which is the plasmid with target sequences for proviral DNA and human IFN-γ, were amplified at the same time. We generated the standard curve by the use of a plasmid that encodes one copy of the target proviral sequence and one copy of IFN-γ gene sequence. The amplification efficiency of the retrovirus transgene and human IFN-γ was equivalent (Supplementary Fig. S1). Each DNA concentration of IFN-γ or proviral vector for MAGE-A4 TCR expression was calculated from the standard curve. The copy number of the MAGE-A4-TCR DNA in the PBMCs was represented by the ratio of proviral DNA and IFN-γ DNA values.

Tetramer analysis and ELISPOT assays

CD8+ T cells were sorted from patients' PBMCs collected at each time point after transfer, and they then were cultured with MAGE-A4 peptide–pulsed non-CD8+ T cells with peptide concentration of 10 nM. The cells were cultured in the presence of IL-2 (10 U/mL), IL-7 (20 μg/mL) and 10% human AB serum. After 8 days in culture, the stimulated CD8+ T cells were used as effector cells in the tetramer analysis and ELISPOT assay.

Using MAGE-A4 peptide/HLA-A*24:02 tetramer, the frequencies of MAGE-A4 TCR–positive T cells were analyzed by flow cytometry. MAGE-A4-tetramer+/CD8+ T cells were subjected to phenotypic analysis using monoclonal antibodies, CD45RO, CD45RA, CCR7, and CD62L. In parallel with the tetramer analysis, ELISPOT assays were performed by

targeting T2A24 cells pulsed with MAGE-A4 peptide and the tumor-cell lines 11-18 (MAGE-A4+, HLA-A*24:02+) and QG-56 (MAGE-A4+, HLA-A*24:02-). T2A24 cells are T2 cells transduced with the HLA-A*24:02 gene.

The ELISPOT assay was done as described previously with some modifications (13, 24). Briefly, ELISPOT plates (MAHA S4510, Millipore) were coated with anti-human CCL4 (MIP-1β) monoclonal antibody (R&D Systems). A total of 5×10^4 sensitized CD8+ T cells and 1×10^5 peptide-pulsed T2A24 cells, non-pulsed T2A24 cells, 11-18 cells or QG56 cells were placed in each well of the ELISPOT plate. After incubation for 22 h at 37°C, the plate was washed, and then supplemented with 0.2 μg/mL biotinylated capture antibody and incubated overnight at 4°C. After washing, the cells were reacted with 1 μg /mL streptavidin-alkaline phosphatase conjugate, and then stained with an alkaline phosphatase conjugate substrate kit (Bio-Rad). The spots were counted using an ELISPOT Plate Reader (ImmunoSpot, CTL-Europe GmbH).

In addition, we prepared 7 peptides with amino-acid sequences similar to that of the MAGE-A4 peptide, by screening the BLAST database program, bastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Supplementary Table S1). Cells of the MAGE-A4–specific CTL clone #2-28, from which the TCR-α and -β genes were cloned for this study, were tested as effector cells. ELISPOT assays were performed using T2A24 cells pulsed with each of these 7

peptides. T2A24 cell were pulsed with the EBNA-3A peptide as a negative control target.

Results

Preparation of TCR-transduced T cells

The specificity of engineered TCR has become a matter of utmost concern in clinical trials. To determine whether the MAGE-A4–targeting TCR could cross-react with other peptides with amino-acid sequences similar to that of the MAGE-A4 peptide, we performed ELISPOT assays using MAGE-A4 CTL clone #2-28, using target cells pulsed with analogous peptides derived from known human proteins (Supplementary Table S1). As shown in Supplementary Fig. S2, we observed no cross-reactivity of clone #2-28 with any of the 7 analogous peptides.

After preparing cells, we analyzed their cell-surface phenotypes (Table 1). More than 89% were CD3+ T cells. Two patients had CD4+ T cell dominance, 2 patients had CD8+ T cell dominance, and the remaining 6 had equal distributions of CD4+ and CD8+ T cells. Among the CD8+ T cells, 9.7–43.1% of lymphocytes responded to MAGE-A4 peptide, approximating the percentage of T cells transduced with the MAGE-A4–specific TCR gene. MAGE-A4 tetramer-positive CD8+ T cells ranged from 1.8% to 12.6% (Table 1, Supplementary Fig. S3).

Esophageal cancer patients receiving transfer of MAGE-TCR–transduced T cells

From May 2010 to November 2012, 15 patients were enrolled in the clinical trial. They underwent apheresis, from which TCR-transduced lymphocytes were prepared. Five of these patients were withdrawn before lymphocyte transfer, because their general condition deteriorated due to rapid disease progression. The remaining patients were treated with TCR-transduced T cells at one of 3 doses: 2×10^8 cells/dose (Cohort 1, three patients), 1×10^9 cells/dose (Cohort 2, four patients), or 5×10^9 cells (Cohort 3, three patients). One patient in Cohort 2, TCR-MA-209, experienced rapid disease progression complicated by worsened performance status; he was removed from the clinical trial without peptide vaccinations, and was assessed for clinical events and cell kinetics in the peripheral blood. Another patient, TCR-MA-212, was entered to Cohort 2 (Table 2).

As shown in Table 2, all patients had esophageal carcinoma that was recurrent or metastatic after standard treatment, including chemotherapy, radiotherapy, and/or surgery. In all 10 patients, the tumors were pathologically diagnosed as squamous cell carcinomas. MAGE-A4 expression was examined by PCR in 9 patients. For TCR-MA-315, the antigen expression was assessed by IHC. MAGE-A4 expression levels varied from low (in patient TCR-MA-314) to high (in TCR-MA-104 and -208) (Table 2). Seven patients (TCR-MA-102, -104, -106, -209, -210, -314, and -315) had

definite tumor lesions that could be assessed according to the RECIST criteria. Three patients (TCR-MA-208, -212, and -213) had minimal lesions, which were assessed by progression-free periods.

Cell kinetics and phenotypes of MAGE TCR–transduced T cells after transfer

By analyzing the TCR-transgene copies, the infused cells were detected in peripheral blood in all 10 patients (Fig. 1A), appearing soon after transfer, whereas MAGE-A4 tetramer-positive T cells were detected in peripheral blood in 5 patients who were given $1x10^9$ or $5x10^9$ cells (Fig. 3A). The number of cells was dependent on the initial dose during the first 14 days, reaching peak and plateau levels on days 3–7, and then decreasing over 14 days (Fig. 1B). Considering that the average retroviral vector copy number in infused cells was approximately 7 copies per cell, the transferred cells reached over 11–17% of the PBMCs in patients of cohort 3 during the first 14 days. The frequency was calculated at the actual TCR-transgene numbers divided by 7 copies per cell. This means that in TCR-MA-315, who received cells that were 45.3% $CD8^+$, including 43.1% of the responding population (Table 1), TCR-engineered cells may have constituted 2–3% of CD8+ T cells in total PBMCs. After the MAGE-A4 peptide vaccinations on days 14 and 28, the levels of TCR-transduced lymphocytes decreased. In 7 of the patients, the

transferred cells persisted in vivo at stable levels in peripheral mononuclear cells for 63 days.

Ex vivo phenotypic analysis of MAGE-A4-tetramer+/CD8+ T cells was performed at the timing of 12 hours and 14 days after the cell transfer. Although effector/effector memory T cells (CD8+/CD45RO+/CCR7-) were dominant in PBMCs at the timing of 12hrs, terminally differentiated effector memory CD8+ T cells (CD8+/CD45RA+/CCR7+/CD62L-) became a dominant population in patients' PBMCs at 14 days in both TCR-MA-314 and 315 (Supplementary Fig. S4) (25, 26).

Tumor samples from 3 patients were biopsied on day 35 (TCR-MA-104, -106, and -210). In TCR-MA-104, TCR-transduced T cells were detected in the tumor tissues, where they constituted 10% of the PBMCs. In the other 2 patients, no TCR-transduced T cells were detected. At the same time, we re-assessed MAGE-A4 expression levels by quantitative PCR, demonstrating that antigen expression was still high in the cases where TCR-transduced T cells could be detected (Supplementary Fig. S5).

Long-term in vivo persistence of the MAGE-TCR–transduced T cells and immune reactivity against MAGE-A4–expressing tumor cells

Over more than 800 days of observation, 5 patients exhibited long-term persistence of the transduced T cells, as determined by detection of TCR genes by quantitative PCR (Fig. 2). According to the average vector copy

number and antigen-specific response in cells used for transfer (Table 1), these cells represented around 0.01–0.04% of the peripheral CD8+ T cells in patients TCR-MA-102 and -208.

We collected T cells from these patients, stimulated them in vitro with the MAGE-A4 peptide, and performed tetramer and ELISPOT assays. The data from 4 patients, TCR-MA-102, -106, -208 and -212, are presented in Figure 3, in whom the T cells were collected for more than 63 days of study period for tetramer analyses. In TCR-MA-102, the lymphocytes were detected as late as day 105, at which time large numbers of tetramer+ CD8+ T cells were detected following in vitro stimulation with MAGE-A4 peptide (Fig. 3B). Concurrently, T-cell clones were established from PBMCs on days 28 and 105. These T cells were derived from the MAGE-A4 tetramer+ and tetramer- fractions. The tetramer+ CTLs were all originated from the transferred TCR-transduced cells (Fig. 3C). In patients TCR-MA-106, -208, and -212, tetramer+ CD8+ T cells were detected until days 119, 287, and 91, respectively (Fig. 3D).

To determine whether the transferred TCR-transduced CD8+ T cells could react to MAGE-A4–expressing tumor cells, lymphocytes collected from 3 patients (TCR-MA-106, -208, and -212) were assayed by ELISPOT targeting the 11-18 tumor cell line, as well as cells pulsed with MAGE-A4 peptide. In all of these patients, the PBMCs were able to react to MAGE-A4–expressing tumor cells (Fig. 4) in an HLA class I–restricted manner.

Clinical course after transfer of MAGE TCR–transduced T cells

None of the 10 patients experienced any adverse events for the first 14 days after T-cell transfer. In 4 patients, we observed skin reactions such as redness and induration, graded as 1, at the peptide vaccine sites (Table 2). During the 63-day study period, 7 patients developed PD (progressive disease) within 2 months (Supplementary Fig. S6). Among them, 6 patients with tumor progression received subsequent rounds of chemotherapy. Seven patients survived for a median of 9 months (range, 3–15 months).

In TCR-MA-208, -212, and -213, who had minimal lesions at baseline, no disease progression was observed at 21, 26+, and 24+ months, respectively (Supplementary Fig. S6). TCR-MA-208 had a lymph node tumor in the mediastinum, which was treated with chemotherapy and radiotherapy. At the time of cell transfer, the tumor size was minimal, and no tumor activity was visible on an FDG ([^{18}F]fluoro-2-deoxyglucose)-PET (positron emission tomography) scan. The tumor did not enlarge for 21 months, at which time CT (computed tomography) and FDG-PET scans revealed tumor relapse at another lymph node. Prior to entering our study, TCR-MA-212 had experienced multiple tumor recurrences in the lymph nodes and bones, all of which developed soon after chemotherapy and radiotherapy. At the time of cell transfer, no measurable tumors were detectable by CT scan, and FDG-PET scan revealed no active tumor uptake. To date, the patient has

been free from disease progression for 26 months. A small tumor was seen in TCR-MA-213 along the esophageal-gastric anastomotic site (Supplementary Fig. S7). The tumor has not enlarged at the time of this writing, 24 months after the transfer of TCR gene–transduced lymphocytes. The uptake of FDG was still active 3 months after the transfer.

Discussion

In this clinical study, we tested the hypothesis that TCR gene–engineered T cells would exhibit the capacity to persist for long periods in cancer patients not subjected to lymphodepleting pretreatment. Peripheral blood–derived T cells transduced with MAGE-A4–specific TCRs were safely transferred into patients with esophageal cancer and persisted for long periods in vivo. Although the transferred cells maintained tumor-specific reactivity in patients, objective tumor regression was not observed. Dose-dependent appearance of the transferred cells was found in the peripheral blood in the first 14 days followed by the immediate decline and the long-term persistence in the patients at the low but detectable level, although the number of the transgene evaluated should be practical rather than absolute. In clinical trials with T cells genetically engineered to express tumor-reactive receptors, a high level of persistence of the infused cells seems to be necessary but not sufficient for tumor regression. In a study of MART-1 specific TCR–engineered T cells in patients with metastatic

melanoma, 2 patients who experienced definite tumor regressions had persistent T cells in their peripheral blood for more than 1 year, as determined by measurements of DNA levels of the genetically marked cells (6). In that study, patients with clinical responses received T cells that had been subjected to shorter periods of in vitro culture than those administered to the patients that did not exhibit a response. Subsequent trials with high-affinity TCRs specific for the melanoma differentiation antigens MART-1 and gp100 (27), or the cancer-testis antigen NY-ESO-1 (7), utilized relatively briefly cultured cells for transfer, but reported either a mild correlation with some exceptions (27), or no correlation (7) between high-level persistence of transferred T cells and the clinical responses. A study of GD2-reactive CAR showed no correlation between tumor response and the dose of transferred cells or their detection level in peripheral blood (28). Recently, however, a successful trial of CD19-CAR therapy for hematopoietic malignancy reported sustained in vivo expansion and persistence of transferred cells in patients (29). However, the dose effects and threshold for durable clinical effects await further analyses. In this study, we observed discordance between the persistence of transferred cells and tumor regression, consistent with the idea that prolonged persistence of the transferred cells is not a sufficient condition for efficient tumor control in adoptive therapy. Given that our protocol achieved a considerable degree of T-cell survival without lymphodepletive pretreatment, our data also support

the idea that pretreatment enhances anti-tumor responses via multiple mechanisms, such as depletion of immunosuppressive cell populations, reduced competition for activating cytokines between endogenous and transferred cells, or increased functionality of APCs, as suggested in animal models (30, 31). Another possible explanation for the lack of tumor regression is that lymphodepletion was not required for persistence of infused cells, but in this case the cells simply did not reach the numerical threshold for tumor destruction. The transferred cells reached 2–3% of PBMCs at most, declined after day 14, and persisted at much lower levels (e.g., 0.04%) after 1 month. Although some CAR trials reported clinical responses with similar levels of detectable infused cells in peripheral blood (8 - 12), other trials with TCR-engineered cells reported clinical responses in patients with higher levels of infused cells (6, 7, 27).

It has been demonstrated that the inhibitory tumor microenvironments in various tumor types impact their clinical prognoses. In esophageal tumors, PD-L1 or -L2 was expressed in approximately 40% in esophageal cancer (32) and M2 macrophages (CD68+CD163+ cells) infiltrated to more than half of esophageal tumor tissues (33). Although we did not analyze these inhibitory factors in patients' tumor samples, they might have played a role in tumor responses in this study. The other issue we should consider is loss or decrease of tumor-antigen expression during tumor progression over the clinical course, although they were all positive at baseline in this study. In

fact, in TCR-MA-104 the TCR-transferred T cells infiltrated a tumor site showing high expression of MAGE-A4 antigen, whereas in TCR-MA-210, whose tumor expressed lower antigen levels, no TCR transference was detected in the tumor.

We have initiated a clinical trial utilizing the same MAGE-A4 TCR–engineered cells accompanied by lymphodepletive pretreatment as a therapy for esophageal, head/neck, ovarian cancer, and melanoma patients. In the trial, we will evaluate whether the pretreatment regimen enhances the clinical response irrespective of whether the infused cells persist for increased periods of time.

In this study, 7 patients had measurable tumors, and none of these patients exhibited tumor shrinkage. Even after the tumors started to progress, the patients survived for a median of 9 months (range, 3–15 months) while receiving chemotherapy. Because the patients who entered this clinical trial had therapy-refractory disease, their survival times are considered to be quite long given their disease status (34, 35). Among the 10 patients, 3 have exhibited a long-term lack of disease progression. Although they had minimal disease at the time of cell transfer, they have all remained progression-free for more than a year without any treatment other than T-cell transfer and MAGE-A4 peptide vaccine. This finding suggests that this sort of TCR-transduced T-cell therapy might be beneficial for patients bearing minimal tumors. In one patient, tumor activation was observed by

FDG-PET uptake; this patient has exhibited stable disease for 24 months. Based on these observations, we conclude that MAGE-A4–targeting TCR-transduced T cells exert constant in vivo anti-tumor activity and may have clinical potential.

Nine of the patients received MAGE-A4 vaccines both on days 14 and 28. Although the patients' derived MAGE-A4-peptide specific T cells were much expanded with in vitro peptide stimulation, these vaccinations did not increase the levels of infused cells in the peripheral blood; indeed, the levels declined in many cases. Because the MAGE-A4 peptide vaccine promoted inhibition of tumor growth when it was administered along with MAGE-A4 TCR–transduced T cells in our pre-clinical studies in NOD-SCID mice (36), we initially expected that the vaccine would stimulate TCR-transduced T cells, and that the levels of the infused cells would be elevated in peripheral blood. However, this was not the case. One possible explanation of this unexpected observation is that cognate peptide vaccine induced T-cell apoptosis at injection sites, consequently reducing the total number of TCR-transduced T cells (37, 38).

Although we did not observe any toxicity related to the TCR-transduced lymphocyte transfer we performed in this study, other studies have reported toxicities induced by TCR-transduced T-cell transfer (27, 39 - 42). In a study using high-affinity TCR against MART-1 and mouse-derived TCR against gp100, melanocyte-related toxicities (including skin, eye, and ear toxicities)

occurred frequently (27). Moreover, an on-target effect on a normal organ was observed in a study in which CEA-targeting mouse-derived TCR was used to treat colorectal cancer patients (39). Additionally, affinity-enhanced MAGE-A3-specific TCR-transferred T cells caused serious cardiac toxicity with unexpected recognition of titin, a cardiac muscle antigen, in patients treated for melanoma and myeloma (40, 41). It is theoretically possible that a TCR could induce toxicity by reacting to peptides from a different antigen that includes sequences similar to the target peptide. For example, in a previous study, T-cell transfer using mouse-derived TCR targeting MAGE-A3 induced severe toxicity in the central nervous system (42); this toxicity was caused by a reaction to a similar peptide of the MAGE-A12 antigen, which is expressed at low levels in normal brain tissues. In this study, we first investigated whether the MAGE-A4-TCR we used could induce a cross-reaction; however, we did not observe cross-reactions to any of 7 peptides similar to the MAGE-A4 peptide. Because it is technically challenging to predict the unexpected cross-reactivity of artificially affinity-enhanced TCRs that have not undergone in vivo negative selection, the use of wild-type TCRs with physiologically high avidity may represent a safe approach for selecting TCRs for clinical use.

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