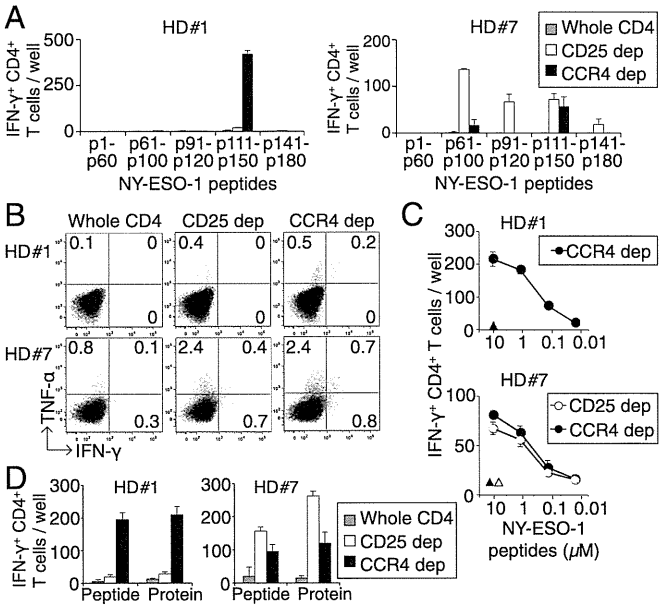

Fig. 3. Induction of cancer/testes antigen-specific CD4$^+$ T cells by depletion of CCR4- or CD25-expressing T cells in healthy donors. (*A*) CD4$^+$ T-cell responses to NY-ESO-1 peptides after depletion of CCR4$^+$ or CD25$^+$ T cells. CD4$^+$ T cells prepared from PBMCs of healthy donors were presensitized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

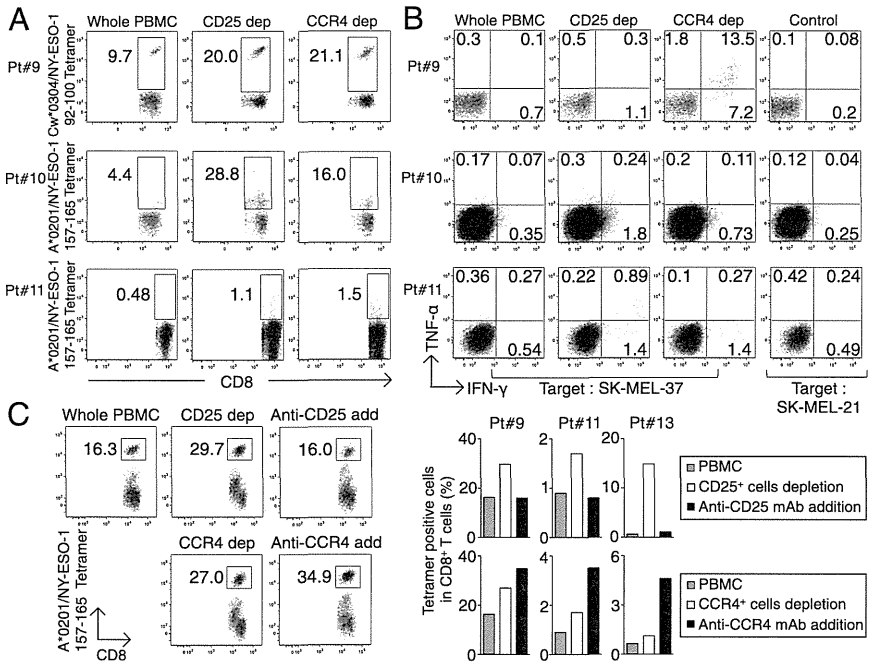

Fig. 4. Augmentation of NY-ESO-1–specific $CD8^+$ T-cell induction in melanoma patients by in vitro $CCR4^+$ T-cell depletion. (*A*) Induction of NY-ESO-1–specific $CD8^+$ T cells. Unfractionated PBMCs, or PBMCs depleted of $CD25^+$ or $CCR4^+$ cells, were prepared from melanoma patients (n = 6), and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

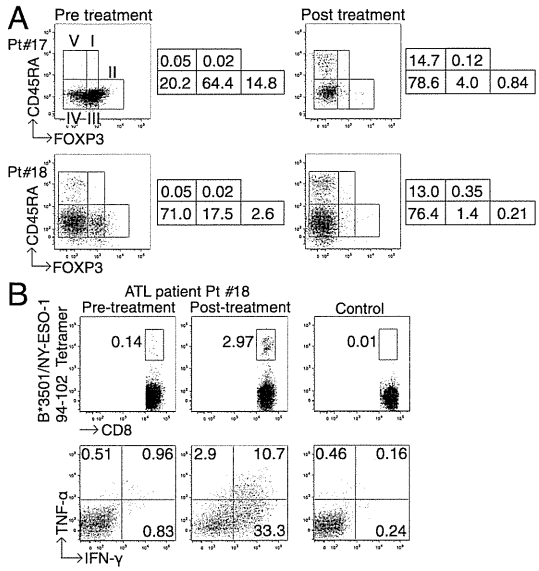

Fig. 5. Reduction of $CD4^+FOXP3^{hi}CD45RA^-$ T cells and augmentation of NY-ESO-1–specific $CD8^+$ T-cell responses in ATL patients after anti-CCR4 mAb (Mogamulizumab) therapy. (*A*) $FOXP3^+$ Treg-cell subpopulations in PBMCs from two ATL patients (Pt. #17: acute type, HLA-A*2402/-, B*3901/5401, C*0102/0702 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 × 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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Impact of Graft-versus-Host Disease on Allogeneic Hematopoietic Cell Transplantation for Adult T Cell Leukemia-Lymphoma Focusing on Preconditioning Regimens: Nationwide Retrospective Study

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Mogamulizumab

A B S T R A C T

Allogeneic hematopoietic cell transplantation (HCT), but not autologous HCT, can provide long-term remission in some patients with adult T cell leukemia-lymphoma (ATL). We retrospectively analyzed the effects of acute graft-versus-host disease (GVHD) among the 616 patients with ATL who survived at least 30 days after allogeneic HCT with other than cord blood grafts. Multivariate analyses treating the occurrence of GVHD as a time-varying covariate demonstrated an association between grade I-II acute GVHD and favorable overall survival (OS) (hazard ratio [HR], 0.634; 95% confidence interval [CI], 0.477 to 0.843), whereas grade III-IV acute GVHD showed a trend toward unfavorable OS (HR, 1.380; 95% CI, 0.988 to 1.927) compared with nonacute GVHD. In subsequent multivariate analyses of patients who survived at least 100 days after HCT (n = 431), the presence of limited chronic GVHD showed a trend toward favorable OS (HR, 0.597; 95% CI, 0.354 to 1.007), and extensive chronic GVHD had a significant effect on OS (HR, 0.585; 95% CI, 0.389 to 0.880). There were no significant interactions between myeloablative conditioning or reduced-intensity conditioning with OS even when acute GVHD was absent or present at grade I-II or grade III-IV or when chronic GVHD was absent, limited, or extensive. This study demonstrates the actual existence of graft-versus-ATL effects in patients with ATL regardless of whether myeloablative conditioning or reduced-intensity conditioning is used.

INTRODUCTION

Adult T cell leukemia-lymphoma (ATL) is an aggressive peripheral T cell neoplasm caused by human T cell lymphotropic/leukemia virus type 1 (HTLV-1). It has a very poor prognosis, and it has been generally accepted that conventional chemotherapeutic agents alone, even including zidovudine/IFN-α, yield few or no long-term remissions or potential cures in patients with ATL [1-6]. Although early experience in myeloablative chemoradiotherapy together with autologous hematopoietic cell rescue for ATL has been associated with high incidences of relapse and fatal toxicities [7], allogeneic hematopoietic cell transplantation (HCT) has been explored as a promising alternative treatment that can provide long-term remission in a proportion of patients with ATL [8-10].

We previously performed a nationwide retrospective study of patients with ATL who underwent allogeneic HCT in Japan, with special emphasis on the effect of the graft source. We concluded that allogeneic HCT using currently available sources is an effective treatment in selected patients with ATL, but that the use of unrelated cord blood as a stem cell source is associated with lower survival [11]. Our results suggest that allogeneic bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT) could be considered the more standard transplantation forms compared with unrelated cord blood transplantation (CBT) for ATL.

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As the next step, we conducted a nationwide retrospective study of patients with ATL who underwent allogeneic HCT other than CBT, with special emphasis on the effects of the preconditioning regimen, whether conventional myeloablative conditioning (MAC) or reduced-intensity conditioning (RIC). No significant difference in overall survival (OS) was observed between patients receiving MAC and those receiving RIC, but a trend toward RIC contributing to better OS in older patients was noted. Thus, we conclude that allogeneic HCT not only with MAC, but also with RIC, is an effective treatment resulting in long-term survival in selected patients with ATL [12].

ATL has a long latency and occurs in older individuals at a median age of nearly 66 years. The median age at diagnosis of ATL has been increasing over the last few decades [13]. Accordingly, the proportion of patients with ATL undergoing HCT with RIC is currently increasing in relation to HCT with MAC. It is thought that compared with HCT with MAC, allogeneic HCT with RIC depends more on donor cellular immune effects after transplantation and less on the cytotoxic effects of the conditioning regimen to eradicate residual tumor cells. In this context, RIC might be suitable for ATL, given that several reports have indicated the high immunogenicity of ATL cells [14-18] and even the existence of graft-versus-HTLV-1 and/or graft-versus-ATL effects [19-21].

Although we previously reported the impact of post-transplantation immune reactions, graft-versus-host disease (GVHD), on outcomes in patients with ATL [21], our cohort included CBT recipients whose OS curve had a quite different trajectory from that of BMT and PBSCT recipients [12]. Thus, in the present study, we evaluated whether acute and chronic GVHD affect outcomes in patients with ATL undergoing allogeneic HCT other than unrelated CBT, with special emphasis on the effects of the preconditioning regimen. Our present analysis included the previous cohort (1996 to 2005) [21] with updated clinical information, as well as data on 1 patient who underwent allogeneic HCT in 1995 and patients who underwent allogeneic HCT between 2006 and 2010.

PATIENTS AND METHODS

Data Collection

Data on patients with ATL who had undergone a first allogeneic BMT, PBSCT, or BMT + PBSCT were collected from nationwide survey data of the Japan Society for Hematopoietic Cell Transplantation (JSHCT). Cases with missing preconditioning information, acute GVHD, or survival data were excluded, leaving 679 patients. Because the association between the occurrence of acute GVHD and disease-associated mortality was difficult to evaluate in the event of early toxic death, patients who died within 30 days or were censored within 29 days of transplantation (n = 63) were excluded; thus, 616 patients who underwent HCT between March 1995 and December 2010 were included in our analysis.

Data collected for analysis included clinical characteristics, such as age at HCT, sex, disease status at HCT, date of HCT, time from diagnosis of ATL to HCT, performance status (PS) according to the Eastern Cooperative Oncology Group criteria at transplantation, stem cell source, donor–recipient relationship, ATL clinical subtype [22], preconditioning regimen, type of GVHD prophylaxis, date alive at last follow-up, date and cause of death, date of occurrence of acute GVHD and maximum grade of acute GVHD, and grade and date of occurrence of chronic GVHD. The study was approved by the Data Management Committees of the JSHCT, as well as by the Institutional Ethics Committee of Nagoya City University Graduate School of Medical Sciences.

Definitions

OS was defined as the time from HCT until death, and patients who remained alive at the time of the last follow-up were censored. Reported causes of death were reviewed and categorized into ATL-related mortality or treatment-related mortality (TRM). ATL-related mortality was defined as death caused by relapse or progression of ATL based on the judgment of each institution. TRM was defined as any death other than ATL-related mortality.

Table 1
Patient and Transplantation Characteristics by Type of Conditioning Regimen

Characteristic	MAC	RIC	*P* Value
Total patients, n (%)	284 (46.1)	332 (53.9)	
Age at HCT, y, n (%)			
<50	178 (62.7)	43 (13.0)	<.0001
51-55	79 (27.8)	91 (27.4)	
56-60	20 (7.0)	125 (37.7)	
61+	7 (2.5)	73 (22.0)	
Sex, n (%)			
Male	159 (56.0)	160 (48.2)	.0628
Female	125 (44.0)	172 (51.8)	
Disease status at HCT, n (%)			
CR	104 (36.6)	128 (38.6)	.1013
Not in CR	161 (56.7)	194 (58.4)	
Unknown	19 (6.7)	10 (3.0)	
GVHD prophylaxis, n (%)			
CyA + MTX	129 (45.4)	112 (33.7)	<.0001
FK506 + MTX	142 (50.0)	147 (44.3)	
CyA	6 (2.1)	58 (17.5)	
FK506	5 (1.8)	13 (3.9)	
Unknown	2 (0.7)	2 (0.6)	
Stem cell source, n (%)			
BM	216 (76.1)	213 (64.2)	.0015
PBSCs	68 (23.9)	117 (35.2)	
BM + PBSCs	0 (0.0)	2 (0.6)	
Donor–recipient relationship, n (%)			
HLA-matched related	98 (34.5)	120 (36.1)	.3649
HLA-mismatched related	24 (8.5)	40 (12.0)	
Unrelated	160 (56.3)	171 (51.5)	
Unknown	2 (0.7)	1 (0.3)	
PS at HCT, n (%)			
0	111 (39.1)	144 (43.4)	.0012
1	127 (44.7)	154 (46.4)	
2	26 (9.2)	27 (8.1)	
3	3 (1.1)	5 (1.5)	
4	1 (0.4)	1 (0.3)	
Unknown	16 (5.6)	1 (0.3)	
ATL clinical subtype, n (%)			
Chronic/smoldering	11 (3.9)	10 (3.0)	.5278
Acute	171 (60.2)	189 (56.9)	
Lymphoma	80 (28.2)	97 (29.2)	
Unknown	22 (7.7)	36 (10.8)	
Time from diagnosis to HCT, d, n (%)			
16-153	82 (28.9)	72 (21.7)	.0632
154-204	64 (22.5)	88 (26.5)	
205-307	75 (26.4)	78 (23.5)	
308-4355	63 (22.2)	91 (27.4)	
Unknown	0 (0.0)	3 (0.9)	
Time of HCT, n (%)			
March 1995 to March 2005	75 (26.4)	79 (23.8)	.3119
April 2005 to May 2007	75 (26.4)	79 (23.8)	
June 2007 to February 2009	73 (25.7)	81 (24.4)	
March 2009 to December 2010	61 (21.5)	93 (28.0)	
Grade of acute GVHD, n (%)			
No acute GVHD	80 (28.2)	128 (38.6)	.0111
Grade I-II	148 (52.1)	159 (47.9)	
Grade III-IV	56 (19.7)	45 (13.6)	

Acute GVHD was diagnosed and graded using traditional criteria [23] by the physicians who performed HCT at each institution, as was chronic GVHD [24]. Among the 487 patients who survived at least 100 days after HCT, 431 patients with complete information on the grade and the day of occurrence of chronic GVHD were included in the analysis for chronic GVHD.

Patients undergoing allogeneic BMT or PBSCT were divided into 2 groups, MAC and RIC, based on the preconditioning regimen. MAC and RIC were defined according to Giralt et al. [25] and Bacigalupo et al. [26] with slight modifications. In the present study, MAC was defined as any regimen that includes (1) ≥5 Gy of total body irradiation (TBI) as a single fraction or ≥8 Gy fractionated, (2) busulfan >8 mg/kg orally or the i.v. equivalent, or (3) melphalan >140 mg/m^2. All other regimens were classified as RIC.

Statistical Analysis

Comparisons among the groups were performed using Fisher's exact test as appropriate for categorical variables. The probability of survival was

estimated by the Kaplan-Meier method. TRM and ATL-related mortality were estimated using cumulative incidence curves to accommodate the competing events ATL-related mortality for TRM and TRM for ATL-related mortality [27]. Semilandmark plots were used to illustrate the effects of GVHD on survival and the cumulative incidence of ATL-related mortality and TRM. This landmark method was used to exclude bias that might have arisen from including patients who died too early to develop GVHD in the group without GVHD [28,29]. For patients with acute or chronic GVHD, the probability of survival and the cumulative incidences of ATL-related mortality and TRM were plotted as functions of time from the onset of acute or chronic GVHD. Day 25, the median day of onset for acute GVHD (range, 6 to 166 days), was designated the landmark day for acute GVHD. Day 126, the median day of onset for chronic GVHD (range, 52 to 1203 days), was designated the landmark day for chronic GVHD.

Multivariate Cox proportional hazards regression models were used to evaluate variables potentially affecting OS, and Fine and Gray proportional subdistribution hazards models [30] were used to evaluate variables potentially affecting ATL-related mortality and TRM. In these regression models, the occurrence of acute and chronic GVHD was treated as a time-varying covariate [31]. In the analysis of acute GVHD, patients were assigned to the no acute GVHD group at the time of HCT and then transferred to the grade I-II acute GVHD group or to the grade III-IV acute GVHD group at the onset of acute GVHD. In the analysis of chronic GVHD, patients were assigned to the no chronic GVHD group at the time of HCT and then transferred to the limited chronic GVHD group or to the extensive chronic GVHD group at the onset of chronic GVHD. We also assessed the interaction between acute and chronic GVHD and the preconditioning regimen in the multivariate models.

The heterogeneities of the effects of grade I-II or III-IV acute GVHD on OS according to background transplantation characteristics were evaluated by forest plots stratified by variables included in the regression analyses. Results are expressed as hazard ratio (HR) with 95% confidence interval (CI). All tests were 2-sided, and a P value <.05 was considered to indicate statistical significance. All statistical analyses were performed by Kureha Special Laboratory (Tokyo, Japan) using SAS 9.3 (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

Among the 616 patients in the study cohort, 284 received MAC and the remaining 332 received RIC. Characteristics of these patients are summarized in Table 1. Compared with MAC recipients, significantly fewer RIC recipients belonged to the youngest age group (<50 years), and significantly more were in the 2 oldest age groups (56 to 60 and 61+ years). In addition, significantly fewer RIC recipients received cyclosporin A (CyA) + methotrexate (MTX), but significantly more received CyA without MTX. PBSCT was significantly more frequent in RIC recipients compared with MAC recipients. There was no significant difference between MAC and RIC recipients regarding PS distribution from 0 to 4, but an unknown PS was observed significantly more frequently in the MAC recipients. A significantly greater number of RIC recipients did not have acute GVHD, and significantly fewer had grade III-IV acute GVHD.

Effects of Acute GVHD on Survival

In the 208 patients with ATL and no acute GVHD, the unadjusted 1-year and 3-year probabilities of survival from the landmark day for acute GVHD were 45.4% (95% CI, 38.3 to 52.2%) and 37.3% (95% CI, 30.3 to 44.4%), respectively. The unadjusted 1-year and 3-year probabilities of survival from the onset of acute GVHD were 60.1% (54.2 to 65.5%) and 49.1% (43.0 to 55.0%), respectively, in the 307 patients with grade I-II acute GVHD and 36.4% (26.9 to 46.0%) and 21.7% (13.9 to 30.6%), respectively, in the 101 patients with grade III-IV acute GVHD (Figure 1A).

Forest plots revealed that the development of grade I-II acute GVHD was associated with longer OS compared with the absence of acute GVHD in patients with the following characteristics: age <56 years, either male or female, not in complete remission (CR), receiving FK506 + MTX,

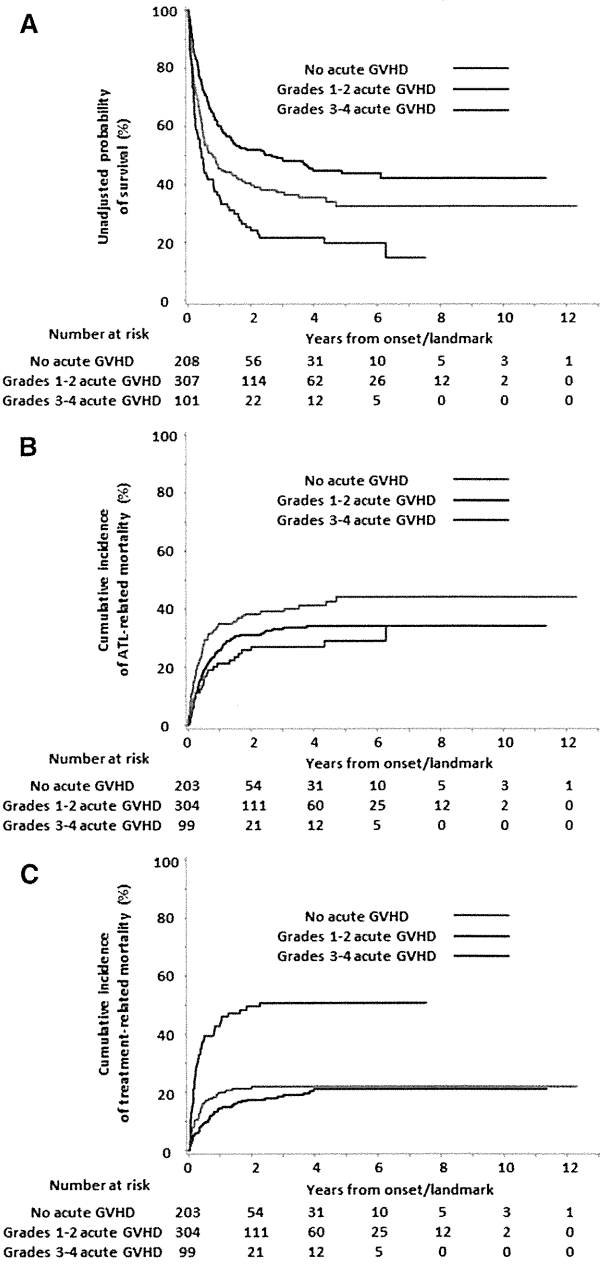

Figure 1. Semi-landmark plots illustrating the effects of acute GVHD on survival, ATL-related mortality, and TRM. (A) HR for survival in patients with grade I-II and grade III-IV acute GVHD compared with patients with no acute GVHD: 0.681 (95% CI, 0.537 to 0.863) versus 1.437 (95% CI, 1.082 to 1.910). (B) HR for ATL-related mortality in patients with grade I-II and grade III-IV acute GVHD compared with patients with no acute GVHD: 0.729 (95% CI, 0.540 to 0.984) versus 0.624 (95% CI, 0.403 to 0.967). (C) HR for TRM in patients with grade I-II and grade III-IV acute GVHD compared with patients with no acute GVHD: 0.824 (95% CI, 0.552 to 1.232) versus 2.793 (95% CI, 1.884 to 4.229).

undergoing either BMT or PBSCT, having an unrelated donor, PS 1 at transplantation, acute type of ATL, interval between ATL diagnosis and HCT of 308 to 4355 days, and date of HCT between June 2007 and February 2009. The development of grade I-II acute GVHD was also significantly associated with longer OS compared with the absence of acute GVHD regardless of whether the patient received MAC or RIC. On the other hand, this comparison revealed a shorter OS in the patients with lymphoma type ATL (Figure 2). These plots also revealed that the development of grade III-IV acute GVHD

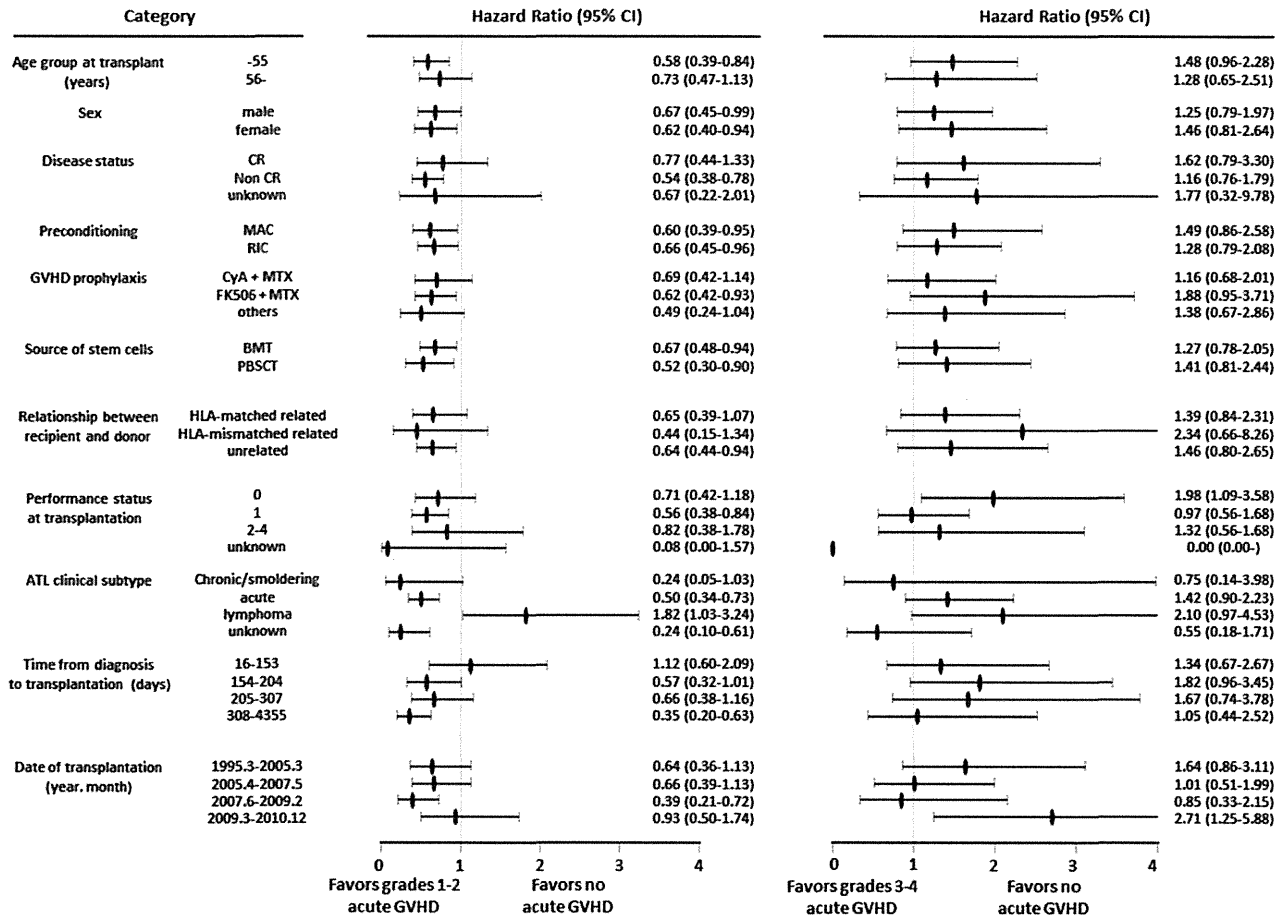

Figure 2. Impact of the grade of acute GVHD on OS in each stratified category. Effects of grade I-II (A) and grade III-IV acute GVHD (B) on OS are shown as forest plots. Closed ellipses on lines indicates HRs compared with the no acute GVHD group, and horizontal lines represent the corresponding 95% CI.

was significantly associated with shorter OS compared with the absence of acute GVHD in patients with PS 0 and who underwent HCT between March 2009 and December 2010. However, this comparison revealed no significant findings for OS according to whether the patient received MAC or RIC (Figure 2).

Multivariate analysis of the 616 study patients was performed to examine whether acute GVHD affects OS using the following variables: age (15 to 55 or 56 to 72 years), sex, disease status at HCT (CR, not CR, or unknown), preconditioning regimen (MAC or RIC), GVHD prophylaxis (CyA + MTX, FK506 + MTX, or other/unknown), relationship between recipient and donor (HLA-matched related, HLA-mismatched related, or unrelated), PS (0, 1, 2 to 4, or unknown), ATL clinical subtype (chronic/smoldering, acute, lymphoma, or unknown), time from diagnosis to HCT (16 to 153, 154 to 204, 205 to 307, or 308 to 4355 days or unknown), date of HCT (March 1995 to March 2005, April 2005 to May 2007, June 2007 to February 2009, or March 2009 to December 2010), and source of stem cells (bone marrow [BM], peripheral blood stem cells [PBSCs], or BM + PBSCs), as well as acute GVHD as a time-dependent covariate (no, grade I-II, or grade III-IV). There was a significant positive impact of grade I-II acute GVHD on OS (HR, 0.634; 95% CI, 0.477 to 0.843) compared with no acute GVHD (Table 2).

To further investigate the clinical significance of acute GVHD for OS, we divided acute GVHD into 5 categories (none or grade I, II, III, or IV) and then performed multivariate analysis in the same manner as described above. HRs for OS of patients with grade I, II, III, and IV acute GVHD compared with the absence of acute GVHD were 0.568 (95% CI, 0.402 to 0.801), 0.688 (95% CI, 0.501 to 0.946), 1.199 (95% CI, 0.831 to 1.730), and 2.245 (95% CI, 1.354 to 3.722), respectively.

Interactions of the Preconditioning Regimen with Acute GVHD for OS

We tested statistical interactions between the preconditioning regimens and acute GVHD with regard to OS by adding an interaction term to the multivariate analysis. This analysis included the same variables as the multivariate Cox proportional hazards regression models for OS. Among the 616 patients, when the HR for death of MAC recipients with no acute GVHD was set as 1.000, the HRs in MAC recipients with grade I-II acute GVHD and in RIC recipients with no GVHD and with grade I-II acute GVHD were 0.659, 0.971, and 0.592, respectively ($P_{\text{interaction}} = .7962$), and the HRs in MAC and RIC recipients with grade III-IV acute GVHD were 1.343 and 1.387, respectively ($P_{\text{interaction}} = .7603$) (Figure 3A).

Effects of Acute GVHD on ATL-Related Mortality and TRM

Among the 616 patients receiving allogeneic BMT or PBSCT, 10 patients could not be assigned to either the ATL-related mortality or TRM category because of missing detailed information on the cause of death. The cumulative incidences of ATL-related mortality at 1 year and 3 years from the landmark day for acute GVHD were 35.0% (95% CI,

Table 2
Effect of Acute GVHD on OS, ATL-related Mortality, and TRM after Allogeneic HCT

Outcome	HR (95% CI)	*P* Value
OS*		
No acute GVHD	1.000	Reference
Grade I-II acute GVHD	0.634 (0.477-0.843)	.0017
Grade III-IV acute GVHD	1.380 (0.988-1.927)	.0590
ATL-related mortality†		
No acute GVHD	1.000	Reference
Grade I-II acute GVHD	0.833 (0.566-1.224)	.3511
Grade III-IV acute GVHD	0.599 (0.373-0.964)	.0347
TRM‡		
No acute GVHD	1.000	Reference
Grade I-II acute GVHD	0.645 (0.407-1.023)	.0624
Grade III-IV acute GVHD	2.474 (1.495-4.095)	.0004

* Other than acute GVHD, the following 4 variables significantly affected OS: older age (56 to 72 yr compared with 15 to 55 yr: HR, 1.356; 95% CI, 1.033 to 1.781), male sex (HR, 1.404; 95% CI, 1.127 to 1.750), not in CR compared with CR (HR, 1.877; 95% CI, 1.459 to 2.416), and worse PS (1 compared with 0: HR, 1.486; 85% CI, 1.168 to 1.889; 2 to 4 compared with 0: HR, 2.691; 95% CI, 1.918 to 3.777).

† Other than acute GVHD the following 2 variables significantly affected ATL-related mortality: not in CR compared with CR (HR, 2.633; 95% CI, 1.818 to 3.814) and worse PS (2 to 4 compared with 0: HR, 3.272; 95% CI, 2.100 to 5.099).

‡ Other than acute GVHD, the following 3 variables significantly affected TRM: older age (56 to 72 yr compared with 15 to 55 yr: HR, 1.663; 95% CI, 1.025 to 2.697), male sex (HR, 1.545; 95% CI, 1.078 to 2.214), and transplantation from an unrelated donor compared with an HLA-matched related donor (HR, 2.098; 95% CI, 1.131 to 3.895).

27.6% to 42.5%) and 39.2% (95% CI, 31.1% to 47.2%), respectively, in the 203 patients with no acute GVHD. Those of TRM were 20.0% (95% CI, 13.5% to 27.5%) and 22.0% (95% CI, 14.8% to 30.1%), respectively (Figure 1B and C). The cumulative incidences of ATL-related mortality at 1 year and 3 years from the onset of acute GVHD were 25.8% (95% CI, 20.5% to 31.4%) and 33.0% (95% CI, 26.9% to 39.3%), respectively, in the 304 patients with grade I-II acute GVHD, whereas those of TRM were 14.5% (95% CI, 10.1% to 19.6%) and 18.5% (95% CI, 13.0% to 24.6%), respectively (Figure 1B and C). In the 99 patients with grade III-IV acute GVHD, the cumulative incidences of ATL-related mortality at 1 year and 3 years from the onset of acute GVHD were 21.2% (95% CI, 10.8% to 33.8%) and 27.0% (95% CI, 14.2% to 41.5%), respectively, and those of TRM were 42.7% (95% CI, 31.8% to 53.3%) and 50.7% (95% CI, 38.6% to 61.7%), respectively (Figure 1B and C).

We next applied the Fine and Gray proportional hazards model to the 606 patients. The analysis included the same variables as in the multivariate Cox proportional hazards regression models for OS. There were significant associations between grade III-IV acute GVHD and lower ATL-related mortality (HR, 0.599; 95% CI, 0.373 to 0.964) and higher TRM (HR, 2.474; 95% CI, 1.495 to 0.964) compared with no acute GVHD (Table 2).

In investigating the clinical significance of acute GVHD for ATL-related mortality or TRM, we divided acute GVHD into 5 categories (none and grade I, II, III, and IV) and conducted the analysis in the same manner as described above. HRs for ATL-related mortality in patients with grade I, II, III, and IV acute GVHD compared with the absence of acute GVHD were 0.809 (95% CI, 0.517 to 1.268), 0.857 (95% CI, 0.558 to 1.315), 0.585 (95% CI, 0.347 to 986), and 0.654 (95% CI, 0.298 to 1.435), respectively. HRs for TRM in patients with grade I, II, III, and IV acute GVHD compared with the absence of acute GVHD were 0.519 (95% CI, 0.282 to 0.955), 0.747 (95% CI, 0.455 to 1.227), 2.153 (95% CI, 1.267 to 3.659), and 4.114 (95% CI, 2.033 to 8.326), respectively.

Effects of Chronic GVHD on Survival

Among the 431 patients evaluable for chronic GVHD, 199 received MAC and 232 received RIC. In the MAC group, limited and extensive chronic GVHD occurred in 26 (13.1%) and 67 patients (33.7%), respectively, and in the RIC group, limited and extensive chronic GVHD occurred in 35 (15.1%) and 65 patients (28.0%), respectively. Regarding the incidence and grade of chronic GVHD, there were no significant differences between MAC and RIC recipients. In the 214 patients with no chronic GVHD, the unadjusted 1-year and 3-year probabilities of survival from the landmark day for chronic GVHD were 58.7% (95% CI, 51.6 to 65.1%) and 51.0% (95% CI, 43.6 to 57.9%), respectively,. Those probabilities from the onset of chronic GVHD were 77.4% (64.2 to 86.2%) and 61.7% (46.7 to 73.6%), respectively, in the 60 patients with limited chronic GVHD and were 70.4% (61.7 to 77.5%) and 55.1% (45.5 to 63.7%), respectively, in the 132 patients with extensive chronic GVHD. Twenty-five patients were excluded from this semilandmark plot because they were censored or died before the landmark day for chronic GVHD (Figure 4A).

We performed a multivariate analysis of data on 431 patients to examine whether chronic GVHD affects OS using the following variables: age, sex, disease status, preconditioning regimen, GVHD prophylaxis, donor–recipient relationship, PS, ATL clinical subtype, time from diagnosis to

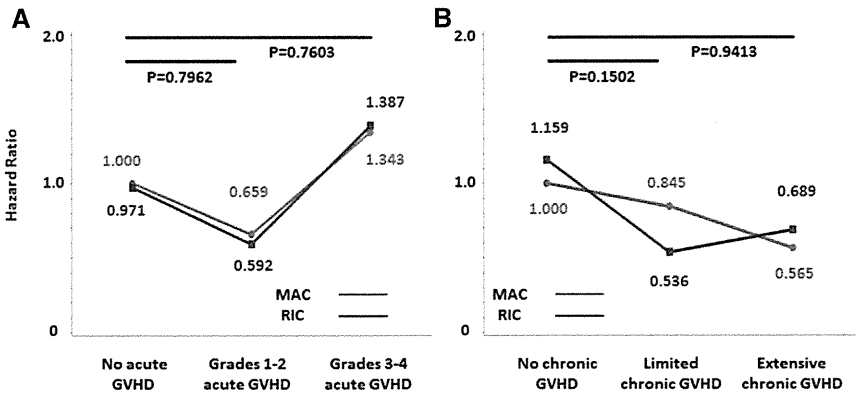

Figure 3. Interactions of the preconditioning regimen with acute GVHD for overall survival. Statistical interactions between the preconditioning regimens (MAC or RIC) and acute GVHD (absent versus grade I-II or grade II-IV; A) and chronic GVHD (absent versus limited or extensive type; B) for overall survival were analyzed.

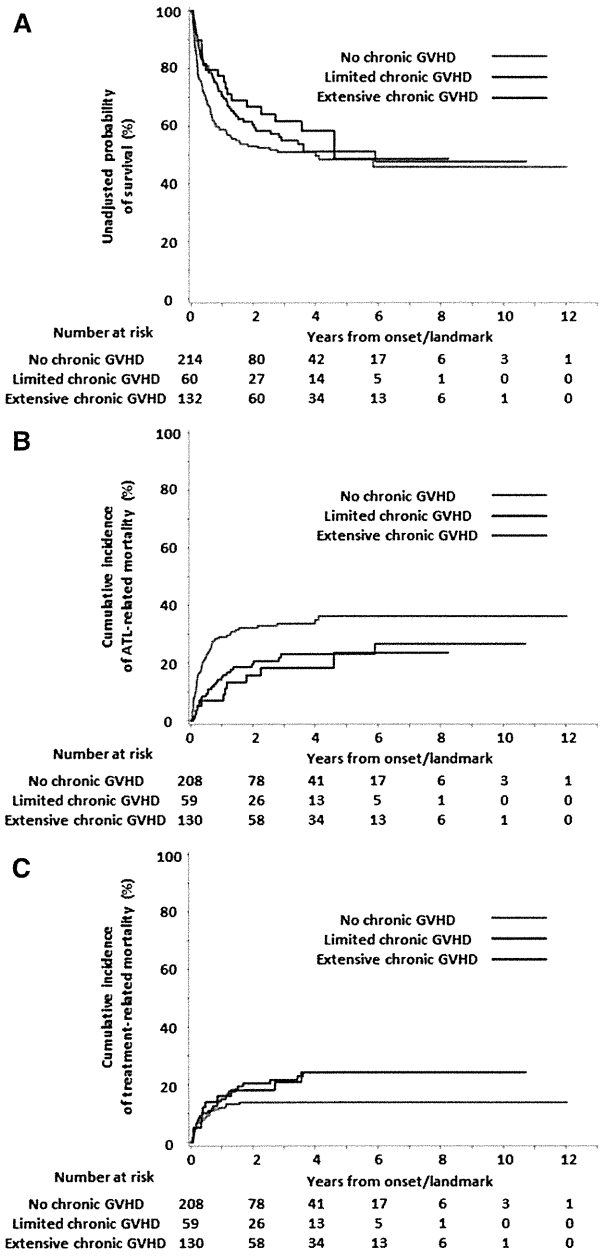

Figure 4. Semilandmark plots illustrating the effects of chronic GVHD on survival, ATL-related mortality, and TRM. (A) HR for survival in patients with limited and extensive chronic GVHD compared with patients with no chronic GVHD: 0.719 (95% CI, 0.457 to 1.131) versus 0.796 (95% CI, 0.576 to 1.100). (B) HR for ATL-related mortality in patients with limited and extensive chronic GVHD compared with patients with no chronic GVHD: 0.461 (95% CI, 0.237 to 0.897) versus 0.570 (95% CI, 0.366 to 0.886). (C) HR for TRM in patients with limited and extensive chronic GVHD compared with patients with no chronic GVHD: 1.609 (95% CI, 0.815 to 3.175) versus 1.620 (95% CI, 0.955 to 2.749).

HCT, date of HCT, and stem cell source, as well as chronic GVHD as a time-dependent covariate. We found a significant positive impact of extensive chronic GVHD on OS compared with no chronic GVHD (HR, 0.585; 95% CI, 0.389 to 0.880) (Table 3).

Interactions of the Preconditioning Regimen with Chronic GVHD for OS

We tested the statistical interactions between the preconditioning regimens and chronic GVHD for OS by adding an interaction term into the multivariate analysis. The analysis included the same variables as the multivariate Cox proportional hazards regression models for OS with chronic GVHD. Among the 431 patients, when the HR for death of MAC recipients with no chronic GVHD was set as 1.000, the HRs in MAC recipients with limited chronic GVHD and RIC recipients with no GVHD and limited chronic GVHD were 0.845, 1.159, and 0.536, respectively ($P_{interaction}$ = .1502), and the HRs in MAC and RIC recipients with extensive chronic GVHD were 0.565 and 0.689, respectively ($P_{interaction}$ = .9413) (Figure 3B).

Table 3
Effect of Chronic GVHD on OS, ATL-related Mortality, and TRM after Allogeneic HCT

Outcome	HR (95% CI)	*P* Value
OS*		
No chronic GVHD	1.000	Reference
Limited chronic GVHD	0.597 (0.354-1.007)	.0533
Extensive chronic GVHD	0.585 (0.389-0.880)	.0100
ATL-related mortality†		
No chronic GVHD	1.000	Reference
Limited chronic GVHD	0.395 (0.184-0.847)	.0170
Extensive chronic GVHD	0.421 (0.240-0.740)	.0026
TRM‡		
No chronic GVHD	1.000	Reference
Limited chronic GVHD	1.549 (0.704-3.409)	.2767
Extensive chronic GVHD	1.204 (0.659-2.201)	.5462

* Other than chronic GVHD, the following 3 variables significantly affected OS: male sex (HR, 1.480; 95% CI, 1.103 to 1.986), not in CR compared with CR (HR, 1.629; 95% CI, 1.171 to 2.266), worse PS (1 compared with 0: HR, 1.446; 95% CI, 1.057 to 1.980, 2 to 4 compared with 0: HR, 2.828; 95% CI, 1.751 to 4.568).

† Other than chronic GVHD, the following 2 variables significantly affected ATL-related mortality: not in CR compared with CR (HR, 2.499; 95% CI, 1.563 to 3.994), worse performance score (1 compared with 0: HR, 1.524; 95% CI, 1.013 to 2.294, 2 to 4 compared with 0: HR, 2.383; 95% CI, 1.216 to 4.669).

‡ The following 4 variables significantly affected TRM: older age (56 to 72 yr compared with 15 to 55 yr: HR, 2.022; 95% CI, 1.045 to 3.913), male sex (HR, 2.254; 95% CI1.322 to 3.844), worse performance score (2 to 4 compared with 0: HR, 3.127; 95% CI, 1.260 to 7.762), and ATL clinical subtype (acute compared with chronic/smoldering type: HR, 0.288; 95% CI, 0.093 to 0.897; lymphoma compared with chronic/smoldering type: HR, 0.249; 95% CI, 0.078 to 0.794).

Effects of Chronic GVHD on ATL-Related Mortality and TRM

Among the 406 patients analyzed by a semilandmark plot for survival, 9 could not be assigned to either the ATL-related mortality or TRM category. The cumulative incidences of ATL-related mortality at 1 year and 3 years from the landmark day for chronic GVHD were 29.0% (95% CI, 22.4% to 35.8%) and 33.7% (95% CI, 26.4% to 41.1%), respectively, in the 208 patients with no chronic GVHD, whereas those of TRM were 12.0% (95% CI, 7.3% to 18.1%) and 13.9% (95% CI, 8.5% to 20.7%), respectively (Figure 4B and C). In the 59 patients with limited chronic GVHD, the cumulative incidences of ATL-related mortality at 1 year and 3 years from the onset of chronic GVHD were 7.0% (95% CI, 2.1% to 16.1%) and 18.3% (95% CI, 7.7% to 32.4%), respectively, and those of TRM were 16.0% (95% CI, 7.5% to 27.4%) and 20.8% (95% CI, 10.1% to 34.1%), respectively (Figure 4B and C). In the 130 patients with extensive chronic GVHD, the cumulative incidences of ATL-related mortalities at 1 year and 3 years from the onset of chronic GVHD were 15.1% (95% CI, 9.0% to 22.6%) and 23.1% (95% CI, 14.7% to 32.6%), respectively, and those of TRM were 15.0% (95% CI, 9.1% to 22.3%) and 21.6% (95% CI, 13.9% to 30.5%), respectively (Figure 4B and C).

We next applied the Fine and Gray proportional hazards model to the 422 patients evaluable for chronic GVHD who could be assigned to either the ATL-related mortality or the TRM category. The analysis included the same variables as the multivariate Cox proportional hazards regression models for OS. Chronic GVHD was significantly associated with reduced ATL-related mortality. HRs for recipients with limited and extensive chronic GVHD compared with the absence of chronic GVHD were 0.395 (95% CI, 0.184 to 0.847) and 0.421 (95% CI, 0.240 to 0.740), respectively (Table 3). On the other hand, chronic GVHD was not significantly associated with TRM.

DISCUSSION

To the best of our knowledge, this is the largest retrospective study reported to date analyzing the impact of acute and chronic GVHD on clinical outcomes in ATL. As shown in Table 1, the associations with no acute GVHD and without grade III-IV acute GVHD were significant in RIC recipients compared with MAC recipients. Those findings are consistent with reports of an association between dose-intensified conditioning, especially regimens including TBI, and acute GVHD [32,33]. Our results also show no significant difference in the occurrence of chronic GVHD between MAC and RIC recipients. This may be because the effects of older age and more frequent PBSCT, which increase the occurrence of chronic GVHD, were counterbalanced by the lower frequency of history of previous acute GVHD, which reduces the incidence of chronic GVHD, in the RIC recipients [32,34].

Forest plots revealed that the development of grade I-II acute GVHD was associated with favorable OS compared with the absence of acute GVHD in most categories, with the exception of lymphoma in the ATL clinical subtype category. The reason for this exception is unclear, however. Our forest plots also show that the occurrence of grade III-IV acute GVHD was associated with unfavorable OS in most categories.

The significant positive impact of grade I-II acute GVHD on OS identified by multivariate analysis confirmed the results presented in our previous report [21]. However, in the present study, we found that grade I-II acute GVHD had no significant association with ATL-related mortality, in disagreement with our previous report showing a significant association between grade I-II acute GVHD and decreased ATL-related mortality in ATL patients undergoing allogeneic HCT [21]. We surmise that the incompatibility might stem from 2 factors, the influence of unrelated CBT, which was included in the previous study [21], and the progress in transplantation-related medicine from 2006 onward. The clear trend of decreased TRM in patients with grade I-II acute GVHD observed here seems a bit puzzling, but we have no suitable explanation. With respect to preconditioning, there were no significant interactions between MAC and RIC for OS even when post-transplantation acute GVHD was absent or present at grade I-II or III-IV.

Our multivariate analysis revealed a clear trend toward a favorable OS with limited chronic GVHD and a significant association with lower ATL-related mortality. These findings are consistent with previous reports by our group [20] and others [35]. The latter report included a variety of hematologic diseases. Even though our univariate analyses revealed a trend toward better survival (but without significance) in patients with extensive chronic GVHD in the semilandmark plots (Figure 4A), our multivariate analysis demonstrated that a significant association between extensive chronic GVHD and a favorable OS. This finding is in disagreement with our previous report [21] and another study demonstrating a negative impact of extensive chronic GVHD on OS [35]. Extensive chronic GVHD had a significant association with lower ATL-related mortality, but not with TRM. The former finding was reasonable and expected, but the latter was not consistent with our previous report demonstrating significant associations between extensive chronic GVHD and greater TRM [21]. Although the present study found a significant association between extensive chronic GVHD and favorable OS in the patients with ATL, we also must pay special attention to the fact that quality of life after HCT is highly compromised by chronic GVHD [36]. With respect to preconditioning, there were also no significant interactions between MAC and RIC with OS even when chronic GVHD was absent, limited, or extensive.

Several promising new agents for treating ATL are currently under development [37-40]. These novel treatments should increase the number of patients with a sufficient disease control status and who have maintained a good PS who could become suitable candidates for HCT [12]. These agents will also contribute to the establishment of better rescue strategies for patients relapsing after HCT [41]. Among the novel agents, we should pay special attention to mogamulizumab (humanized anti-CCR4 monoclonal antibody) [42], which was approved for the treatment of ATL in Japan in 2012, because of its potent activity that depletes regulatory T (Treg) cells, leading to enhanced antitumor activity [38,43,44]. The occurrence and severity of GVHD are closely associated with low Treg frequency [45]; thus, a decrease in Treg cells caused by mogamulizumab not only may lead to enhanced GVHD, but also may provoke an anti-HTLV-1/ATL immune effect.

Although this study reports significant novel findings on GVHD in patients with ATL, it also has inherent limitations common to observational retrospective studies. First, eligibility for HCT as well as choice of transplantation protocol, including the selection of MAC or RIC, were determined by physicians at each institution. Second, regarding analysis of mortality, it was not always easy to determine whether death after allogeneic HCT was an ATL-related mortality or TRM, in part because patients with relapsed ATL sometimes achieve partial or complete remission after decreasing or discontinuing immunosuppressive agents, donor lymphocyte infusions, or chemotherapy, which can result in long-term remission and survival [20]. Third, acute GVHD is occasionally induced in some patients considered at high risk for relapse by treating clinicians. Finally, the evaluation of chronic GVHD according to the 2005 National Institutes of Health consensus criteria [46] is not possible in this study, which was based on nationwide survey data of the JSHCT.

In conclusion, we found that the development of mild to moderate (grade I-II) acute GVHD was significantly associated with favorable OS, as was the development of both limited and extensive chronic GVHD. Regarding preconditioning, we found no difference in the clinical impact of acute GVHD and chronic GVHD on OS between patients receiving MAC and those receiving RIC. These findings confirm the actual existence of graft-versus-HTLV-1 and/or graft-versus-ATL effects in recipients of HCT for ATL regardless of whether MAC or RIC was used. New strategies that enhance the post- transplantation allogeneic anti–HTLV-1 effect targeting HTLV-1–associated antigens, such as Tax and/or HBZ [14-17], and/or the anti-ATL effect targeting tumor-specific antigens, such as cancer testis antigens [18],

which do not provoke GVHD, lead to improved outcomes in patients undergoing allogeneic HCT for ATL.

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Authorship statement: T.I., M.H., K.K., R.T., and A.U. designed the research, organized the project, and wrote the manuscript. T.I. helped with statistical analysis. H.S. and R.S. collected data from the JSHCT, and Y.M. collected data from the JMDP. All authors interpreted data and reviewed and approved the final manuscript.

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LETTER TO THE EDITOR

Successful salvage therapy using lenalidomide in a patient with relapsed multiple myeloma after allogeneic hematopoietic stem cell transplantation

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A 34-year-old Japanese man was diagnosed in 2008 as having stage III symptomatic multiple myeloma (IgD-lambda type) as defined by the Durie Salmon and International staging system. Chromosomal analysis revealed a normal karyotype, although overexpression of Cyclin D1 was detected by reverse-transcription/real-time polymerase chain reaction assay. After three cycles of induction chemotherapy with vincristine, doxorubicin, and dexamethasone, he received high-dose melphalan therapy with adjunct autologous peripheral blood stem cell transplantation (PBSCT). A very good partial response (VGPR) was achieved, although he relapsed at 11 months after PBSCT. Four cycles of bortezomib and dexamethasone therapy were subsequently given, resulting in a second VGPR. In March 2010, he underwent allogeneic bone marrow transplantation (BMT) from an HLA-matched unrelated donor. The conditioning regimen consisted of fludarabine (30 mg/m^2/day on days −8 to −3) and intravenous busulfan (3.2 mg/kg/day on days −6 to −5) and total body irradiation (4 Gy on day −2). Prophylaxis for graft-versus-host disease (GVHD) included tacrolimus and short-term methotrexate. Neutrophil engraftment was observed on day 16 after BMT, and 92 % donor chimerism was confirmed in the bone marrow on day 32. He developed grade 1 skin GVHD on day 31, which resolved with topical corticosteroids. On day 120, as monoclonal IgD levels increased to 502 mg/dl, tacrolimus was reduced in dose to relieve immunosuppression. However, IgD levels continued to increase up to 968 mg/dl 2 weeks later. Lenalidomide (Len) (10 mg/day per-oral [PO] for 21 days of a 28-day cycle) was then given on day 154. No symptoms of GVHD developed, and tacrolimus was discontinued before the second cycle of Len began. The patient's IgD levels promptly decreased, and after the forth cycle of Len, the monoclonal protein was undetectable on immunofixation electrophoresis of serum and urine, which correlated with resolution of immunoparesis. On day 840, stringent complete remission was confirmed by serum-free light chain assay and bone marrow examination (Fig. 1).

As the patient relapsed just 4 months after allogeneic BMT, his myeloma cells were considered refractory to the prior treatment. However, Len alone was highly effective and has yielded a durable response for nearly 2 years without any serious adverse events. Currently, there is no standard regimen of Len as salvage treatment for patients with relapsed myeloma after allogeneic hematopoietic stem cell transplantation (HSCT) [1]. Len has direct anti-tumor and anti-angiogenic properties in addition to immunomodulatory effects including enhanced T cell and NK-cell activation [2], which could be clinically useful to enhance graft-versus-myeloma (GVM) effects.

The results of Len treatment for multiple myeloma using salvage or maintenance treatment following allogeneic HSCT are summarized in Table 1. Lioznov et al. retrospectively investigated the efficacy and toxicity of Len (25 mg/day or 15 mg/day PO on day 1 to 21 of a 28-day cycle) in 24 heavily pretreated patients who relapsed after allogeneic HSCT. They reported an overall response rate of 66 %, a median time to progression of 9.7 months and 13 % of patients developed mild grade 1–2 GVHD [3]. More recently, Coman et al. also reported the efficacy and tolerance of Len in 52 relapsed multiple myeloma patients after allogeneic HSCT. Most of these patients were treated

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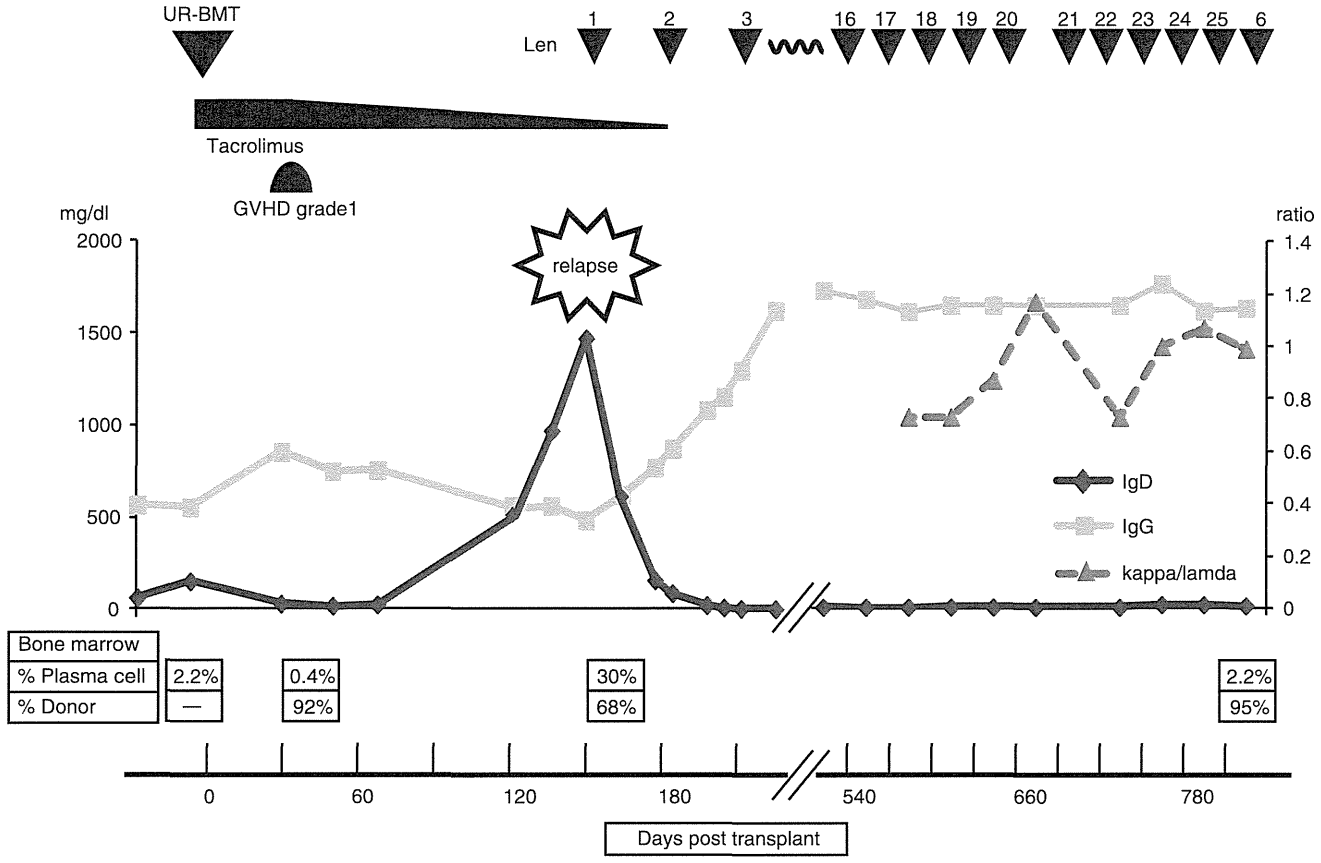

Fig. 1 Clinical course after allogeneic bone marrow transplantation. *UR-BMT* unrelated bone marrow transplantation, *Len* lenalidomaide 10 mg/day PO on days 1–21 of a 28-day cycle, *GVHD* graft-versus-host disease

using a reduced intensity conditioning regimen and peripheral blood stem cells from HLA-matched sibling donor. In approximately 40 % of the transplants, in vivo T-cell depletion was conducted. Salvage treatment composed mainly of Len (25 mg/day) combined with dexamethasone (40 mg/day). With the median follow-up time of 16.3 months, the median progression-free survival (PFS) and overall survival were 18.0 and 30.5 months, respectively. In some cases, Len was discontinued due to the development of progressive diseases (44 %) and GVHD (22 %) [4]. As compared with the results of these two retrospective studies of salvage Len treatment, our case has an interesting clinical course that a relatively low dose of Len alone induced long-term complete remission even in a refractory myeloma patient.

The role of Len as maintenance treatment after allogeneic HSCT is controversial. The HOVON group investigated Len (10 mg/day PO on days 1–21 of a 28-day cycle) as maintenance treatment after allogeneic HSCT from an HLA-matched sibling donor in a prospective phase 2 study. Patients who developed acute GVHD of grade 2 or higher at the time of registration were excluded from the study. Because 37 % of the patients developed GVHD of grade 2 or higher, which was thought to correlate strongly with Len treatment, and the dropout rate was 43 %, it was concluded that Len was not feasible as maintenance therapy after allogeneic HSCT [5]. However, Kroger et al. reported that Len (median dose of 5 mg/day PO on days 1–21 of a 28-day cycle) is feasible as maintenance therapy after non-myeloablative allogeneic HSCT. Acute GVHD (grade 2–3) rate after Len administration and dropout rates due to toxicity were 28 and 30 %, respectively. Seven patients with partial responses and one patient with stable disease after transplantation achieved complete response after Len treatment and 3-year PFS was 52 % [6]. It is difficult to simply compare the results of these studies due to the different conditioning regimens used, GVHD prophylaxis, donor sources and start timing of Len treatment shown in Table 1.

Although in the current case report we used the same dose and schedule of Len monotherapy adopted by the HOVON group, long-term complete remission was obtained even in a patient with early relapsed myeloma after allogeneic HSCT. He did not experience any GVHD symptoms after Len, but we speculate that the GVM effects may have been enhanced by Len treatment. Further well-designed studies are warranted to evaluate optimal patient

Table 1 Summarized results of lenalidomide treatment for multiple myeloma using salvage or maintenance treatment following allogeneic transplantation

Author	Lioznov et al.	Coman et al.	Kneppers et al.	Kroger et al.
Study design	Retrospective	Retrospective	Prospective	Prospective
Total number of patient	$n = 24$	$n = 52$	$n = 30$	$n = 24$
Treatment setting of Len	Salvage	Salvage	Maintenance	Maintenance
Dose of Len	25 mg/day 83 %, 15 mg/day 17 %	25 mg/day 79 %, others 21 %	10 mg/day	Median 5 mg/day (range 5–15)
Combination of Dexa	NA	Yes (77 %)	No	No
Median start time of Len	29 months[a]	24 months	3 months	6 months
After allo-HSCT		(range 1.0–97.0)	(range 1.0–6.8)	(range 2.8–15.3)
Donor source	Related 50 %	Matched sibling 77 %	Matched sibling 100 %	Matched sibling 18 %
PBSC	NA	PBSC 87 %	NA	PBSC 100 %
Conditioning intensity	NA	RIC 85 %	RIC 100 % (TBI 2 Gy)	MAC 100 %
Other	NA	In vivo T cell depletion 44 %	Unmanipulated graft	ATG 100 %
	NA	16.3 months	22 months	19 months
Median follow-up time		(range 3.7–49.6)	(range 8–34)	(range 3–58)
Median cycles of Len	5 (range 2–17)	6 (range 0.2–23)	3 (range 1–24)	6 (range 1–30)
PFS	Median 9.7 months	median 18 months	2 year estimated 60 %	3 year estimated 52 %
OS	median 19.9 months	Median 30.5 months	2 year estimated 93 %	3 year estimated 79 %
GVHD after Len	grade 2, 16 %	grade 1–2, 20 %; grade 3–4, 12 %	grade 2–3, 37 %	grade 2–3, 28 %
Discontinuation of Len	NA	52 % (27/52)	86 % (26/30)	54 % (13/24)
Reasons of discontinuation				
Progressive disease	NA	44 % (12/27)	20 % (5/26)	46 % (6/13)
GVHD	NA	22 % (6/27)	50 % (13/26)	23 % (3/13)
Other	NA	34 % (9/27)	30 % (8/26)	31 % (4/13)

Len lenalidomide, *Dexa* dexamethasone, *HSCT* hematopoietic stem cell transplantation, *PBSC* peripheral blood stem cell, *PFS* progression free survival, *OS* overall survival, *GVHD* graft-versus-host disease, *NA* not available, *RIC* reduced intensity conditioning, *MAC* myeloablative conditioning, *TBI* total body irradiation, *ATG* anti-thymocyte globulin

[a] Data as follows: the median interval between allo-HSCT and relapse was 18 months (range 2–71), the median interval between relapse after allo-HSCT and lenalidomide was 11 months (range 1–56)

selection, dosing and start timing of Len, in addition to combination drugs to establish safer and highly effective regimens after allogeneic HSCT. In conclusion, Len can be an attractive choice for salvage treatment of relapsed myeloma after allogeneic HSCT.

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