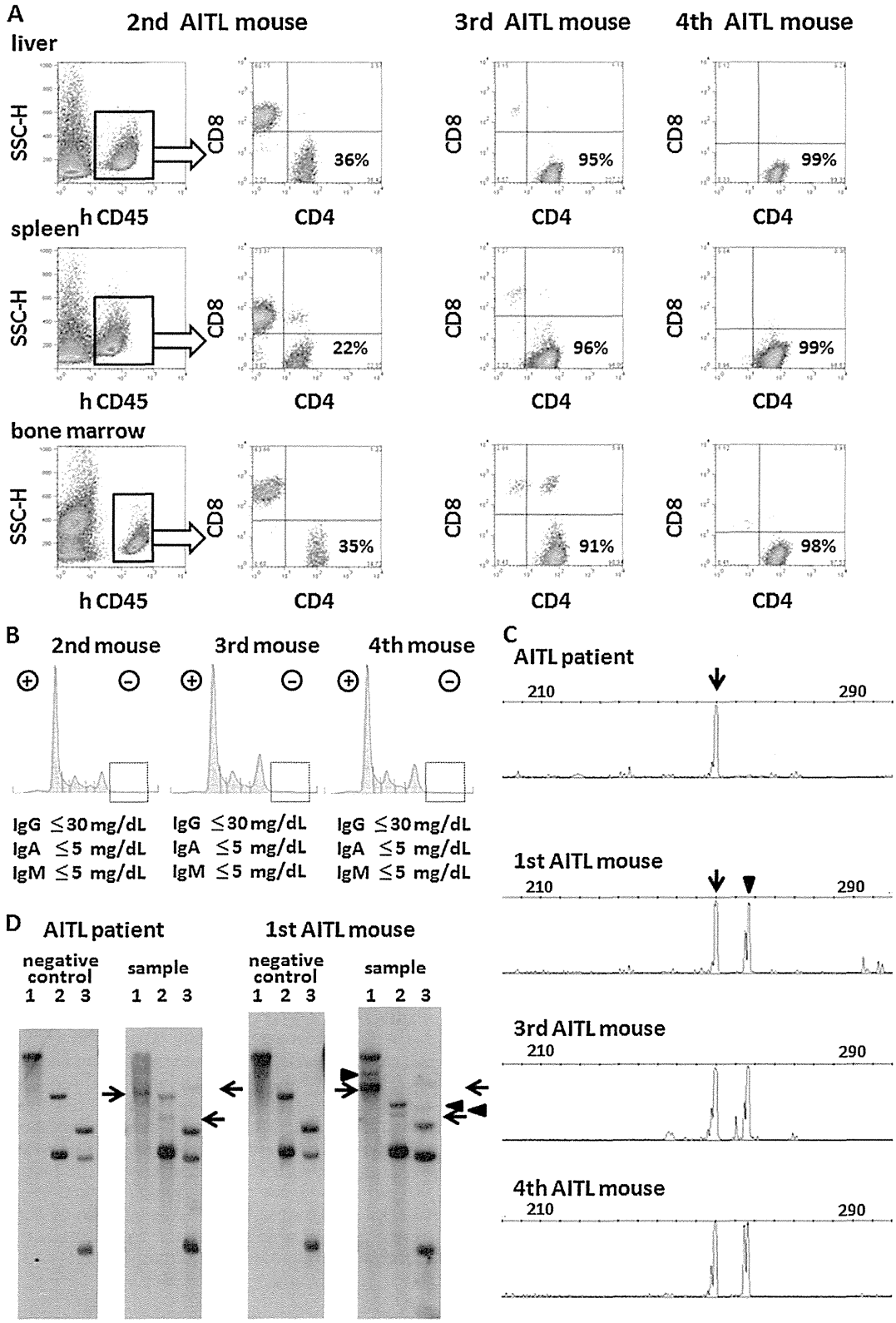

Fig. 4. Serial transplantations of spleen cells from AITL NOG mice. (A) The presence of human CD45-positive cells in the liver, spleen and bone marrow of the 2nd, 3rd, and 4th AITL NOG mice was determined by human CD4 and CD8 expression. (B) Serum protein fraction of 2nd, 3rd, and 4th AITL NOG mice. (C) Clonality analysis by PCR. Arrow and arrowhead indicate the clonal rearrangement of T cell receptor. (D) Clonality analysis by Southern blotting of T cell receptor Cβ1 gene. 1, 2, and 3 indicate BamH I, EcoR V, and Hind III, respectively. Arrow and arrowhead indicate the rearrangement band.

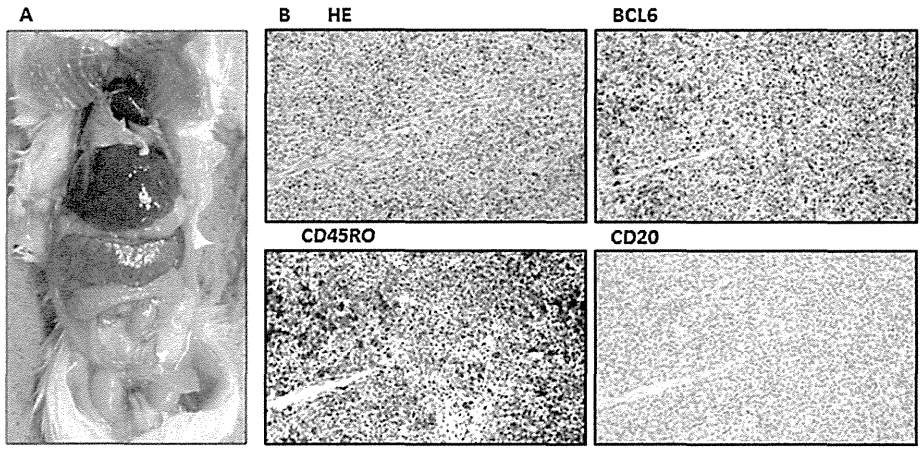

Fig. 5. Macroscopic and microscopic findings of 4th AITL mice. (A) Macroscopic image of a 4th AITL mouse. (B) Immunohistochemical images of the 4th AITL mouse spleen with hematoxylin and eosin staining, and staining by anti-BCL6, CD45RO, and CD20 antibodies.

vascular proliferation in the spleen. Most of the infiltrated cells were positive for CD45RO and BCL6. In contrast to the 1st AITL NOG mice, there were no CD20- (Fig. 5B) or CD138-positive reactive cells (data not shown), which were consistent with the results of flow cytometric analyses (Fig. 4A).

4. Discussion

The recent identification of $CD4^+$ TFH cell as the cell of origin of AITL provides a rationale to explain some of the clinical and histological features of AITL. A fundamental function of TFH cells is regulation of B cell-mediated humoral immunity. It has been known that in humanized NOG mice reconstituted with human $CD34^+$ hematopoietic stem cells, there was little IgG production because of the inappropriate differentiation of human B cells in the mouse environment [17–20]. Considering this fact, it was striking that the present AITL NOG mice produced polyclonal human Ig including IgG. This was direct evidence that $CD45RO^+BCL6^+$ AITL tumor cells functioned as TFH cells, and to the best of our knowledge, this is the first report to reconstitute TFH function in AITL cells in an experimental model either in vitro or in vivo. This could also explain one of the characteristic clinical features of AITL patients, hypergammaglobulinemia. In the AITL mice, human B cells were observed in the spleen and bone marrow, but not in blood, suggesting that antibody production mediated by T cells might need a suitable microenvironment like the germinal center of lymph nodes.

Serial transplantations of spleen cells of AITL NOG mice resulted in the reduction of reactive components such as B cell lineage and CD8-positive cells. CD4-positive AITL neoplastic cells can survive for a long period of time only by interacting with mouse environment cells. As a result, they failed to interact with human B or plasma cells, leading to the absence of human Ig production in the 2nd, 3rd, and 4th AITL NOG mice.

In general, not only monoclonal T cell receptor rearrangement, but also oligoclonal rearrangements were detected in AITL cases [1]. In the present study, although only one T cell clone (clone #1) was detected in an AITL patient 1, another T cell clone (clone #2) was also detected in the AITL NOG mice. We surmise that there were two neoplastic clones in the patient's affected lymph node, although the level of clone #2 was below the detectable limit. Because NOG mice have severe multiple immune dysfunctions, clone #2 was able to increase in the mice to a detectable level.

The immunohistological findings of the present AITL mice were almost identical to those of AITL patients; i.e., only a fraction of AITL neoplastic cells, which were small to medium-sized cells with clear cytoplasms and minimal cytologic atypia, were admixed with a reactive population of small lymphocytes including B and T cells, and plasma cells, and the spleen showed prominent vascularization. On the other hand, there was a lack of myeloid lineage cells such as eosinophils, histiocytes, and follicular dendritic cells, in the background inflammatory components, probably due to their fundamentally short life span. There was also a lack of EBV-positive B cells in the infiltrate in the present AITL mice, which could be explained by the fact that there was a lack of EBV-positive B cells in the background inflammatory components in the affected lymph node of both donors. In this type of analysis, attention should be paid to cross-reaction of antihuman antigens antibodies to mouse cells. The antihuman CD3, CD20, PD1, CD138, BCL6, CD45RO, immunoglobulin kappa and lambda light chain antibodies in the present study did not react with hematopoietic cells of mice origin (data not shown), probably due to the lack of mice T, B, and NK cells in NOG mice [7,8].

In conclusion, primary AITL tumor cells and reactive components engrafted NOG mice, and AITL cells interacted with B and plasma cells, and functioned as TFH cells. Human Igs including IgG were produced in the mice. The present observations strongly support the recent identification of TFH cell as the cell of origin of AITL. The present procedures using NOG mice would be a powerful tool to understand the immunopathogenesis of AITL.

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Conflicts of interest

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Authors' contributions. F.S., T.I., R.U., and H.I. designed the research. F.S., T.I., A.I., F.M., A.M., and H.T. performed the experiments. All of the authors analyzed and interpreted the data. F.S. and T.I. wrote the paper, and all of the other authors contributed to writing the paper.

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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

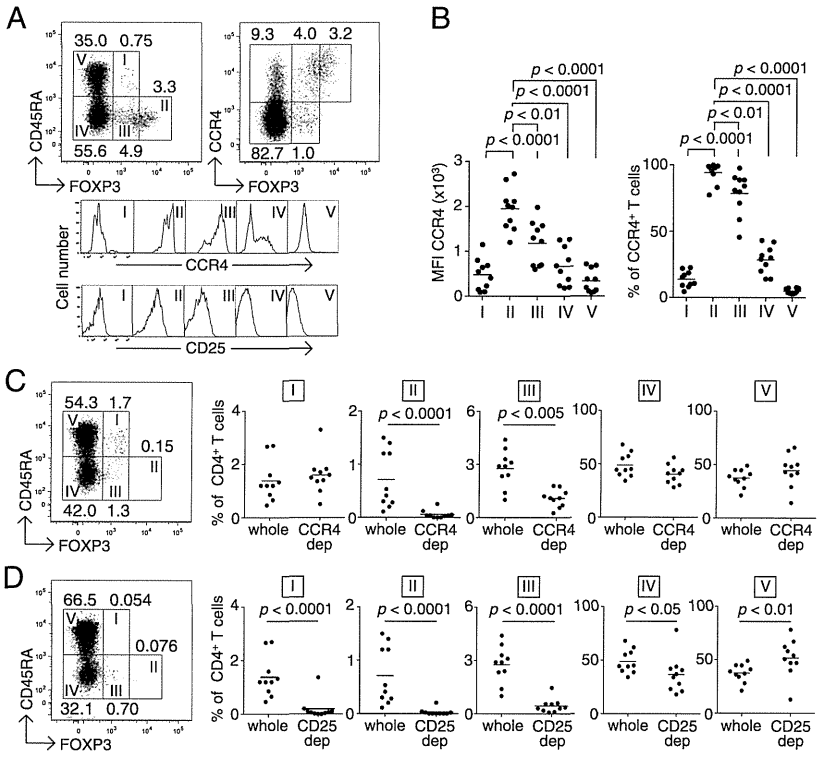

Fig. 1. Reduction of eTreg cells by in vitro depletion of CCR4-expressing T cells. (*A*) CCR4 and CD25 expression by subpopulations of FOXP3$^+$ Treg cells in PBMCs from healthy donors. CCR4 and CD25 expression levels were evaluated for each fraction. Representative data from 10 healthy donors are shown.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

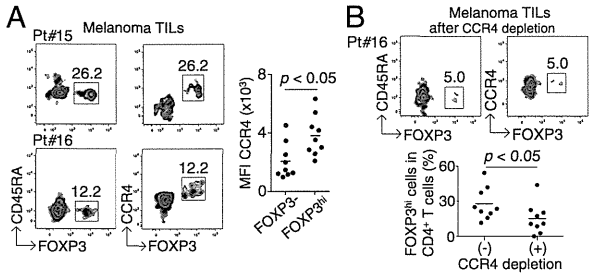

Fig. 2. Predominant infiltration of CCR4$^+$ eTreg cells into melanoma tissues. (*A*) CCR4 expression by melanoma-infiltrating T cells. CD4$^+$ T cells from melanoma sites were fractionated into subpopulations based on the expression of CCR4, CD45RA, and FOXP3; CCR4 expression by each fraction was analyzed.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

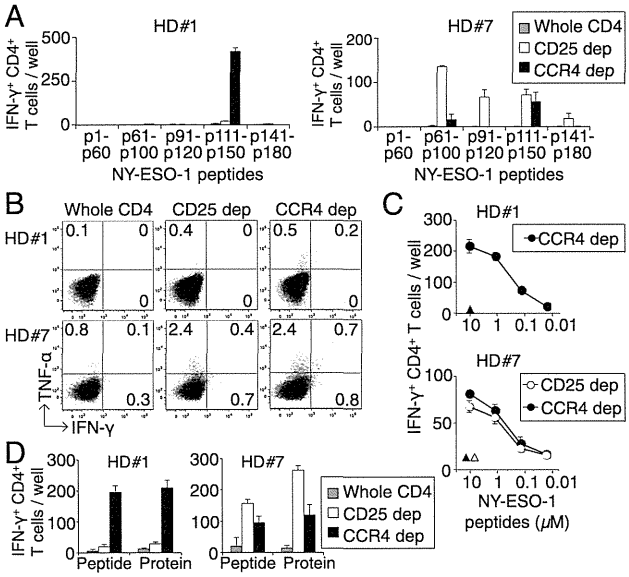

Fig. 3. Induction of cancer/testes antigen-specific CD4$^+$ T cells by depletion of CCR4- or CD25-expressing T cells in healthy donors. (*A*) CD4$^+$ T-cell responses to NY-ESO-1 peptides after depletion of CCR4$^+$ or CD25$^+$ T cells. CD4$^+$ T cells prepared from PBMCs of healthy donors were presensitized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

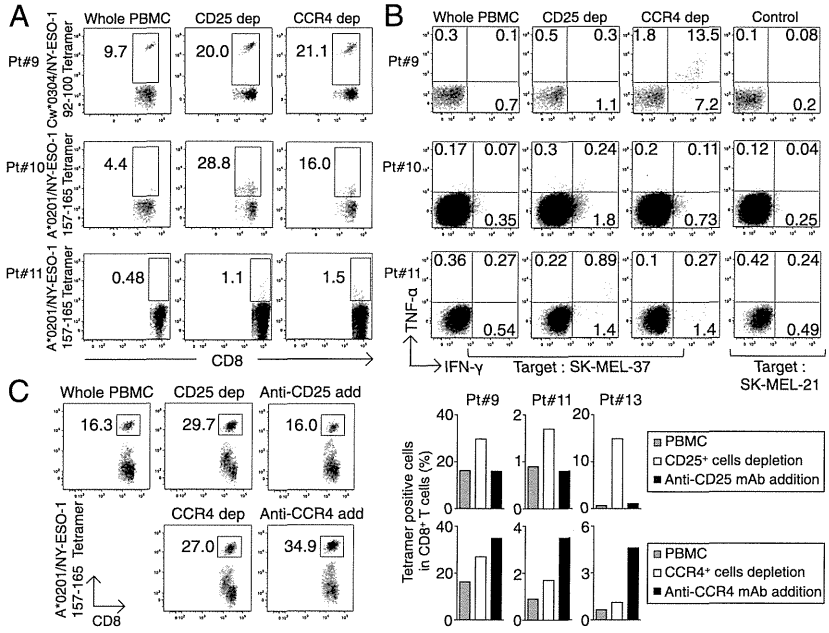

Fig. 4. Augmentation of NY-ESO-1–specific $CD8^+$ T-cell induction in melanoma patients by in vitro $CCR4^+$ T-cell depletion. (*A*) Induction of NY-ESO-1–specific $CD8^+$ T cells. Unfractionated PBMCs, or PBMCs depleted of $CD25^+$ or $CCR4^+$ cells, were prepared from melanoma patients (n = 6), and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

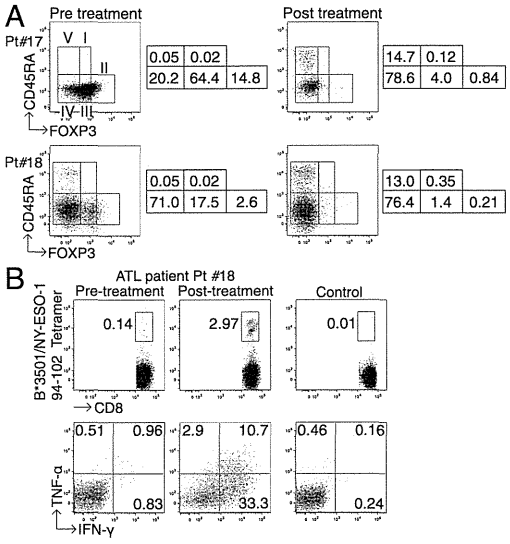

Fig. 5. Reduction of $CD4^+FOXP3^{hi}CD45RA^-$ T cells and augmentation of NY-ESO-1–specific $CD8^+$ T-cell responses in ATL patients after anti-CCR4 mAb (Mogamulizumab) therapy. (*A*) $FOXP3^+$ Treg-cell subpopulations in PBMCs from two ATL patients (Pt. #17: acute type, HLA-A*2402/-, B*3901/5401, C*0102/0702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

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Keywords: surgical treatment; detection marker; follow-up marker; recurrence; prognosis

NY-ESO-1 antibody as a novel tumour marker of gastric cancer

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Background: NY-ESO-1 antibodies are specifically observed in patients with NY-ESO-1-expressing tumours. We analysed whether the NY-ESO-1 humoral immune response is a useful tumour marker of gastric cancer.

Methods: Sera from 363 gastric cancer patients were screened by enzyme-linked immunosorbent assay (ELISA) to detect NY-ESO-1 antibodies. Serial serum samples were obtained from 25 NY-ESO-1 antibody-positive patients, including 16 patients with curative resection and 9 patients who received chemotherapy alone.

Results: NY-ESO-1 antibodies were detected in 3.4% of stage I, 4.4% of stage II, 25.3% of stage III, and 20.0% of stage IV patients. The frequency of antibody positivity increased with disease progression. When the NY-ESO-1 antibody was used in combination with carcinoembryonic antigen and CA19-9 to detect gastric cancer, information gains of 11.2% in stages III and IV, and 5.8% in all patients were observed. The NY-ESO-1 immune response levels of the patients without recurrence fell below the cutoff level after surgery. Two of the patients with recurrence displayed incomplete decreases. The nine patients who received chemotherapy alone continued to display NY-ESO-1 immune responses.

Conclusion: When combined with conventional tumour markers, the NY-ESO-1 humoral immune response could be a useful tumour marker for detecting advanced gastric cancer and inferring the post-treatment tumour load in seropositive patients.

Gastric cancer is the second most common cause of cancer-related death worldwide (Health and Welfare Statistics Association: Tokyo, 2006; Katanoda and Yako-Suketomo, 2009). Although complete removal of the tumour by surgical resection is an ideal treatment option for patients with gastric cancer, many patients with advanced-stage gastric cancer need to be treated with intensive chemotherapy. Gastric cancer patients exhibit high relapse rates even after curative surgery and unresponsiveness to chemotherapy, resulting in dismal survival rates (Sasako *et al*, 2011). Several methods for the prediction and early detection of subclinical 'minimal residual cancer' after surgery (Austrup *et al*, 2000; Klein *et al*, 2002) or relapse have been developed, for example, peritoneal lavage, positron emission tomography, gene profiling, and so on. (Motoori *et al*, 2006; Makino *et al*, 2010; Graziosi *et al*, 2011), reliable markers that can specifically reflect gastric cancer disease status have not been determined.

Analysing serum level of tumour markers is employed for cancer detection, monitoring patients' disease status, and prognosis prediction. Several organ-specific tumour markers are used in the clinic, for example, prostate-specific antigen and prostatic acid

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phosphatase for prostate cancer (Seamonds *et al*, 1986; Ferro *et al*, 1987) and protein induced by vitamin K absence-II for liver cancer (Fujiyama *et al*, 1986). As no gastric cancer-specific markers have been determined, a combination of several nonspecific tumour markers, for example, carcinoembryonic antigen (CEA), CA19-9,and so on, is merely applicable for monitoring treatment efficacy, but not the diagnosis of gastric cancer (Takahashi *et al*, 1995, 2003). Carcinoembryonic antigen and CA19-9 are found in the sera of 20–60% of gastric cancer patients, and their expression levels in gastric cancer are related to clinical events, such as relapse (Kodera *et al*, 1996). Carcinoembryonic antigen value, in particular, is indicative of the formation of a large tumour, liver or peritoneal metastasis, and/or a high risk of relapse and poor prognosis (Ikeda *et al*, 1993; Yamamoto *et al*, 2004). However, as CEA, a cell surface-anchored glycoprotein, is expressed in normal cell membranes, 5% of CEA-positive cases are pseudopositives, that is, caused by heavy smoking, endometriosis, and ageing, and so on. (Alexander *et al*, 1976), suggesting the importance of novel markers for gastric cancer.

NY-ESO-1 antigen, a cancer/testis (CT) antigen, was originally identified in oesophageal cancer by serological expression cloning using autologous patient serum and has been shown to be strongly immunogenic. Spontaneous NY-ESO-1 antibody production is often observed in patients with NY-ESO-1-expressing tumours, for example, 9.4% of melanoma patients, 12.5% of ovarian cancer patients, 7.7–26.5% of breast cancer patients, 4.2–20.0% of lung cancer patients, and 52% of prostate cancer patients, but has not been detected in non-cancerous donors (Stockert *et al*, 1998; Nakada *et al*, 2003; Türeci *et al*, 2006; Chapman *et al*, 2007; Isobe *et al*, 2009; Gati *et al*, 2011). Thus, it is possible that the NY-ESO-1 humoral immune response could be used as a serological marker for detecting these cancers and to facilitate the clinical management of some patients with particular types of cancer (Gnjatic *et al*, 2006). Jäger *et al* (1999) found that the change in the NY-ESO-1 humoral immune response reflected the overall tumour load in 10 out of 12 patients with various cancers. However, there is ongoing controversy regarding the association between the NY-ESO-1 immune response and prognostic criteria (Yuan *et al*, 2011). To address these issues in gastric cancer, we investigated the clinical usefulness of the NY-ESO-1 humoral immune response for diagnosis, monitoring, and relapse prediction in gastric cancer patients.

MATERIALS AND METHODS

Serum sample and tissue specimen collection from gastric cancer patients. In all, 363 patients with histologically confirmed gastric cancer, who underwent surgical resection or chemotherapy at one of four institutions between 2004 and 2011, were included in this study after providing written informed consent. Serum samples were obtained from the 363 patients during their admission to hospital for surgical treatment and/or chemotherapy, and afterwards, serial serum samples were obtained at each follow-up visit from 25 patients who displayed NY-ESO-1 humoral immune responses. All serum samples were collected as surplus samples after routine blood tests and stored. Fixed and frozen gastric cancer tissue samples were obtained from 60 out of 363 patients during surgery and stored. The samples were subsequently subjected to expression analysis. Information regarding blood test results, tumour stage, histological type, depth of invasion, lymph node metastasis, and distant metastasis, which were obtained from pathological examinations and CT scans, were collected from the relevant patient databases. Serum samples obtained from 50 healthy donors were used as controls. This study was approved by the institutional review boards of Osaka University Hospital, Toyonaka Municipal Hospital, Ikeda City Hospital, and Minoh City Hospital.

Reverse transcription–polymerase chain reaction. Total cellular RNA was extracted from the frozen tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The total RNA (1 μg) was subjected to the reverse transcription (RT) in 20 μl buffer with oligo-$(dT)_{15}$ primer using a RT system (Promega, Madison, WI, USA). Conventional polymerase chain reaction (PCR) was performed in a 25-μl reaction mixture containing 1 μl of cDNA template, 500 nM of each primer, and 1 U of *Taq* DNA polymerase (AmpliTaq Gold, Roche Molecular Systems, Pleasanton, CA, USA) in the following conditions: one cycle of 95 °C for 12 min; followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min; and then a final step of 72 °C for 10 min. The sequences of the primers for *NY-ESO-1* were as follows: ESO1-1, 5′-AGTTC TACCTCGCCATGCCT-3′; and ESO1-2, 5′-TCCTCCTCCAGC GACAAACAA-3′. The integrity of each RNA sample was verified by performing RT–PCR for *porphobilinogen deaminase* (*PBGD*). The PCR products were subjected to electrophoresis on a 2% agarose gel and visualised with ethidium bromide.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were used for the immunohistochemistry (IHC) analyses. Slides were incubated with the primary antibody overnight at 4 °C. The monoclonal antibody E978, which was previously generated by our group, was used to detect NY-ESO-1. The slides were then subjected to a heat-based antigen retrieval technique by immersing them in a preheated buffer solution (hipH solution; Dako, Carpinteria, CA, USA). A polymer-based antibody detection system (PowerVision; Leica Microsystems, Buffalo Grove, IL, USA) was used as the secondary reagent, and 3,3-diaminobenzidine tetrahydrochloride (Liquid DAB; Biogenex, San Ramon, CA, USA) was used as the chromogen. Normal adult testis tissue as a positive control and appropriate negative controls were included for each case.

Enzyme-linked immunosorbent assay. A measure of 100 μl of 1 μg ml^{-1} recombinant protein in coating buffer (pH 9.6) were added to each well of 96-well PolySorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The plates were then washed with PBS and blocked with 200 μl per well of 5% FCS/PBS for 1 h at room temperature. After being washed again, 100 μl of serially diluted serum were added to each well and incubated for 2 h at room temperature. Then, after extensive washing, goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells as a secondary antibody, and the plates were incubated for 1 h at room temperature. The plates were washed again, and the signals were developed with 100 μl per well of 0.03% *o*-phenylene diamine dihydrochloride, 0.02% hydrogen peroxide, and 0.15 M citrate buffer, and absorbance was read at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). Ovalbumin (OVA; Sigma, St Louis, MO, USA) was used as the control protein. Levels of NY-ESO-1 humoral response were assessed using optical density (OD) values.

CEA and CA19-9. Serum CEA and CA19-9 levels were measured at each hospital's clinical laboratory department. Carcinoembryonic antigen and CA19-9 positivity were defined as serum levels of CEA and CA19-9 of >5.0 ng ml^{-1} and >37 U ml^{-1}, respectively.

Statistical analysis. Fisher's exact test was used to assess the associations between NY-ESO-1 antibody expression and clinicopathological parameters. Kaplan–Meier curves were plotted to assess the effect of the NY-ESO-1 antibody on overall survival. Survival curves were compared using the log-rank test.

Table 1. Frequencies of NY-ESO-1 antibody, CEA, and CA19-9 in gastric cancer patients

Stage	NY-ESO-1 Ab	CEA	CA19-9	CEA and/or CA19-9	CEA and/or CA19-9 and/or NY-ESO-1 Ab
I	6/176 (3.4)	24/176 (13.6)	6/176 (3.4)	27/176 (15.3)	31/176 (17.6)
II	2/45 (4.4)	8/45 (17.8)	7/45 (15.6)	11/45 (24.4)	12/45 (26.6)
III	17/67 (25.3)	22/67 (32.9)	11/67 (16.4)	25/67 (37.3)	35/67 (52.2)
IV	16/75 (20.0)	23/75 (30.7)	30/75 (40.0)	40/75 (53.3)	46/75 (61.3)
I + II	8/221 (3.6)	32/221 (14.5)	13/221 (5.9)	38/221 (17.2)	43/221 (19.5)
III + IV	33/142 (23.2)	45/142 (31.7)	41/142 (28.9)	65/142 (45.8)	81/142 (57.0)
Total	41/363 (11.1)	77/363 (21.2)	54/363 (14.9)	103/363 (28.4)	124/363 (34.2)

Abbreviations: Ab = antibody; CA = carbohydrate antigen; CEA = carcinoembryonic antigen. Values within parentheses are percentages.

RESULTS

Determination of NY-ESO-1 humoral immune response positivity. We first determined the OD cutoff value for NY-ESO-1 humoral immune response positivity. When the serum samples from the 50 healthy donors were examined for reactivity to the NY-ESO-1 recombinant protein by ELISA, their OD values ranged from 0.08 to 0.20, and their mean and standard deviation values were 0.15 and 0.05, respectively, at a dilution of 1 : 200. Thus, NY-ESO-1 humoral immune response positivity was defined as an OD value of >0.25 at a dilution of 1 : 200 (95% accuracy level) and >3 times of the OD value against control protein (OVA).

NY-ESO-1 humoral immune responses of gastric cancer patients. Serum samples were obtained from 363 gastric cancer patients, including 176 stage I, 45 stage II, 67 stage III, and 75 stage IV patients at admission (Table 1). The NY-ESO-1 antibody was detected in 3.4% (6 of 176) of stage I, 4.4% (2 of 45) of stage II, 25.3% (17 of 67) of stage III, and 20.0% (16 of 75) of stage IV gastric cancer patients, resulting in an overall detection rate of 11.1% (41 of 363). An analysis of the gastric cancer patients' characteristics found that NY-ESO-1 antibody positivity was significantly correlated with gender (male > female) and tumour progression (Table 2). In particular, the patients with progressive gastric cancer involving deeper tumour invasion, positive lymph node metastasis, positive distant metastasis, or a higher clinical stage tended to produce the NY-ESO-1 antibody.

Analysis of NY-ESO-1 antigen expression. *NY-ESO-1* mRNA and NY-ESO-1 protein expression were analysed by RT–PCR and IHC, respectively, in gastric cancer tissues obtained from 60 patients for whom both frozen and formalin-fixed specimens were available, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients (Table 3). *NY-ESO-1* mRNA was detected in six specimens. NY-ESO-1 was immunohistochemically detected in 19 specimens, including 6 and 13 that were positive and negative for *NY-ESO-1* mRNA, respectively. Most of the specimens displayed a heterogeneous staining pattern (data not shown).

NY-ESO-1 antibody and antigen expression. We analysed the frequency of NY-ESO-1 antibody positivity in gastric cancer patients in whom NY-ESO-1 antigen expression was or was not detected by RT–PCR or IHC. As shown in Table 3, 9 out of the 60 gastric cancer patients whose specimens were available for expression analysis possessed the NY-ESO-1 antibody in their sera. The NY-ESO-1 antibody was detected in 8 of 19 (42.1%) patients with IHC-positive gastric cancer and 5 of 6 (83.3%) patients with RT–PCR (and IHC)-positive gastric cancer, whereas only 1 of 41 patients in whom both RT–PCR and IHC analysis produced negative results displayed an NY-ESO-1 humoral immune responses.

Table 2. Relationship between NY-ESO-1 antibody positivity and clinicopathological features in gastric cancer patients

Variable	NY-ESO-1 Ab		*P*-value*
	Negative	Positive	
Gender			
Male	223 (86.4)	35 (13.6)	0.04307
Female	99 (94.3)	6 (5.7)	
Age (years)			
>65	178 (88.6)	23 (11.4)	0.9209
<65	144 (88.9)	18 (11.1)	
Histological type			
Differentiated	143 (89.4)	17 (10.6)	0.5605
Undifferentiated	132 (87.4)	19 (12.6)	
Depth of tumour invasion			
cT1–T2	193 (92.8)	15 (7.2)	0.0044
cT3–T4	129 (83.2)	26 (16.8)	
Lymph node metastasis			
Negative	196 (97.0)	6 (3.0)	<0.001
Positive	126 (78.3)	35 (21.7)	
Distant metastasis			
Negative	277 (91.1)	27 (8.9)	<0.001
Positive	45 (76.3)	14 (23.7)	
Stage			
I–II	213 (96.4)	8 (3.6)	<0.001
III–IV	109 (76.8)	33 (23.2)	

Abbreviations: Ab = antibody. Fisher's exact test was used for the statistical analysis. Values within parentheses are percentages.

Frequencies of NY-ESO-1 humoral immune responses and conventional tumour markers in gastric cancer patients. The frequency of the NY-ESO-1 humoral immune response was compared with those of conventional tumour markers in gastric

Table 3. Frequency of NY-ESO-1 antibody positives in gastric cancer patients in whom the NY-ESO-1 antigen was or was not detected by IHC or RT–PCR

	IHC		
	Positive	**Negative**	**Total**
mRNA			
Positive	5/6 (83.3)	0/0 (0.0)	5/6 (83.3)
Negative	3/13 (23.1)	1/41 (2.4)	4/54 (7.4)
Total	8/19 (42.1)	1/41 (2.4)	9/60 (15.0)

Abbreviations: IHC = immunohistochemistry; RT–PCR = reverse transcription–polymerase chain reaction. Frozen and formalin-fixed tissue specimens from 60 patients, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients, were analysed. All stage IV patients had previously undergone surgical treatment. Values within parentheses are percentages.

cancer patients. The serum CEA and CA19-9 levels of 363 gastric cancer patients were measured at admission (Table 1). Carcinoembryonic antigen and CA19-9 positivity were observed in 21.2% (77 of 363) and 14.9% (54 of 363) of the gastric cancer patients, respectively, and, except for CA19-9 in the stage III patients, they displayed higher frequencies than the NY-ESO-1 humoral immune response in all stages of the disease. We then analysed whether the addition of the NY-ESO-1 humoral immune response to CEA and CA19-9 increased the diagnostic frequency of gastric cancer. The combined use of CEA and CA19-9 tests produced positivity rates of 15.3% (27 of 176) in stage I, 24.4% (11 of 45) in stage II, 37.3% (25 of 67) in stage III, and 53.3% (40 of 75) in stage IV gastric cancer patients, resulting in an overall positivity rate of 28.4% (103 of 363). When the NY-ESO-1 humoral immune response was added to these two conventional tumour markers, the positivity rates of all stages increased, resulting in information gains of 14.9% (from 25 to 35 patients; 10 of 67) in stage III and 11.2% (from 65 to 81 patients; 16 of 142) in stage III and IV gastric cancer patients.

Changes in the NY-ESO-1 humoral immune responses of the patients during their clinical courses. Serial serum samples were obtained from 25 gastric cancer patients who displayed positive NY-ESO-1 antibody at admission, and the changes in their NY-ESO-1 humoral immune responses were examined throughout their clinical courses. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgical treatment, and 14 did not suffer recurrence. The NY-ESO-1 immune response levels of the patients who did not suffer recurrence decreased after treatment and had fallen below the cutoff level by 9 months after surgery in most cases and did not subsequently increase (Figure 1). The half-lives of their NY-ESO-1 humoral immune response levels were 1.5, 1.6, 2.1, 3.2, and 6.6 months in the stage I patients; 3.0 and 4.0 months in the stage II patients; and 1.6, 1.9, 2.3, 3.0, 3.2, 4.1, and 6.7 months in the stage III patients (mean: 3.0 months). On the other hand, the two patients who underwent curative surgery but subsequently suffered recurrence, M-2 (stage I) and M-11 (stage III), displayed not only incomplete decreases in their NY-ESO-1 humoral immune response levels but also their subsequent restoration to pretreatment levels (Figure 1 and Figure 2A and B). In a comparison between the patients' conventional tumour marker levels and their NY-ESO-1humoral immune response levels, we found that the changes in their CEA and CA19-9 levels were consistent with their NY-ESO-1 immune response levels in patient M-2, whereas patient M-11 was negative for both CEA and CA19-9 throughout their clinical course. Nine stage IV patients who received chemotherapy alone maintained high NY-ESO-1 humoral immune response levels throughout their clinical courses,

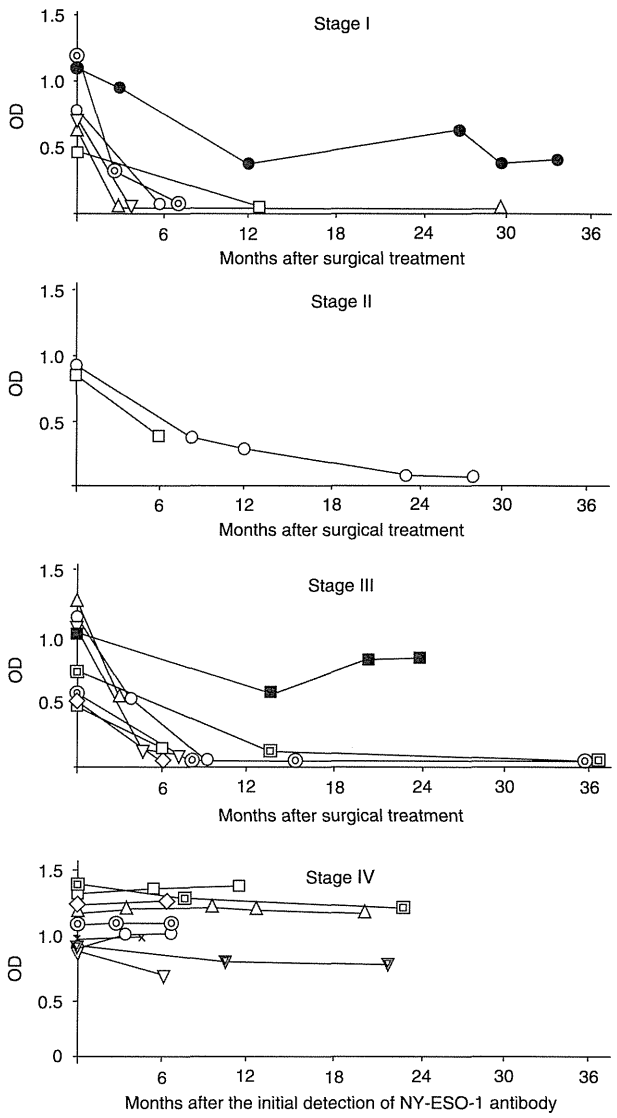

Figure 1. Change in the NY-ESO-1 humoral immune responses of gastric cancer patients after treatment. The serum NY-ESO-1 humoral immune responses of patients with stage I, II, III, or IV gastric cancer in whom NY-ESO-1 antibody production was detected before surgical treatment or chemotherapy were serially analysed. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgery, and only 2 patients (●, ■) suffered recurrence. Other 14 patients did not suffer recurrence. Nine patients with stage IV gastric cancer received chemotherapy alone after the initial detection of NY-ESO-1 antibody. Each mark represents a patient. Optical density (OD) values were measured at a serum dilution of 1 : 200.

including some patients who achieved partial tumour responses after chemotherapy (Figure 1).

Prognostic value of the NY-ESO-1 humoral immune response in gastric cancer. The prognostic value of the NY-ESO-1 immune response was evaluated in gastric cancer patients. An analysis of the cumulative overall survival of the gastric cancer patients indicated that there was no difference in the survival rates of the patients who did and did not display positive NY-ESO-1 humoral immune responses (Figure 3A). However, among the patients with higher stage gastric cancer, overall survival was better in the patients in whom NY-ESO-1humoral immune responses were

detected, although the difference was not significant (Figure 3B). NY-ESO-1 protein expression, as detected by IHC, did not affect the overall survival rate (data not shown).

DISCUSSION

NY-ESO-1 antibody was detected in 23.2% of stage III and IV gastric cancer patients, and the combinatorial use of the NY-ESO-1 antibody with CEA and CA19-9 as tumour markers increase the percentage of tumour detection from 45.8 to 57.0%. As the frequency of NY-ESO-1 humoral immune response was relatively low in the patients with early-stage gastric cancer, analysing serum NY-ESO-1 antibody levels alone might not be useful for screening for early-stage gastric cancer. Nevertheless, the expression of NY-ESO-1, a CT antigen, is restricted to tumour tissues and NY-ESO-1 antibody is only detectable in patients with NY-ESO-1-expressing tumours (Stockert *et al*, 1998), indicating the highly specific nature of NY-ESO-1 humoral immune responses in cancer patients. Given that NY-ESO-1 expression by malignant cells is required for antibody induction (Stockert *et al*, 1998), the detection of NY-ESO-1 antibody would be helpful for diagnosing malignancy, although extensive analysis of serum samples from patients with non-cancerous disease, for example, liver or renal disorders, autoimmune diseases, and so on, would be necessary to confirm. In our expression analysis, more NY-ESO-1-positive cases were detected by IHC (19 of 60) than by RT–PCR (6 of 60). This was probably due to the heterogeneous expression of NY-ESO-1 in gastric cancer and the fact that a limited number of biopsy samples were used for the RT–PCR, whereas multiple slices from whole tumour specimens were used for the IHC. Extensive IHC analysis should be used for NY-ESO-1 expression studies of gastric cancer.

We detected a correlation between the NY-ESO-1 humoral immune response levels and the clinical outcome after therapy in gastric cancer patients. The patients who underwent surgery and did not suffer a subsequent relapse displayed consistent decreases in their NY-ESO-1 humoral immune response levels or even the complete disappearance of the NY-ESO-1 antibody from their sera. It is generally accepted that constant immunological stimulation is necessary to maintain a strong humoral immune response (Jager *et al*, 1999). Thus, reduction of antigen doses by the removal of NY-ESO-1-expressing tumour is one possible reason for the observed decreases in these patients' NY-ESO-1 humoral immune response levels after surgery. Patients M-2 and M-11, in whom NY-ESO-1 humoral immune responses remained high for 1 year after surgery and increased thereafter, may have a subclinical residual disease of the so-called 'minimal residual cancer' (Austrup *et al*, 2000; Klein *et al*, 2002) after curative surgery. Local recurrent tumours of 23 and 25 mm in diameter subsequently developed in M-2 and M-11, respectively, suggesting that even a small tumour burden is sufficient to stimulate antibody production. Patient M-2 showed a partial decrease in their NY-ESO-1 humoral immune response levels after the resection of the relapsed tumour, and we are carefully observing the progression of this tumour.

Nine patients with stage IV gastric cancer received chemotherapy alone. Among them, six patients displayed stable disease, two

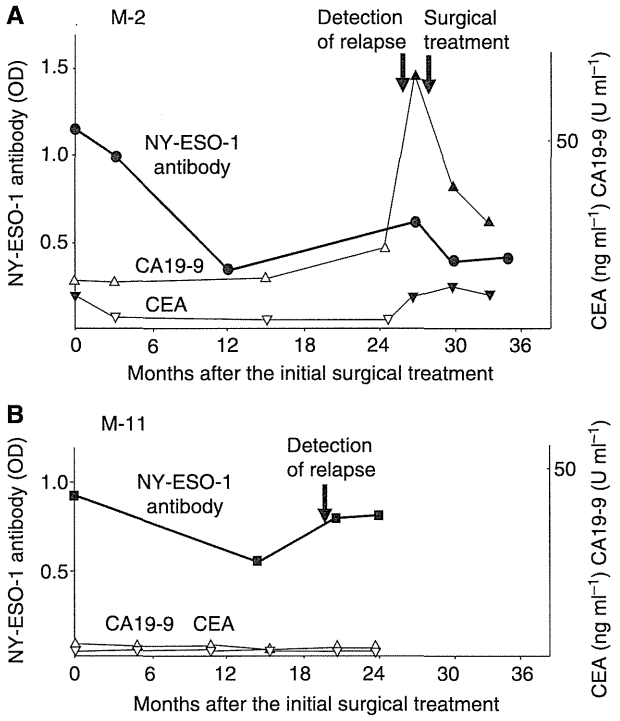

Figure 2. NY-ESO-1 humoral immune response, CEA, and carbohydrate antigen (CA)19-9 levels of patients who relapsed after curative surgery. The NY-ESO-1 humoral immune response (●, ■; Figure 1), CEA (▽), and CA19-9 (△) levels of two patients, M-2 (stage I) (**A**) and M-11 (stage III) (**B**), who underwent curative surgery but subsequently suffered recurrence, were serially analysed. OD values were measured at a serum dilution of 1 : 200. The closed marks indicate CEA or CA19-9 positivity.

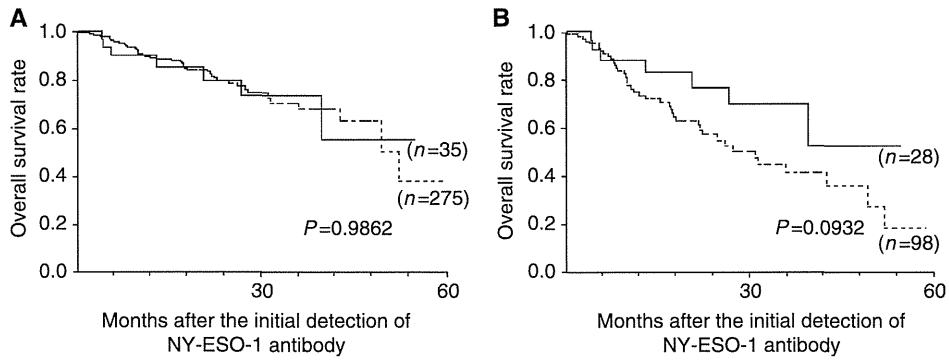

Figure 3. Prognostic role of NY-ESO-1 antibody in gastric cancer patients. The cumulative overall survival rate was analysed in all patients ($n=310$; **A**) and stage III and IV ($n=126$; **B**) gastric cancer patients in whom NY-ESO-1 antibodies were (continuous line) and were not detected (dotted line). The detection of NY-ESO-1 protein by IHC analysis did not affect the overall survival rate (data not shown). Survival curves were plotted using the Kaplan–Meier method. The log-rank test was used for comparisons between groups. *P*-values <0.05 were considered significant.

patients displayed progressive disease, and one patient (M-19) achieved a partial response. Serial analysis of the NY-ESO-1 humoral immune responses of these nine patients including M-19 showed that they barely changed throughout their clinical courses, suggesting that even small tumours are enough to provoke strong NY-ESO-1 humoral immune responses. In this regard, the NY-ESO-1 humoral immune response might not be suitable as a clinical marker for palliative therapy.

We have performed serial cancer vaccine clinical trials with NY-ESO-1 because of its strong immunogenicity and high specificity (Uenaka *et al*, 2007; Wada *et al*, 2008; Kakimi *et al*, 2011). The NY-ESO-1humoral immune response could be a reliable marker of the induction of immune response, as well as for predicting clinical responses in these trials. Furthermore, antibody-based examinations detected both intra- and intermolecular antigen spreading in the sera of patients who had been vaccinated with NY-ESO-1 protein (Kawada *et al*, 2012), suggesting the possible correlation of NY-ESO-1 humoral immne responses and clinical status. In addition, we have started a phase I study of vaccination with NY-ESO-1 protein mixed with Hiltonol (Poly ICLC), Picibanil (OK-432), and Montanide (ISA-51) in patients with NY-ESO-1-expressing cancers (UMIN000007954). Furthermore, NY-ESO-1 vaccine involving modulators of immune checkpoints, for example, anti-CTLA4 antibody and anti-PD-1 antibody, and reagents that are antagonistic to regulatory T cells, for example, anti-CCR4 antibody (Pardoll, 2012) should be considered.

Recently, the antibody against p53, another tumour antigen, has been recognised as a useful tumour marker (Lubin *et al*, 1995). Shimada *et al* (2000)) reported that p53 antibody was detected in 35% of serum samples from patients with *in situ* oesophageal cancer and that it disappeared after endoscopic mucosal resection, proposing that p53 antibody is useful for the early detection and subsequent monitoring of oesophageal cancer. In addition, Müller *et al* (2006) reported that p53 antibody was found in 23.4% of serum samples from cancer patients with 100% accuracy and was correlated with poor prognosis in hepatocellular carcinoma and breast cancer.

Here, we have demonstrated that the NY-ESO-1 humoral immune response could also be valuable as a marker for detecting advanced gastric cancer and inferring whether residual tumour cells remain after treatment, although its frequency in gastric cancer is not very high. We have started a prospective multi-institutional clinical study of NY-ESO-1 humoral immune responses in higher stage gastric cancer patients. In this new study, the NY-ESO-1 humoral immune responses of approximately 100 patients who relapsed after curative surgery will be serially analysed and then followed up. This trial has been registered as UMIN000007925 in Japan.

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