Serum samples

To analyze antigen-specific antibody responses, sera were collected at baseline and two weeks after each vaccination. All sera were stored at −80°C until analysis.

Antibody responses to NY-ESO-1 antigen

NY-ESO-1-specific antibodies in the sera were measured by ELISA as described previously [15]. Briefly, recombinant NY-ESO-1 proteins (His-tag and GST-tag) and NY-ESO-1 peptides were absorbed onto immunoplates (442404; Nunc, Roskilde, Denmark) at a concentration of 10 ng/50 μL/well at 4°C. The collected serum samples were diluted from 1:400 to 1:102,400. After washing and blocking the plate, the sera were added and incubated for 10 h. After washing, goat anti-human IgG (H + L chain) (MBL, Nagoya, Japan) conjugated with peroxidase (The Binding Site, San Diego, CA) was added. After adding the TMB substrate (Pierce, Rockford, IL), the plate was read using a Microplate Reader (model 550; Bio-Rad, Hercules, CA).

Serum samples for 80 healthy volunteers were evaluated to determine a cut-off level for the anti-NY-ESO-1 antibody based on the optical density $(OD)_{450-550}$ absorption value. The cut-off level of anti-NY-ESO-1 IgG was 0.182. A sample was considered to be positive for anti-NY-ESO-1 antibodies if the optical density $(OD)_{450-550}$ absorption value in the ELISA was at the cut-off level or higher at a serum dilution of 1:400. The immune responses of patients with pre-existing anti-NY-ESO-1 antibodies were judged as augmentation if the serum diluted 4-fold or more remained positive.

Statistical analysis

Rates of the immune responses between the patients in Cohort 1 and Cohort 2 were compared by Fisher's exact test, and the survival curve was estimated using the Kaplan–Meier method and compared by the log-rank test. In order to adjust the confounding factors, Cox proportional hazards model was applied. All analyses were done using SAS 9.2 (SAS Institute Inc., Cary, NC).

Results and discussion

Patient characteristics and clinical safety

A total of 25 patients were enrolled in the clinical trial. All patients had unresectable, advanced, or refractory esophageal cancers. The tumor cells in all of these patients were NY-ESO-1-positive, in which the positivity was determined by immunohistochemistry and qRT-PCR for 24 patients and one patient, respectively. All patients received standard chemotherapy and/or other cancer therapies including radiotherapy and surgery, which were ultimately ineffective (Table 1).

Cohort 1 consisted of 13 patients who were given 100 μg of the vaccine; Cohort 2 consisted of 12 patients who were given 200 μg of the vaccine. The patients in Cohort 1 and Cohort 2 received 2 to 27 vaccinations with a median of 8 doses and 3 to 21 vaccinations with a median of 9.5 doses, respectively (Table 1). No dose-limiting toxicity (DLT) was observed. All the patients except one developed transient, grade 1 skin reactions at the injection sites. Other adverse events included swallowing disturbance (n = 8), diarrhea (n = 3), and fever (n = 2), in which events of grade 3 or 4 were included. These events were considered unrelated to the CHP-NY-ESO-1 vaccination. Based on the laboratory data, decreased lymphocyte counts were observed (n = 10), which were all grade 3. These patients had lymphopenia at baseline, probably due to the previous chemotherapies. During the course of the vaccinations, they developed grade 3 lymphopenia, which were shifted from the other grade of the pre-vaccine lymphopenia. Other changes included decreased Na levels (n = 4), decreased hemoglobin levels (n = 3), elevated transaminase levels (n = 2) and elevated uric acid (n = 2) (Table 2). These adverse events were changed from the decreased or elevated levels at baseline. They did not affect the vaccine continuation. Therefore, the changes were considered not related or unlikely related to the vaccination.

Table 1 Patients demographics

	100 μg	200 μg
No. patients enrolled	13	12
Sex		
Male	13	11
Female	0	1
Age		
Median	69	64.5
Range	49-72	53-79
Prior therapy		
Surgery	6	5
Radiotherapy	11	7
Chemotherapy	13	12
Pre-existing antibody to NY-ESO-1 antigen	3	7
No. vaccinations		
Median	8	9.5
Range	2-27	3-21

Immune responses to NY-ESO-1 protein

As shown Table 3, 3 out of the 13 patients, and 7 out of 12 patients had pre-existing antibodies to NY-ESO-1, while the remaining 10 and 5 patients did not have this reactivity in Cohort 1 and Cohort 2, respectively.

To evaluate the antibody responses after vaccination, serum samples collected at the serial vaccinations were analyzed using an antigen-specific IgG ELISA. In three patients of 100–02, 100–3 and 200–7 who were vaccinated three times, the serum samples from 1st and 2nd

Table 2 Adverse events during CHP-NY-ESO-1 vaccinations

Adverse event	100 μg(n = 13)						200 μg(n = 12)						Total
	Grade						Grade						
	1	2	3	4	5	Subtotal	1	2	3	4	5	Subtotal	
Skin reaction	12	0	0	0	0	12	12	0	0	0	0	12	24
Swallowing disturbance	0	0	3	0	0	3	0	0	4	1	0	5	8
Diarrhea	0	0	2	0	0	2	1	0	0	0	0	1	3
Fever	2	0	0	0	0	2	0	0	0	0	0	0	2
Decreased lymphocytes count	0	0	7	0	0	7	0	0	3	0	0	3	10
Decreased Na level	0	0	2	0	0	2	0	0	2	0	0	2	4
Decreased Hb level	0	0	3	0	0	3	0	0	0	0	0	0	3
Elevated ALT/AST level	0	0	2	0	0	2	0	0	0	0	0	0	2
Elevated uric acid level	0	0	1	1	0	2	0	0	0	0	0	0	2

NOTE: Events occurring more than once are listed. Events of disease progression are not listed.

vaccination were assayed. In Cohort 1, out of 10 pre-antibody-negative patients, 5 became seropositive. Two out of 3 pre-antibody-positive patients had augmented antibody responses. In total, 7 of 13 (53.8%) patients exhibited immune responses. Five pre-antibody-negative and 7 pre-antibody-positive patients in Cohort 2 became positive or were augmented, yielding 12 out of 12 or 100% responsiveness. The 200-μg dose was more immunogenic than the 100-μg dose ($p = 0.015$, Fisher's exact test). In Cohort 1, immune reactions were observed after a median of 2 cycles, with a range of 1 to 4 vaccine cycles. In Cohort 2, the immune responses were also evident after a median of 2 cycles with a range of 1 to 5 cycles (Table 3). The chronological appearance of the immune responses and antibody titers are shown in Figure 1. The antibody intensities appeared more quickly and at a higher titer in patients in Cohort 2 (200 μg) than those in Cohort 1(100 μg). In addition to His-tag NY-ESO-1 protein, we tested serum reactivities to GST-tag NY-ESO-1 protein and NY-ESO-1 peptides. We confirmed specific reactions to NY-ESO-1 antigen in these sera.

Clinical responses and long-term follow-up

There were no cases of tumor shrinkage with partial response (PR) or complete response (CR) in any of the 25 patients. At the assessment that occurred every 6 weeks after vaccination, stable disease (SD) was observed in 3 patients in Cohort 1 and 6 patients in Cohort 2 (Table 4). There was no discordance in the evaluations between RECIST ver1.1 [11] and its modified version [12].

Table 3 Antibody responses in patients vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1

100 μg				200 μg			
pt No.	Vaccination cycle	Baseline (dilution titer)	Antibody response (cycle*)	pt No.	Vaccination cycle	Baseline (dilution titer)	Antibody response (cycle*)
100-01	9	negative	responded(4)	200-01	15	negative	responded(2)
100-02	3	negative	no response**	200-02	9	negative	responded(2)
100-03	3	negative	no response**	200-03	8	positive (x1,600)	responded(5)
100-04	7	negative	no response	200-04	21	negative	responded(2)
100-05	2	negative	no response	200-05	3	negative	responded(2)
100-06	16	positive (x6,400)	responded(1)	200-06	10	positive (x400)	responded(1)
100-07	9	positive (x25,600)	no response	200-07	3	positive (x25,600)	responded(2)**
100-08	10	negative	responded(1)	200-08	11	positive (x400)	responded(1)
100-09	5	negative	no response	200-09	18	positive (x400)	responded(3)
100-10	27	positive (x400)	responded(3)	200-10	11	positive (x400)	responded(2)
100-11	8	negative	responded(2)	200-11	3	positive (x400)	responded(2)
100-12	8	negative	responded(2)	200-12	9	negative	responded(1)
100-13	26	negative	responded(2)				
antibody response rate			53.8%***			100%***	

*vaccine cycles with which antibody responses appeared. **antibody responses assayed after two vaccinations.***p = 0.015(Fisher's exact test).

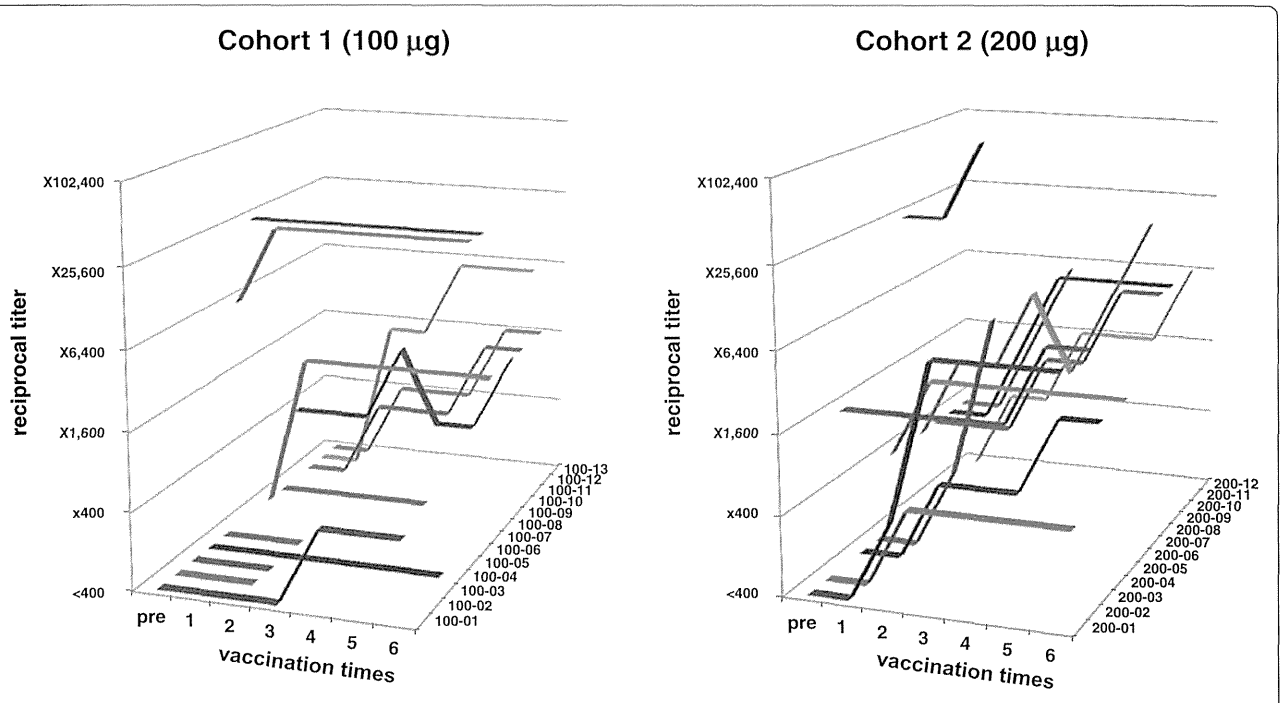

Figure 1 Antibody responses to recombinant NY-ESO-1 protein as determined by ELISA. *Sera from 13 patients in Cohort 1 (100-μg dose) and 12 patients in Cohort 2 (200-μg dose) were collected at each vaccination, and serially diluted by 400, 1,600, 6,400, 25,600 and 102,400. Reciprocal titers were determined based on the maximally diluted sera, which showed a higher OD (optical density of 450–500) value than the cut-off level for NY-ESO-1 antibody positivity.*

The disease progression-free survival time was 11 weeks on average, with a median of 6 weeks and range of 4 to 52 weeks. In Cohort 1 (n = 13), patients who were vaccinated with 100 μg of CHP-NY-ESO-1 survived without disease progression for 11 weeks on average, with a median of 6 weeks and range of 4 to 52 weeks. In Cohort 2 (n = 12) in which patients received the 200-μg dose, the patients were progression-free for 10 weeks on average, with a median of 8.5 weeks and range of 6 to 18 weeks (Table 4). There was no difference between the two cohorts (p = 0.748, Figure 2-A).

The overall survival time was 33 weeks on average, with a median of 31 weeks and range of 4 to 72 weeks. In Cohort 1 (n = 13), the patients survived for 25 weeks on average, with a median of 23 weeks and range of 4 to 60 weeks. In Cohort 2 (n = 12), they survived for 41 weeks on average, with a median of 41 weeks and range of 8 to 72 weeks (Table 4). The patients vaccinated with 200 μg of CHP-NY-ESO-1 had statistically longer survival than those who received the 100-μg dose (p = 0.050, Figure 2-B). Each cohort included three patients who were vaccinated three times or less because of early disease progression, and were withdrawn from this study, respectively. Having excluded those 6 patients, the patients vaccinated with 200 μg-vaccine still had longer survival than those with 100 μg-vaccinations (data not shown).

When the survival of patients who had responded to previous therapies (n = 12) was compared to non-responders (n = 13), the responders lived longer than the non-responders after vaccination (p = 0.005, Figure 2-C). The patients who never responded to previous therapies and received the 200-μg dose (n = 6) significantly lived longer than those who received the 100-μg dose (n = 7) (p = 0.029, Figure 2-D).

When the survival of patients who had tumors with a maximal diameter of 30 mm or less, including non-measurable lesions (n = 13) was compared with those with diameters more than 30 mm (n = 12), the patients with higher tumor burdens had shorter life spans (p = 0.021, Figure 2-E). Among patients with higher tumor burdens, patients who were vaccinated with the 200-μg dose (n = 4) lived longer than those who received the 100-μg dose (n = 8), (p = 0.037, Figure 2-F).

Using Cox proportional hazards models, the vaccine dose and the responsiveness to previous therapy were independent factors that influenced the overall survival, which showed p = 0.011 with HR 3.595 (95%CI 1.335-9.678) and p = 0.002 with HR 0.194 (95%CI 0.068-0.553), respectively. Also, the vaccine dose and the tumors sizes including non-measurable disease independently affected the overall survival, showing p = 0.040 with HR 2.630 (95%CI 1.045-6.614) and p = 0.020 with HR 0.322 (95%CI 0.124-0.833), respectively.

Table 4 Baseline clinical profiles and responses after CHP-NY-ESO-1 vaccinations

100 μg						200 μg					
pt No.	Response to previous therapies (duration time, weeks)	*sum of tumor diameters (mm)	Tumor response (BOR)	Time-to-progression (weeks)	Survival (weeks)	pt No.	Response to previous therapies (duration time, weeks)	*sum of tumor diameters (mm)	Tumor response (BOR)	Time-to-progression (weeks)	Survival (weeks)
100-01	PR (4)	NA	PD	6	31	200-01	PR (29)	24	SD	17	70
100-02	SD	53	NE	4	6	200-02	NE	25	SD	18	33
100-03	NE	144	NE	4	6	200-03	PR (32)	55	PD	6	37
100-04	PD	182	PD	5	17	200-04	PR (30)	NA	PD	6	50
100-05	CR (38)	101	NE	4	4	200-05	PR (32)	NA	PD	6	72
100-06	SD	69	PD	6	31	200-06	NE	32	SD	18	54
100-07	CR (15)	78	PD	6	29	200-07	NE	205	NE	6	8
100-08	NE	39	SD	18	23	200-08	PR (12)	16	SD	11	33
100-09	SD	18	PD	6	8	200-09	CR (96)	88	PD	6	45
100-10	CR (24)	NA	SD	11	60	200-10	SD	NA	SD	12	48
100-11	SD	31	SD	12	20	200-11	SD	NA	NE	6	12
100-12	PR (9)	NA	NE	16	28	200-12	SD	NA	SD	12	33
100-13	PR (16)	NA	NE	52	59						

*target lesions determined based on RECIST criteria.

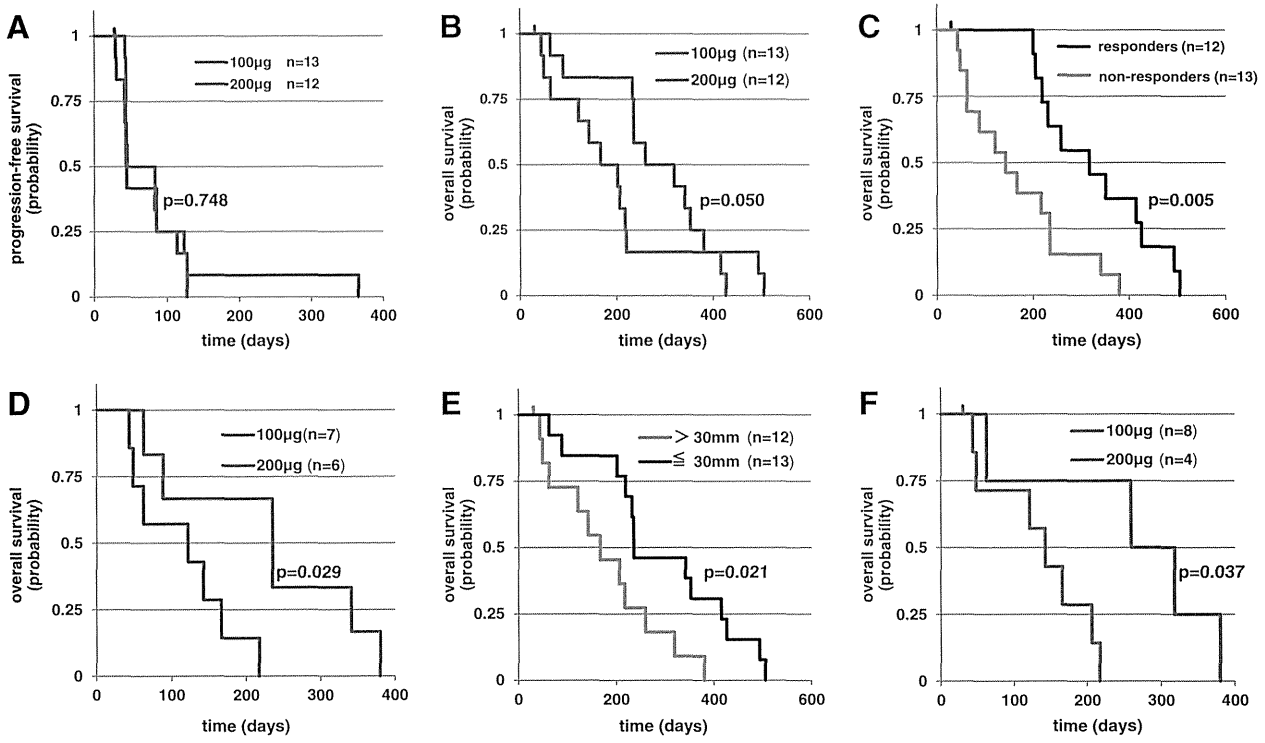

Figure 2 Kaplan-Meier survival curves of patients who were alive after the CHP-NY-ESO-1 vaccinations. A: Progression-free survival (PFS) of patients assigned to Cohort 1 (100-μg dose, n = 13) or Cohort 2 (200-μg dose, n = 12). The probability of PFS of the patients who received 200 μg of the vaccine and those who received the 100-μg dose were similar (log-rank test, p = 0.748). **B**: Overall survival (OS) of patients assigned to Cohort 1 or Cohort 2. The patients who received 200 μg of the vaccine survived longer than those who received the 100-μg dose. (log-rank test, p = 0.050). **C**: OS of patients vaccinated with either 100 μg or 200 μg of CHP-NY-ESO-1 compared between patients who responded to previous therapies and those who never responded. The patients who responded to previous therapies and were vaccinated with CHP-NY-ESO-1 survived longer than non-responders. (log-rank test, p = 0.0050). **D**: OS of patients vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1 who never responded to previous therapies. The patients who received 200 μg of the vaccine survived longer than those who received the 100-μg dose (log-rank test, p = 0.029). **E**: OS of patients vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1 compared with either baseline tumor burdens of >30 mm (sum of the tumor diameters) or 30 mm or less including non-measurable lesions. The patients with a tumor burden higher than 30 mm had reduced survival compared to those with smaller burdens (log-rank test, p = 0.021). **F**: OS of patients who received 100 μg or 200 μg of CHP-NY-ESO-1 who had baseline tumor burdens greater than 30 mm. The patients who received 200 μg of the vaccine survived longer than those who received the 100-μg dose (log-rank test, p = 0.037).

This study was a phase 1 dose-escalating clinical trial that examined two doses of the CHP-NY-ESO-1 vaccine in esophageal cancer patients. The primary goals were to evaluate the vaccine safety and immune responses to the NY-ESO-1 antigen, and we further explored the clinical effects on esophageal cancer patients with a poor prognosis.

CHP consists of a hydrophobic polysaccharide pullulan containing chemically introduced cholesterol groups, which spontaneously aggregate to form nano-sized particles that can contain antigen proteins. Using this system as a vaccine, tumor antigen proteins delivered to antigen-presenting cells can stimulate both antigen-specific CD4+ T cells and CD8+ T cells. In a pre-clinical study, dendritic cells pulsed with the CHP-NY-ESO-1 complex could induce both NY-ESO-1-specific CD4+ and CD8+ T cells [4]. Previous clinical studies using CHP-HER2 and CHP-NY-ESO-1 vaccines have shown that these vaccines can induce antigen-specific $CD4^+$ and $CD8^+$ T cell immunity in cancer patients [5-7].

In the current study, we found that CHP-NY-ESO-1 was clinically safe and that the immune responses to the NY-ESO-1 antigen, which were evaluated based on IgG antibody titers, showed a dose-dependent effect between the 100-μg dose and 200-μg. Furthermore, the survival rates of patients who were vaccinated with the 200-μg dose were superior to those who received the 100-μg dose. The patients had recurrent or metastatic esophageal tumors that exhibited clinical resistance to chemotherapy or radiotherapy. The first 13 patients were enrolled to Cohort 1, and the next 12 patients were included in Cohort 2. As the clinical backgrounds of the two cohorts were similar, it was reasonable to make a comparative consideration.

As the previous NY-ESO-1 protein vaccine trials have demonstrated, the toxicity of the CHP-vaccine was very

mild. Grade 3 swallowing disturbances were seen, which were likely related to the progression of esophageal cancer. The other grade 3 events included diarrhea, which was not related to the vaccine. The only related events were grade 1 skin reactions at the injection sites.

Previous vaccine trials have used recombinant full-length NY-ESO-1 protein with various adjuvants. Melanoma patients were divided into three cohorts that were vaccinated with 10 μg, 30 μg or 100 μg of the NY-ESO-1 protein in combination with the saponin adjuvant ISCOMATRIX [10]. The 100-μg dose of NY-ESO-1 induced more immune responses than the other two doses. The responses were evaluated based on IgG antibody titers and delayed-type hypersensitivity (DTH) of skin reactions. In the CHP system, a single 100-μg dose of CHP-NY-ESO-1 was examined with or without the adjuvant OK-432 [6,7,16]. These reports suggested that the 100-μg dose of CHP-NY-ESO-1 is sufficient to induce immune responses. The current trial was designed to determine whether the NY-ESO-1 protein vaccine has potential dose-dependent effects on immunogenicity in patients with homogeneous backgrounds. By assessing humoral immune responses in the cohorts that received 100 μg and 200 μg of the vaccine, the responses appeared in the early phases. We initially intended to analyze antibodies using samples from patients who were vaccinated for at least 4 cycles, as we thought it could take at least 4 cycles to detect immune responses. In the overall data acquisition, samples from all 25 patients were analyzed, which included sera from at least two vaccinations. In conclusion, we found that the 200-μg dose was more efficient than the 100-μg dose.

The other reports included vaccine studies using recombinant NY-ESO-1 protein in combination with Imiquimod and CpG [17,18]. In these studies, the NY-ESO-1 protein was given at doses of 100 μg, and 100 μg or 400 μg, respectively. Based on the patients' sera, the 400-μg dose might have induced more antibody responses than the 100-μg dose, but this was not statistically analyzed. Combined with these reports, the NY-ESO-1 protein might be immunogenic at increasing doses of 10 μg, 30 μg, 100 μg and 200 μg. Since dose-limited toxicity (DLT) was not observed at the higher dose of 200 μg in this study, additional dose increments might be acceptable to determine whether higher doses can induce stronger immune responses.

In this study, we explored a long-term clinical outcome of the NY-ESO-1 protein vaccine. This study was not initially designed to detect a statistical significance of the clinical effect between the 2 cohorts. Instead, we made a comparison to find out if there might include a positive signal for further clinical trials of this vaccine. The NY-ESO-1 protein vaccine with the adjuvant ISCOMATRIX suggested that melanoma patients who were vaccinated after standard therapy tended to have fewer relapses [10], which were not statistically analyzed. The other studies reported that vaccinations with NY-ESO-1-expressing poxvirus vectors and NY-ESO-1 overlapping peptides both prolonged progression-free survivals in ovarian cancer patients who did not have measurable disease after standard therapy [19,20]. In this study, most of the patients developed disease-progression in 6 months, and there was no difference between the patients vaccinated with 100 μg and 200 μg of the CHP-NY-ESO-1, as the previous studies demonstrated that disease-progression occurs in the early phase of vaccinations [12,21].

In contrast, we found that dose-dependent effects of the CHP-NY-ESO-1 vaccine on overall survival of patients with advanced/metastatic esophageal cancer. Analyzing other clinical categories, both the baseline tumor sizes and the tumor responsiveness to previous therapies were significant factors influencing the overall survival. Using Cox proportional hazards models, it was indicated that the tumor sizes and the vaccine doses independently influenced the survival. In the same way, the responsiveness to previous therapies and the vaccine doses independently affected the survival. Therefore, it is suggested that the higher dose of CHP-NY-ESO-1 vaccine played a role in prolongation of the overall survival in the esophageal cancer patients.

In addition, the higher-dose of the vaccine provided significant survival benefit in patients who never responded to the previous therapies or had larger tumor burdens than the lower dose vaccinations. It is difficult to discuss why the patients with a poorer prognosis were more benefited from the 200-μg dose of the vaccine than 100-μg. It might be speculated that the dose-dependency clinical benefits were more often observable in patients with a poorer prognosis, because they might have needed more immune responses in order to survive longer by preventing disease deterioration.

In the previous CHP-NY-ESO-1 vaccine study, which was a phase 1 study that enrolled various types of NY-ESO-1-expressing cancer patients, tumor regression was observed in two out of four esophageal cancer patients [6]. However, tumor shrinkage is rarely observed in cancer vaccine therapies, although some disease stabilization is seen. This study shows that clinical benefits, such as long-term survival, can be detected if a clinical trial is designed in a comparative way. The results were not compared to unvaccinated controls, and it is not possible to directly determine the effects of the vaccine, but is possible to reasonably interpret the effects of immune response on the clinical outcomes.

Conclusions

The safety and immunogenicity of the CHP-NY-ESO-1 vaccine were confirmed in the patients with antigen-expressing esophageal cancer. The 200-μg dose efficiently induced antigen-specific immune responses and suggested better survival benefits, even for patients with a poorer prognosis. In future clinical trials, 200 μg will be the recommended dose.

Abbreviations
BOR: Best overall response; NA: Not available; NE: Not evaluable.

Competing interests
This study is supported by ImmunoFrontier, Inc. and Naozumi Harada is an employee, and Mami Ohnishi and Tadashi Hishida are former employees of ImmunoFrontier, Inc. Hiroshi Shiku is a stockholder of ImmunoFrontier, Inc.

Authors' contributions
SK, HW, KM, YN, SU, HM, ST and YD treated patients and provided the clinical data. SHS and YM worked on immune responses. HI, NI and ES evaluated tumor antigen expression. TY, MOs and MOh worked on the study statistics. NH and TH were responsible for manufacturing the study drug. SK and HS wrote the manuscript. All authors read and approved the final manuscript.

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Regular Article

LYMPHOID NEOPLASIA

Development of a novel redirected T-cell–based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia

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Key Points

- The efficacy and safety of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for patients with adult T-cell leukemia.
- hTERT-specific T-cell receptor gene-transduced $CD8^+$ T cells lyse ATL cells, but not normal cells, both in vitro and in vivo.

Although adult T-cell leukemia (ATL) has a poor prognosis, successful allogeneic hematopoietic stem cell transplantation (allo-HSCT) in some cases suggests that a cellular immune-mediated strategy can be effective. So far, however, no effective target for anti-ATL immunotherapy has been defined. Here we demonstrated for the first time that *human telomerase reverse transcriptase* (hTERT) *is a promising therapeutic target for* ATL, and we developed a novel redirected T-cell–based immunotherapy targeting hTERT. *hTERT* messenger RNA was produced abundantly in ATL tumor cells but not in steady-state normal cells. Rearranged human leukocyte antigen-A*24:02 (HLA-A*24:02) –restricted and $hTERT_{461-469}$ nonameric peptide-specific T-cell receptor (TCR) α/β genes were cloned from our previously established cytotoxic T lymphocyte clone (K3-1) and inserted into a novel retroviral TCR expression vector encoding small interfering RNAs for endogenous *TCR* genes in redirected T cells (hTERT-*siTCR* vector). Consequently, allogeneic or autologous gene-modified $CD8^+$ T cells prepared using the hTERT-*siTCR* vector successfully killed ATL tumor cells, but not normal cells including steady-state hematopoietic progenitors, in an HLA-A*24:02-restricted manner both in vitro and in vivo. Our experimental observations support the development of a novel hTERT-targeting redirected T-cell–based adoptive immunotherapy for ATL patients, especially those for whom suitable allo-HSCT donors are lacking. (*Blood.* 2013;121(24):4894-4901)

Introduction

Adult T-cell leukemia (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus I (HTLV-I).[1] It is estimated that there are more than 1 million HTLV-I carriers in Japan, about 5% of whom develop ATL at around 60 years of age or older.[2] Because ATL tumor cells soon acquire chemotherapy resistance and compromise host immunity against infectious pathogens, ATL has a poor prognosis.[3] Although most ATL patients are ineligible for allogeneic hematopoietic stem cell transplantation (allo-HSCT) because of advanced age, age-related comorbidity, or lack of suitable donors,[4] the number of ATL patients who are treated successfully with allo-HSCT and achieve prolonged survival has been increasing.[5] The graft-versus-ATL effect observed in ATL patients treated successfully with allo-HSCT[5] strongly suggests that a cellular immune-mediated approach for ATL can be clinically effective. With regard to cellular immunotherapy for ATL (unlike Epstein-Barr virus [EBV]-associated malignancy[6]), targeting of antigens associated with HTLV-I (the causative virus of ATL) such as Tax[7] and HBZ[8] still remains controversial, and the recently proposed NY-ESO-1[9] (a cancer-testis antigen) still awaits clinical validation. Thus, at this time, no effective therapeutic target antigen for anti-ATL immunotherapy has been clinically defined.

Human telomerase reverse transcriptase (hTERT), which is a component of human telomerase and a catalytic subunit for telomere elongation, is activated in almost all cancer cells, including hematologic malignancies, but not in normal cells.[10] In HTLV-I–infected cells and ATL tumor cells, Tax or interleukin-2 (IL-2) signaling strongly activates the *hTERT* promoter through the nuclear factor-κB or PI3K pathway,[11-13] suggesting that expression of hTERT protein would be upregulated in ATL tumor cells. Clinical trials of anticancer immunotherapy targeting hTERT have already been conducted, and both the safety and induction of immune responses to hTERT have been reproducibly confirmed.[10,14-17] In our previous studies, we defined a [human leukocyte antigen] HLA-A*24:02-restricted $hTERT_{461-469}$ nonameric peptide (VYGFVRACL) that was capable of inducing antileukemia cytotoxic T lymphocytes (CTLs),[18] and we subsequently established a CTL clone, K3-1, specific for this

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epitope.[19] We previously conducted a phase I/II clinical trial of hTERT peptide vaccine for treatment of HLA-A*24:02$^+$ patients with lung cancer and metastatic renal cell cancer.[20] These achievements strongly encouraged us to further explore cellular immune-mediated treatment of ATL targeting hTERT. Because of concern over the potential regulatory T-cell function of ATL tumor cells,[21] in this study we focused on developing a redirected T-cell–based immunotherapy targeting hTERT rather than using an $hTERT_{461\text{-}469}$ peptide vaccine. Recently developed forms of anticancer immunotherapy using gene-modified T cells that redirect defined tumor-associated antigens have been shown to have clinical promise.[22-25] To this end, therefore, we first cloned the rearranged HLA-A*24:02-restricted and $hTERT_{461\text{-}469}$-specific T-cell receptor α/β (*TCR-α/β*) genes from K3-1 and inserted them into a novel *TCR* gene expression vector carrying silencers for endogenous TCRs (*siTCR* vector)[26] in redirected T cells (hTERT-*siTCR* vector). Notably, we used a souped-up second-generation 2A peptide-based *siTCR* vector that achieved an increased level of expression of the introduced TCR.[27]

In this study, we used the newly established hTERT-*siTCR* vector to examine the feasibility of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for treatment of ATL.

Patients and methods

Cell lines, freshly isolated leukemia cells, and normal cells

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was obtained from all patients, healthy volunteers, and parents of cord blood donors in accordance with the Declaration of Helsinki.

B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with EBV. ATN-1,[28] TL-Om1,[29] HUT102[29], and TL-MAT[30] were human T-cell lines established from ATL patients, and TL-Su,[31] MT-1,[32] MT-2[32], and MT-4[33] were human T-cell lines transformed by HTLV-I infection. LCLs, T2-A24,[19] K562 (American Type Culture Collection [ATCC]), and human T-cell lines (except TL-Om1), maintenance of which requires 10 U/mL recombinant human IL-2 (rhIL-2) (R&D Systems), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The HLA-A*24:02 gene-transduced K562 (K562-A24) was maintained in culture medium supplemented with 1.0 μg/mL puromycin (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) from ATL patients and healthy donors and cord blood mononuclear cells (CBMCs) from healthy donors were isolated by density gradient centrifugation and stored in liquid nitrogen until use. All samples from ATL patients contained more than 90% ATL cells. CD4$^+$ T cells, CD14$^+$ cells from PBMCs, and CD34$^+$ cells from CBMCs were isolated by using CD4$^+$ cell-, CD14$^+$ cell-, or CD34$^+$ cell-isolating immunomagnetic beads (MACS beads; Miltenyi Biotec), respectively. IL-2–dependent CD4$^+$ cell lines induced by HTLV-I infection were generated as reported previously.[8]

Cloning of full-length *TCR* α and β chain genes and construction of hTERT-*siTCR* retroviral vector

HLA-A*24:02-restricted and $hTERT_{461\text{-}469}$ nonameric peptide (VYGFVRACL)-specific *TCR-α/β* genes were cloned from our previously established CTL clone, K3-1,[19] by using the 5′ rapid amplification of complementary DNA ends method (Clontech). The rearranged *TCR-α/β* genes of K3-1 expressed the germ line gene segments *TRAV29DV5/TRAJ34/TRAC* and *TRBV20-1/TRBJ2-1/TRBC2*, respectively. The retroviral vector expressing K3-1–derived *TCR* genes was constructed as reported previously.[26,27,34] Briefly, the constant regions of the hTERT-specific *TCR-α/β* genes were codon optimized and then integrated into a novel Splice-b2Aa-siTCR–based retroviral vector encoding small interfering RNAs that complementarily bind to the constant regions of the endogenous *TCR-α/β* genes (hTERT-*siTCR* vector).[27]

Establishment of hTERT-*siTCR*–transduced CD8$^+$ T-cell lines

Isolated CD8$^+$ T cells from PBMCs of healthy volunteers or ATL patients using CD8$^+$ cell-isolating MACS beads and stimulation with 1 μg/mL anti-CD3 monoclonal antibody (mAb; OKT-3; BioLegend) were cultured in GT-T503 (Takara Bio) supplemented with 5% human serum, 0.2% human albumin, 50 U/mL rhIL-2, 5 ng/mL rhIL-7 (R&D Systems), 10 ng/mL rhIL-15 (PeproTech), and 10 ng/mL rhIL-21 (Shenandoah Biotechnology). Then, CD8$^+$ T cells were transfected with the hTERT-*siTCR* retroviral vector using RetroNectin (Takara Bio) -coated plates as described previously.[34] In some experiments, because TRBV20-1 is specifically labeled with anti-Vβ2 mAb (IMGT Web resources: http://www.imgt.org/), Vβ2-positive cells among hTERT-*siTCR*–transduced CD8$^+$ T cells (hTERT-*siTCR*/CD8) were further isolated by using fluorescein isothiocyanate (FITC) –conjugated Vβ2 mAb (Beckman Coulter) and anti-FITC–conjugated MACS beads. To measure the expression levels of the introduced hTERT-specific TCR in gene-modified CD8$^+$ T cells, the cells were labeled with anti-CD8 (BD Biosciences) and anti-Vβ2 mAbs and phycoerythrin-conjugated HLA-A*24:02/$hTERT_{461\text{-}469}$ tetramer or HLA-A*24:02/HIV-1 $Env_{584\text{-}592}$ (RYLRDQQLL) tetramer, as a negative control.[19] Labeled cells were analyzed by using a Gallios flow cytometer (Beckman Coulter) and FlowJo Version 7.2.2 software (TreeStar). To expand the hTERT-*siTCR*/CD8 cells, they were stimulated weekly with mitomycin-C (Kyowa Hakko) –treated and $hTERT_{461\text{-}469}$ peptide–pulsed HLA-A*24:02$^+$ LCLs.

Cytotoxicity assays

Standard ^{51}Cr-release assays were performed as described previously.[35] Briefly, 5×10^3 unpulsed or peptide-pulsed target cells were labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$; MP Bio Japan) and incubated at various ratios with effector cells in 200 μL of culture medium in 96-well round-bottomed plates. To assess HLA class I restriction, target cells were incubated with 10 μg/μL anti-HLA class I framework mAb (clone w6/32; ATCC) or a control anti-HLA -DR mAb (clone L243; ATCC) for 1 hour, then incubated with effector cells for 5 hours. The percentage of specific lysis was calculated as (experimental release cpm – spontaneous release cpm)/(maximal release cpm – spontaneous release cpm) × 100 (%). In some experiments, time-lapse imaging was used. Ten thousand ATL cells lentivirally gene-modified to express monomeric Azami-Green (Amalgaam) were cocultivated with 5×10^4 effector cells expressing hTERT-specific TCR (at an effector:target ratio of 5:1) for 12 hours in culture medium supplemented with 10 μg/mL propidium iodide (Sigma) to label dead cells red by using a glass dish for microscopic observation of live cells (iBIDI-dish1 Hi-Q4; Nikon). Images were acquired by using a systemic bio-imaging tool (BioStation IM; Nikon). To examine the cytotoxicity of these effector cells against early-differentiated and highly proliferating subsets of hematopoietic progenitor cells, CB-CD34$^+$ cells cultured by using a hematopoietic cell expansion medium (StemSpan CC100 and StemSpan SFEM; Stem Cell) for 7 days were subjected to flow-based cytotoxicity assay. 7-Aminoactinomycin D (7-AAD) –positive dead cells in each subset were examined by flow cytometry.

Quantitative analysis of hTERT mRNA expression

Quantitative real-time PCR (qRT-PCR) for *hTERT* messenger RNA (mRNA) was performed as described previously.[36] Briefly, after complementary DNA was synthesized, qRT-PCR for *hTERT* mRNA (NM_198253) was performed by using the QuantiTect SYBR green PCR Kit (QIAGEN) and primers as follows: forward, 5′-TTCTTGTTGGTGACACCTCACCTC-3′; reverse, 5′-CAGCCATACTCAGGGACACCTC-3′ (Takara Bio). Human hypoxanthine phosphoribosyltransferase 1 (*hHPRT1*) mRNA (NM_000194) was prepared and used as an internal control. Samples were analyzed by using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The expression level of *hTERT* mRNA was corrected by reference to that of *hHPRT1* mRNA, and the amount of *hTERT* mRNA relative to that in PBMCs was calculated by the comparative threshold cycle method. K562, which strongly expresses *hTERT* mRNA, was used as an internal control.

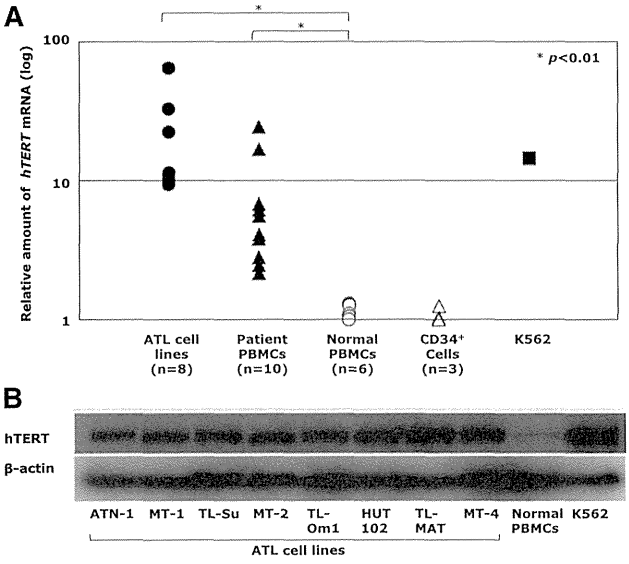

Figure 1. Abundant expression of hTERT in ATL tumor cells. (A) Expression of *hTERT* mRNA in ATL/HTLV-I infected cell lines (■), freshly isolated ATL tumor cells from patients (▲), normal PBMCs (○), and CB-CD34$^+$ cells (△) were examined by qRT-PCR. The level of *hTERT* mRNA expression in the K562 leukemia cell line (●) was used as an internal control. The expression level of *hTERT* mRNA in each sample was calculated relative to that of PBMCs. *hTERT* mRNA expression relative to normal PBMCs was 21.3 ± 17.9 for the ATL/HTLV-I–infected cell line, 7.48 ± 6.89 for freshly isolated ATL tumor cells, and 1.10 ± 0.12 for CB-CD34$^+$ (mean ± standard deviation [SD]). The ATL/HTLV-I–infected cell line and freshly isolated ATL tumor cells expressed *hTERT* mRNA abundantly and significantly (*$P < .01$). (B) Expression of hTERT protein in ATL cell lines and normal PBMCs was confirmed by western blotting.

Western blotting of hTERT protein

For analysis of protein expression, western blotting was performed as described previously.[35] Briefly, cell lysates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (e-PAGEL, ATTO) and blotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). The blots were incubated first with anti-hTERT rabbit mAb (Millipore), then with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G Ab (GE Healthcare). The probed proteins were visualized by using an enhanced chemiluminescence system (GE Healthcare). Subsequently, the blotted membranes were stripped and reprobed with anti–β-actin mouse mAb (Sigma-Aldrich) to confirm equivalent protein loading between samples.

Detection of hTERT$_{461\text{-}469}$–specific CTL precursors in the periphery of ATL patients

PBMCs from HLA-A*24:02$^+$, HLA-A*24:02$^-$ ATL patients, or HLA-A*24:02$^+$ healthy individuals were seeded in 24-well plates at 1.5 × 10^6 per well in the presence of the hTERT$_{461\text{-}469}$ peptide at a concentration of 1 μM in GT-T503 medium supplemented with 5% human serum and 10 U/mL IL-2. After culturing for 14 days, cultured PBMCs were stained with FITC-conjugated anti-CD8 mAb and HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer or control tetramer at a concentration of 20 μg/mL at 4°C for 20 minutes. Subsequently, the stained cells were analyzed by flow cytometry.

IFN-γ secretion assay

hTERT-*siTCR*/CD8 or K3-1 (2 × 10^4) cells were incubated with 2 × 10^4 hTERT$_{461\text{-}469}$ peptide-pulsed (1 μM) or unpulsed K562-A24 or K562 cells for 24 hours. Interferon gamma (IFN-γ) in the culture supernatant was measured by using an enzyme-linked immunosorbent assay kit (Pierce). Enzyme-linked immunospot assays were used to detect the epitope-responsive IFN-γ production mediated by hTERT$_{461\text{-}469}$-specific CTL precursors in the periphery of ATL patients as described previously.[34]

Anti-ATL tumor effect of hTERT-*siTCR*–transduced CD8$^+$ T cells in xenografted mouse models

To assess the in vivo anti-ATL tumor effect mediated by hTERT-*siTCR*/CD8, a bioluminescence assay using a xenografted mouse model was used. First, we lentivirally generated a luciferase gene–transduced HLA-A*24:02$^+$ ATL cell line, ATN-1 (ATN-1/luc). For measurement, anesthetized xenografted mice were given an intraperitoneal injection of 2.5 mg/body VivoGlo luciferin (Caliper Life Science), and images were acquired for 5 to 10 minutes by using an AEQUORIA luminescence imaging system (Hamamatsu Photonics). The acquired photon counts were analyzed by using AQUACOSMOS software (Hamamatsu Photonics).

Six-week-old NOD/scid/γc^{null} (NOG) female mice[37] were purchased from the Central Institute for Experimental Animals and maintained in the institutional animal facility at Ehime University. All in vivo experiments were approved by the Ehime University animal care committee. For the Winn assay, 5 × 10^5 ATN-1/luc cells and 2.5 × 10^6 hTERT-*siTCR*/CD8 or non–gene-modified CD8$^+$ T cells (NGM/CD8) were subcutaneously inoculated into the abdominal wall of NOG mice that had been pretreated with 1 Gy irradiation. Thereafter, 2.5 × 10^6 effector cells of each type were administered weekly to the corresponding mice, respectively, via the tail vein for a total of 3 times. For the adoptive transfer experiments, similarly pretreated mice were intravenously inoculated with 5 × 10^5 ATN-1/luc cells. After 4 days, mice started to receive intravenously infused 5 × 10^6 hTERT-*siTCR*/CD8 or NGM/CD8, respectively, for a total of 5 times. These mice were serially monitored for tumor growth determined by photon counts acquired every 7 days until they were euthanized owing to disease progression.

Statistical analysis

The Mann-Whitney *U* test was used to assess differences between two groups; a *P* value of < .05 was considered significant.

Results

ATL tumor cells abundantly express *hTERT* mRNA and hTERT protein

The expression level of *hTERT* mRNA in the ATL/HTLV-I–infected cell line (n = 8), freshly isolated tumor cells from ATL patients (n = 10), normal PBMCs from healthy individuals (n = 6), and CD34$^+$ cells from normal CBMCs (CB-CD34$^+$) (n = 3) were measured by using the qRT-PCR method. *hTERT* mRNA expression relative to normal PBMCs was 21.3 ± 17.9 for the ATL/HTLV-I–infected cell line, 7.48 ± 6.89 for freshly isolated ATL tumor cells, and 1.10 ± 0.12 for CB-CD34$^+$ cells (mean ± standard deviation). In Figure 1A, the ATL/HTLV-I–infected cell line and freshly isolated ATL tumor cells, but not CB-CD34$^+$, abundantly produced *hTERT* mRNA in comparison with normal PBMCs, the difference being statistically significant. The *P* value was .002 for the ATL/HTLV-I–infected cell line, .001 for freshly isolated ATL tumor cells, and .243 for CB-CD34$^+$ cells. Similarly, western blotting demonstrated abundant expression of hTERT protein in the ATL tumor cells (Figure 1B).

Circulatory hTERT$_{461\text{-}469}$-specific CTL precursors were exclusively detectable in the periphery of HLA-A*24:02$^+$ ATL patients

Next, by using the tetramer assay, we examined circulatory hTERT$_{461\text{-}469}$-specific CTL precursors in PBMCs from HLA-A*24:02$^+$ ATL patients (n = 7), HLA-A*24:02$^-$ ATL patients (n = 3) before chemotherapy, and HLA-A*24:02$^+$ healthy individuals as controls (n = 6). Since freshly isolated PB lymphocytes were almost

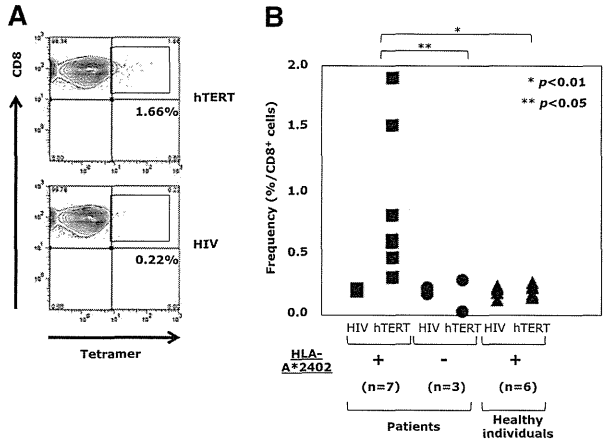

Figure 2. Detection of circulatory hTERT$_{461\text{-}469}$-specific CTL precursors in the periphery of ATL patients. (A) hTERT$_{461\text{-}469}$-specific CTL precursors in PBMCs repetitively stimulated with hTERT$_{461\text{-}469}$ peptide from HLA-A*24:02$^+$ ATL patients were detected by using HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer. A representative case is shown. HLA-A*24:02/HIV tetramer was used as a negative control. (B) In comparison with HLA-A*24:02$^-$ ATL patients (●) (n = 3) and HLA-A*24:02$^+$ healthy individuals (▲) (n = 6), the frequency of hTERT$_{461\text{-}469}$-specific CTL precursors in HLA-A*24:02$^+$ ATL patients (■) (n = 7) was significantly high (*$P < .01$; **$P < .05$). The frequency was 0.88% ± 0.55% for HLA-A*24:02$^+$ ATL patients, 0.11% ± 0.1% for HLA-A*24:02$^-$ ATL patients, and 0.2% ± 0.04% for HLA-A*24:02$^+$ healthy individuals (mean ± SD).

negative for tetramer staining, PBMCs stimulated with hTERT$_{461\text{-}469}$ peptide were analyzed. A representative example of an HLA-A*24:02$^+$ ATL patient is shown in Figure 2A. The frequencies of hTERT$_{461\text{-}469}$-specific CTL precursors in HLA-A*24:02$^+$ and HLA-A*24:02$^-$ ATL patients and HLA-A*24:02$^+$ healthy individuals are summarized in Figure 2B. hTERT$_{461\text{-}469}$-specific CTL precursors were detected at 0.88% ± 0.55% in HLA-A*24:02$^+$ ATL patients, being significantly more frequent than in HLA-A*24:02$^-$ ATL patients (0.11% ± 0.1%; $P < .05$) or HLA-A*24:02$^+$ healthy individuals (0.2% ± 0.04%; $P < .01$). These observations confirmed the presence of primed memory CD8$^+$ T cells with hTERT$_{461\text{-}469}$ epitope/HLA-A*24:02 complex (ie, that the hTERT$_{461\text{-}469}$ epitope must be naturally immunogenic) in HLA-A*24:02$^+$ ATL patients.

hTERT-*siTCR*–transduced CD8$^+$ T cells exert anti-ATL reactivity in vitro

The hTERT-*siTCR* gene was retrovirally introduced into normal CD8$^+$ T cells. Transduction efficiency determined by expression of Vβ2 on the gene-modified T cells was 85% to 95% (data not shown), and almost 50% of the transfectants were positive for HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer (Figure 3A). The cognate epitope specificity and HLA-A*24:02 restriction were examined by using standard ^{51}Cr-release assays (Figure 3B). Because expression of *hTERT* mRNA in LCLs was upregulated (supplemental Figure 2C), hTERT peptide-unpulsed HLA-A*24:02$^+$ LCLs were killed to some extent, reflecting the presence of endogenously processed hTERT (Figure 3B). Such epitope-specific cytotoxicity mediated by hTERT-*siTCR*/CD8 was obviously attenuated by anti-HLA class I mAb, but not by anti-HLA-DR mAb (Figure 3C). The antigen sensitivity to cognate hTERT$_{461\text{-}469}$ peptide mediated by hTERT-*siTCR*/CD8 (shown in Figure 3D) was similar to that of the parental CTL clone, K3-1 (Figure 3E-F).

hTERT-*siTCR*/CD8 dose-dependently killed the HLA-A*24:02$^+$ ATL/HTLV-I–infected cell lines ATN-1, TL-Su, and MT-2, but not the HLA-A*24:02$^-$ TL-Om1, HUT102, and MT-4 (Figure 4A). Additionally, the tumoricidal effect mediated by hTERT-*siTCR*/CD8 was abrogated by anti-HLA class I mAb, but not by anti-HLA-DR mAb (Figure 4B). Furthermore, time-lapse imaging directly demonstrated this tumoricidal activity of hTERT-*siTCR*/CD8 against HLA-A*24:02$^+$ ATN-1, but not that against HLA-A*24:02$^-$ HUT102 or K562 (negative control) (supplemental Fig 1-(1)). We then examined the tumoricidal activity against freshly isolated ATL tumor cells and found that these transfectants also dose-dependently killed HLA-A*24:02$^+$, but not -A*24:02$^-$ freshly isolated ATL tumor cells (Figure 5A).

Conversely, as shown in Figure 5B, neither HLA-A*24:02$^+$ normal CD4$^+$ T cells (the normal counterpart of ATL tumor cells)

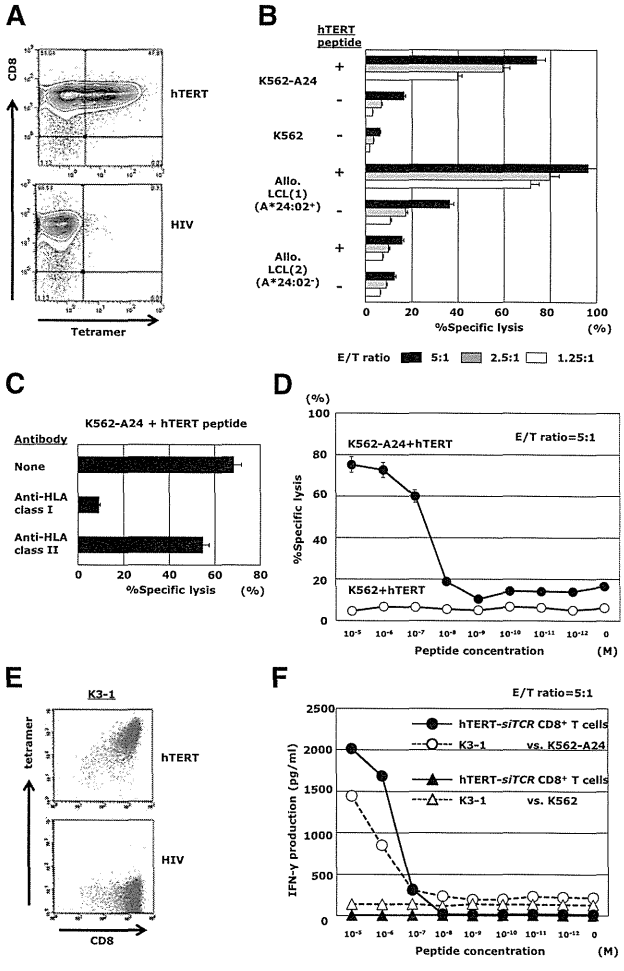

Figure 3. hTERT-*siTCR*–transduced CD8$^+$ T cells display epitope-specific responsiveness. (A) Representative flow cytometry plots showing staining of hTERT-*siTCR*–transduced CD8$^+$ T cells with HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer. HLA-A*24:02/HIV tetramer was used as a negative control. (B) ^{51}Cr-release assays were conducted by using hTERT-*siTCR*–transduced CD8$^+$ T cells with unpulsed or hTERT$_{461\text{-}469}$ peptide-loaded (1 μM) K562-A24, K562, HLA-A*24:02$^+$, or HLA-A*24:02$^-$ allogeneic B-LCLs at the indicated effector:target (E/T) ratios. (C) Effect of HLA class I and class II blockade on the cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against the cognate peptide-pulsed (1 μM) K562-A24 was determined by ^{51}Cr-release assays at an E/T ratio of 5:1. (D) hTERT-*siTCR*–transduced CD8$^+$ T cells were tested in ^{51}Cr release assays against K562 (negative control) and K562-A24 cells pulsed with the indicated concentrations of hTERT$_{461\text{-}469}$ peptide at an E/T ratio of 5:1. Error bars represent SDs. (E) Representative flow cytometry plots showing staining of K3-1 with the HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer (upper) and the irrelevant HLA-A*24:02/HIV-1 Env$_{584\text{-}592}$ tetramer (negative control; bottom). (F) IFN-γ production by hTERT-*siTCR*–transduced CD8$^+$ T cells was measured by using a format similar to that described for panel D. The parental K3-1 CTL clone was tested in parallel.

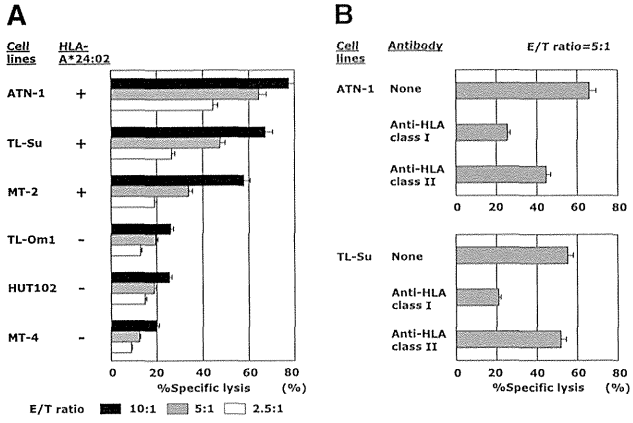

Figure 4. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against ATL/HTLV-I–infected cell lines. (A) Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against HLA-A*24:02$^+$ or HLA-A*24:02$^-$ ATL/HTLV-I–infected cell lines was tested in ^{51}Cr-release assays at the indicated E/T ratios. All tested ATL/HTLV-I–infected cell lines overexpressed *hTERT* mRNA and protein, as shown in Figure 1. (B) Effect of HLA class I and class II blockade on the cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against ATN-1 and TL-Su was tested in ^{51}Cr-release assays at an E/T ratio of 5:1.

nor HLA-A*24:02$^+$ normal CB-CD34$^+$ cells as normal hematopoietic progenitor cells were killed. In the same experiment, newly established IL-2–dependent HTLV-I–infected CD4$^+$ T cells (Patient #1 and Patient #2), but not the corresponding original normal/HTLV-I$^-$ CD4$^+$ T cells (Patient #1 and Patient #2), became to some extent sensitive to the same transfectants as the level of *hTERT* mRNA expression increased (Figure 5B). This observation confirmed that not only ATL tumor cells, but also HTLV-I–infected cells from which ATL tumor cells were derived could be killed by these hTERT-specific effector cells.

Next, because the majority of ATL patients were of an advanced age and were therefore ineligible for allo-HSCT, we examined the tumoricidal activity against autologous ATL tumor cells mediated by gene-modified PB-CD8$^+$ T cells from the patient (Figure 6). Although PB-CD8$^+$ T cells from heavily pretreated ATL patients were sometimes difficult to subject to *TCR* gene modification and ex vivo expansion, hTERT-*siTCR*/CD8 cells generated from HLA-A*24:02$^+$ patients (n = 3) were able to substantially lyse autologous ATL tumor cells in proportion to the corresponding level of *hTERT* mRNA expression. Autologous CD14$^+$ PB monocytes were used as a negative control because they lacked expression of *hTERT* mRNA. These results demonstrated that hTERT-*siTCR*/CD8 cells were able to exert tumoricidal activity against ATL tumor cells through recognition of the hTERT$_{461\text{-}469}$ epitope/HLA-A*24:02 complex, which is naturally presented on the surface of ATL tumor cells.

hTERT-*siTCR*–transduced CD8$^+$ T cells display in vivo anti-ATL reactivity

In vivo anti-ATL reactivity mediated by hTERT-*siTCR*/CD8 cells was assessed by using a xenografted mouse model and bioluminescence assay. Serial bioluminescence assay images were simultaneously acquired.

In the Winn assay (Figure 7A), tumor cell growth in NOG mice treated with hTERT-*siTCR*/CD8 (n = 2) was completely inhibited for longer than 6 months. In contrast, when compared with non-treated NOG mice (n = 2) in which the inoculated ATL tumor mass rapidly enlarged, activated NGM/CD8 (n = 2) did suppress ATL tumor growth to some degree, but eventually huge tumor masses developed within 2 months. In a therapeutic adoptive transfer model (Figure 7B), the tumor cell growth in mice treated with hTERT-*siTCR*/CD8 (n = 2) was obviously suppressed within the 8-week observation period, in contrast to that in mice treated with NGM/CD8 (n = 2) and that in control mice (n = 2).

Discussion

Although ATL still has a poor prognosis, the clinical presence of the graft-versus-ATL in patients treated successfully by allo-HSCT has encouraged the search for a novel cellular immune-mediated treatment of ATL. Unlike EBV-related malignancy,[6] the feasibility of HTLV-I–associated Tax[7] and HBZ[8] proteins as therapeutic targets of anti-ATL immunotherapy still remains controversial. Therefore, in this study, we explored the feasibility of a novel therapeutic target other than one associated with HTLV-I. Consequently, we demonstrated for the first time that hTERT was a promising therapeutic target for anti-ATL adoptive immunotherapy. Freshly isolated ATL tumor cells produced *hTERT* mRNA abundantly, and HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific CTL precursors were detected in the periphery of HLA-A*24:02$^+$ ATL patients. These findings suggested that naturally processed and presented hTERT$_{461\text{-}469}$/HLA-A*24:02 complex on the surface of ATL tumor cells was sufficiently immunogenic to be recognized by the target-specific CTLs in HLA-A*24:02$^+$ ATL patients. Additionally, *hTERT* mRNA expression in newly generated HTLV-I–infected CD4$^+$ T cells was upregulated, and these cells became sensitive to gene-modified hTERT-specific CTLs (Figure 5B). The involvement of Tax[12] and HBZ[38] in upregulation of the *hTERT* gene in

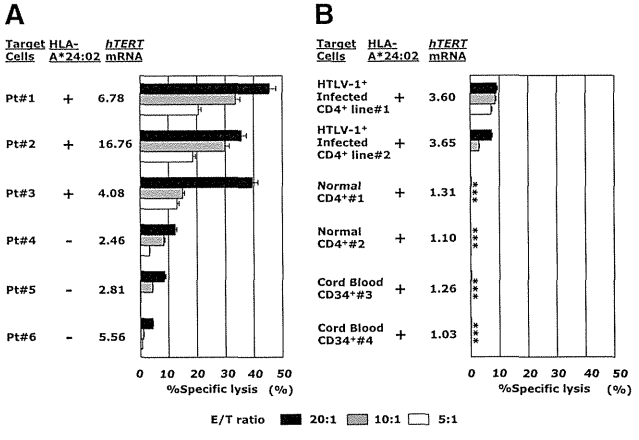

Figure 5. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated ATL cells and newly HTLV-I–infected CD4$^+$ T cells, but not normal cells, in vitro. (A) Freshly isolated HLA-A*24:02$^+$ (n = 3) or HLA-A*24:02$^-$ (n = 3) ATL tumor cells overexpressing *hTERT* mRNA were used as targets in ^{51}Cr-release assays with hTERT-*siTCR*–transduced CD8$^+$ T cells at the indicated E/T ratios. (B) The same hTERT-*siTCR*–transduced CD8$^+$ T cells used in panel A at the same E/T ratios were tested in ^{51}Cr-release assays against newly generated HLA-A*24:02$^+$ HTLV-I–infected CD4$^+$ T cells (n = 2) representing HTLV-I carrier CD4$^+$ T cells, original HLA-A*24:02$^+$ normal CD4$^+$ T cells (n = 2) representing the normal counterpart ATL tumor cells (corresponding number indicating cells from the identical donor), and HLA-A*24:02$^+$ normal CB-CD34$^+$ cells (n = 2) encompassing steady-state normal hematopoietic progenitor cells. Listed levels of expression of *hTERT* mRNA are those relative to the mean levels of expression across 6 PBMC samples from healthy donors determined by qRT-PCR and calculated by using the comparative threshold cycle method. Error bars represent SDs (* indicates less than detectable).

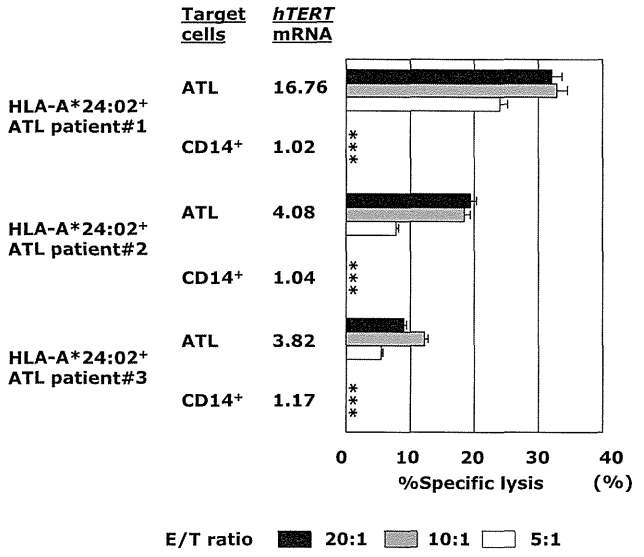

Figure 6. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated autologous ATL tumor cells on the basis of hTERT expression levels. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells obtained from HLA-A*24:02$^+$ ATL patients (n = 3) against autologous freshly isolated ATL tumor cells and autologous peripheral CD14$^+$ cells (negative control) was tested in ^{51}Cr-release assays at the indicated E/T ratios. *hTERT* mRNA in each patient's ATL tumor cells is listed using a format similar to that used in Figure 5. Error bars represent SDs (* indicates less than detectable).

HTLV-I–infected immortalized CD4$^+$ T cells and ATL tumor cells has been reported previously. Initially, it might seem more realistic to develop an hTERT$_{461\text{-}469}$ peptide vaccine for treatment of HLA-A*24:02$^+$ ATL patients. However, because we were concerned that CTL induction of hTERT peptide vaccine might have a tendency to be impeded by the regulatory T-cell function of ATL tumor cells,[21] we focused on developing a redirected T-cell–based adoptive immunotherapy targeting hTERT to allow administration of a number of hTERT-specific CTLs directly.

To this end, we cloned the full-length rearranged *TCR-α/β* genes from K3-1, the HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific CTL clone.[19] With codon optimization of the constant regions, we inserted them into our new souped-up second-generation 2A peptide-based *siTCR* vector to accomplish an increased expression level of the introduced TCR, carrying small interfering RNAs for the endogenous *TCR-α/β* genes in the redirected T cells (hTERT-*siTCR* vector).[26,27,34] The *siTCR* vector system makes it possible to simultaneously accomplish profound suppression of endogenous *TCR* genes and markedly increase the cell-surface expression of the introduced TCR, resulting in upregulated antitumor reactivity,[34] thus leading to inhibition of mispaired TCR formation between the endogenous and introduced TCR-α and -β chains, and lowering the potential risk of lethal graft-versus-host disease.[39] We found that both allogeneic and autologous gene-modified CD8$^+$ T cells using the hTERT-*siTCR* vector successfully killed ATL tumor cells both in vitro and in vivo (Figures 4-7), but not normal cells, including steady-state hematopoietic progenitor cells (Figure 5B). The introduced cytocidal activity against ATL tumor cells mediated by these gene-modified CTLs was actually accomplished through recognition of the HLA-A*24:02/hTERT$_{461\text{-}469}$ complex on the surface of ATL tumor cells (Figures 3 and 4).

Clinical studies of anticancer immunotherapy targeting hTERT have not demonstrated any significant adverse events so far.[14-17,20] However, for clinical application, because a number of activated gene-modified hTERT-specific CTLs would be administered at once, it would again be necessary to be mindful of on-target adverse events against normal tissues that constitutively express the *hTERT* gene.[10,40] Notably, any impairment of hematopoiesis would be the major concern. In this study, both allogeneic and autologous gene-modified effector CD8$^+$ T cells expressing hTERT-specific TCR from adult peripheral lymphocytes, and CB lymphocytes did not kill CB-CD34$^+$ cells representing steady-state hematopoietic progenitors (Figure 5B). By using cytokine-driven myeloid differentiation with CB-CD34$^+$ cells, gene-modified CTLs targeting hTERT showed a slight cytocidal effect against differentiated and highly proliferating subsets of CD34$^+$CD33$^+$ and CD34$^-$CD33$^+$ cells but spared CD34$^+$CD33^{dim} cells (supplemental Fig 2A). Additionally, contrary to resting CD4$^+$ cells and CD19$^+$ cells, highly mitotic polyhydroxic acid–stimulated CD4$^+$ cells and CD19$^+$ EBV LCLs became sensitive to effector CTLs because of increased expression of *hTERT* mRNA, the latter being more salient (Figure 5B and supplemental Fig 2B). Taken together, our findings suggest that gene-modified hTERT-specific CTLs will

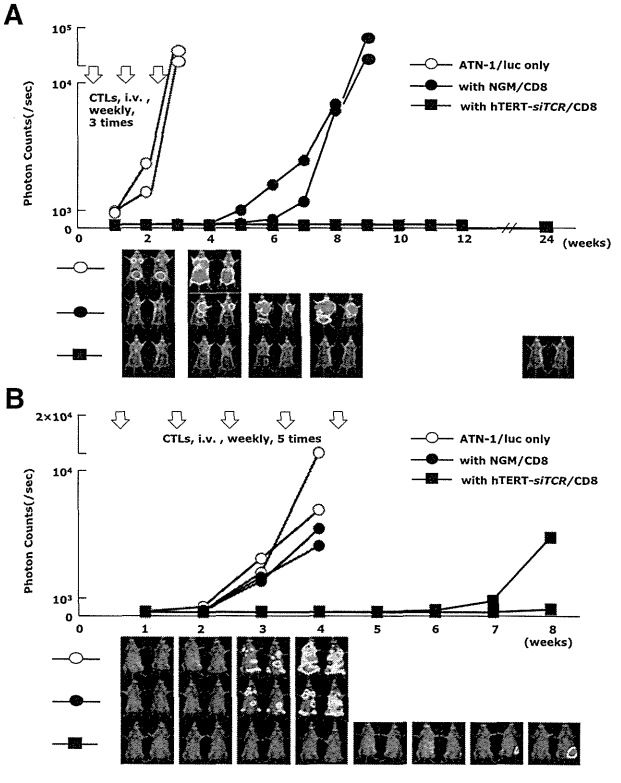

Figure 7. Anti-ATL reactivity of hTERT-*siTCR*–transduced CD8$^+$ T cells in vivo. (A) Winn assay. NOG mice were coinjected with a luciferase-transduced HLA-A*24:02$^+$ ATL cell line (ATN-1/luc) (5 × 10^5) and either 2.5 × 10^6 hTERT-*siTCR*–transduced (hTERT-*siTCR*/CD8) or NGM/CD8$^+$ T cells (n = 2 per group). Subsequently, 3 weekly infusions of the respective CD8$^+$ T-cell populations (2.5 × 10^6 cells per infusion) were administered intravenously (i.v.). Tumor growth was monitored every 7 days by using bioluminescence assay. Nontreated ATN-1/luc cells were similarly inoculated into NOG mice (n = 2) as a control. Although NGM/CD8 activated using OKT-3 and rhIL-2 suppressed tumor growth to some extent, hTERT-*siTCR*/CD8 durably suppressed tumor growth for longer than 6 months. (B) Therapeutic adaptive transfer model. NOG mice were intravenously inoculated with 5 × 10^5 ATN-1/luc cells. Four days later, intravenous administration of either 5 × 10^6 hTERT-*siTCR*/CD8 or NGM/CD8 (n = 2 per group) was started once a week for a total of 5 infusions. NOG mice given only ATN-1/luc cells (n = 2) were used as a control. In comparison with NGM/CD8, therapeutically infused hTERT-*siTCR*/CD8 also obviously suppressed the tumor cell growth within the 8-week observation period. Serial images of the bioluminescence assay demonstrate tumor growth in each group.

spare steady-state hematopoietic progenitor cells. However, to ensure safety, it would be better to avoid the active recovery phase of bone marrow after chemotherapy, notably under granulocyte colony-stimulating factor support, and also the acute infectious period in which immune-cell components are stimulated.

Another likely problem in clinical practice is that heavily pretreated peripheral lymphocytes from ATL patients might fail to proliferate. Proliferative activity of therapeutically infused gene-modified T cells in vivo is an important prerequisite for a successful outcome.[41] In this connection, although the control of treatment-related graft-versus-host disease still remains unsolved, use of CB lymphocytes has been investigated.[42] In this study, gene-modified CB-CD8$^+$ T cells from 2 donors successfully killed ATL tumor cells but spared autologous steady-state CB-CD34$^+$ cells (supplemental Figure 1-(2)). Compelling lack of suitable allo-HSCT donors for patients of advance age with ATL will encourage the application of CB transplantation using reduced-intensity preconditioning in the near future. Genetic redirection of CB lymphocytes using tumor antigen–specific *TCR* gene transfer will also play a considerable role.

Conversely, because hTERT is overexpressed in various kinds of cancer,[10] this approach may have widespread potential clinical application. Furthermore, the clinical availability of a new defucosylated anti-CCR4 mAb for treatment of ATL[43] can be reasonably anticipated to diminish regulatory T cells, the key player in the immunosuppressive microenvironment in patients with cancer,[44] because CCR4 is also expressed on regulatory T cells.[45] Therefore, hTERT-targeting immunotherapy after preconditioning with this anti-CCR4 mAb may become a realistically promising treatment option not only for ATL, but also for other malignancies.

In summary, using a newly established hTERT-*siTCR* vector, we have demonstrated the feasibility of anti-ATL redirected T-cell–based adoptive immunotherapy targeting hTERT, notably for patients who are ineligible for allo-HSCT. Further studies will be needed to investigate the clinical safety and utility of this novel therapy.

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Authorship

Contribution: Y.M. performed the research and wrote the paper; H.F. designed and performed the research, wrote and edited the paper and provided financial support; H.A., F.O., and T.O. performed the research and discussed the experimental results; T.A. interpreted the experimental results and provided financial support; T.I., S.O., J.M., K.K., and H.S. provided materials and discussed the experimental results; and M.Y. discussed and interpreted the experimental results and provided financial support.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Establishment of animal models to analyze the kinetics and distribution of human tumor antigen-specific CD8$^+$ T cells

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ABSTRACT

Many patients develop tumor antigen-specific T cell responses detectable in peripheral blood mononuclear cells (PBMCs) following cancer vaccine. However, measurable tumor regression is observed in a limited number of patients receiving cancer vaccines. There is a need to re-evaluate systemically the immune responses induced by cancer vaccines. Here, we established animal models targeting two human cancer/testis antigens, NY-ESO-1 and MAGE-A4. Cytotoxic T lymphocyte (CTL) epitopes of these antigens were investigated by immunizing BALB/c mice with plasmids encoding the entire sequences of NY-ESO-1 or MAGE-A4. CD8$^+$ T cells specific for NY-ESO-1 or MAGE-A4 were able to be detected by ELISPOT assays using antigen presenting cells pulsed with overlapping peptides covering the whole protein, indicating the high immunogenicity of these antigens in mice. Truncation of these peptides revealed that NY-ESO-1-specific CD8$^+$ T cells recognized D^d-restricted 8mer peptides, NY-ESO-1$_{81\text{-}88}$. MAGE-A4-specific CD8$^+$ T cells recognized D^d-restricted 9mer peptides, MAGE-A4$_{265\text{-}273}$. MHC/peptide tetramers allowed us to analyze the kinetics and distribution of the antigen-specific immune responses, and we found that stronger antigen-specific CD8$^+$ T cell responses were required for more effective anti-tumor activity. Taken together, these animal models are valuable for evaluation of immune responses and optimization of the efficacy of cancer vaccines.

1. Introduction

A number of cancer vaccine strategies targeting tumor antigens recognized by the human immune system have been tested [1–3]. While many of these cancer vaccines elicited measurable immune responses detectable in peripheral blood mononuclear cells (PBMCs), only a subset of treated patients experienced clinical benefits, such as tumor regression [4,5]. Because of the weak clinical effectiveness of currently available cancer vaccines, not only new immunogenic antigens, effective adjuvant formulations, vectors or vaccination methods but also new methodologies to evaluate efficacy of cancer vaccines are required.

NY-ESO-1, a germ line cell protein detected by SEREX (serological identification of antigens by recombinant expression cloning) using the serum of an esophageal cancer patient, is often expressed by cancer cells, but not by normal somatic cells [3,6]. This ideal expression pattern facilitated the study of this antigen; including immuno-monitoring of cancer patients with NY-ESO-1-expressing tumors and clinical trials that focused on NY-ESO-1 [3]. While these studies have revealed that a number of different cancer vaccines, including short and overlapping peptides, protein, viral vectors and DNA, resulted in development of measurable immune responses,

Abbreviations: APC, antigen presenting cells; CTL, cytotoxic T lymphocyte; dLN, draining lymph node; ELISPOT assay, enzyme-linked immunospot assay; IFN, interferon; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells.

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correlations between immunological and clinical responses were often week or difficult to observe [3].

MAGE-A4, another cancer/testis (CT) antigen, elicits MAGE-A4-specific $CD4^+$ and $CD8^+$ T cell responses in some patients with MAGE-A4-expressing cancers, indicating that MAGE-A4 is also an immunogenic protein [7–9]. We have recently reported a novel MAGE-A4 epitope presented by human leukocyte antigen (HLA)-A*2402 using a $CD8^+$ T cell clone 2-28 [9]. As this clone effectively killed tumor cell lines that expressed both MAGE-A4 and HLA-A*2402, this antigen could be a candidate for a cancer vaccine.

Given the poor correlation between the immune responses detected in PBMCs and clinical responses [2,3,5], it is necessary to re-evaluate existing cancer immunotherapy strategies in detail using animal models, namely reverse translational research. To this end, we developed animal models involving human tumor antigens, such as NY-ESO-1 or MAGE-A4 in this study.

2. Materials and methods

2.1. Mice

Female BALB/c mice were purchased from SLC Japan (Shizuoka, Japan) and used at 7–10 weeks of age. They were maintained at the Animal Center of Mie University Graduate School of Medicine (Mie, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine.

2.2. Antibodies and reagents

Anti-H2-K^d (KD40, mouse IgG2a), anti-H2-D^d (DD98, mouse IgG2a), and anti-H2-L^d (30-5-7, mouse IgG2a) were produced and purified from each hybridoma. FITC-conjugated anti-CD8 mAb (53-6.7, rat IgG2a) and APC-conjugated anti-CD4 mAb (GK1.5, rat IgG2b) were purchased from BD Biosciences (Franklin Lakes, NJ). PE-conjugated anti-Foxp3 mAb (Fjk16s, rat IgG2a) was purchased from eBiosciences (San Diego, CA). Synthetic NY-ESO-1 and MAGE-A4 peptides (summarized in Supplementary Table 1) were obtained from Sigma Genosys (Hokkaido, Japan).

2.3. Immunization using a gene gun

Naive BALB/c mice were immunized twice at two-week intervals. Gold particles coated with 1 μg of each plasmid DNA were prepared and delivered into the shaved skin of the abdominal wall of BALB/c mice using a Helios Gene Gun System (BioRad, Hercules, CA) at a helium discharge pressure of 350–400 psi, as described previously [10,11].

2.4. Cell isolation

Spleen cell suspensions were mixed with CD8 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated into $CD8^+$ T cells by positive selection on a MACS column. The isolated $CD8^+$ T cell populations were confirmed to contain >95% $CD8^+$ T cells.

2.5. Enzyme-linked immunospot (ELISPOT) assay

The number of IFN-γ secreting antigen-specific $CD8^+$ T cells was assessed by ELISPOT assay as described previously [10,11]. Briefly, purified $CD8^+$ T cells were cultured for 24 hours with 5×10^5 irradiated CD90-depleted splenocytes pulsed with the indicated peptides in 96-well nitrocellulose-coated microtiter plates (Millipore, Bedford, MA) coated with rat anti-mouse IFN-γ mAb (R4-6A2, BD Biosciences). Spots were developed using biotinylated anti-mouse IFN-γmAb (XMG1.2, BD Biosciences), alkaline phosphatase conjugated streptavidin (MABTECH, Sweden) and alkaline phosphatase substrate kit (BioRad), and subsequently counted.

2.6. ELISA

96-well flat-bottomed microliter plates (Immuno-NUNC) were coated with 20 ng/50 μml of NY-ESO-1 or MAGE-A4 protein, respectively, at 4 °C overnight. Wells were blocked with 1% BSA/PBS for 1 hour at room temperature and washed three times. Serum (1:100 dilution) was added and incubated at 4 °C overnight. After washing, goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Promega, Madison, WI) was added (1:5000 dilution). Two hours later, color was developed with TMB substrate solution (Thermo scientific, IL) and stopped with H_2SO_4. The absorbance was measured at 450 nm and calculated after subtraction of the absorbance value of control wells without sera.

2.7. Flow cytometry and tetramer staining

Tetramer staining was performed as described previously [11]. Briefly, cells were stained with PE-labeled NY-ESO-$1_{81-88}/D^d$ or MAGE-$A4_{265-273}/D^d$ tetramers (prepared at the Ludwig Institute Core Facility, Lausanne, Switzerland) for 10 minutes at 37 °C before additional staining of surface markers for 15 minutes at 4 °C. After washing, the results were analyzed on FACSCanto (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

2.8. Tumors

CT26 is a colon epithelial tumor derived by intrarectal injections of N-nitroso-N-methylurethane in BALB/c mice [12]. CT26 expressing NY-ESO-1 or MAGE-A4, a human cancer/testis antigen were established as described previously [11,13].

2.9. Statistical analysis

Values were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test. *p* values <0.05 were considered statistically significant.

3. Results

3.1. NY-ESO-1-specific $CD8^+$ T cells recognize D^d-restricted NY-ESO-1_{81-88} peptide

We analyzed the minimal epitope recognized by NY-ESO-1-specific $CD8^+$ T cells after immunization with NY-ESO-1. To this end, we employed a Helios Gene Gun System as we have previously detected NY-ESO-1-specific $CD8^+$ T cell responses [10,11]. To identify minimal epitopes, naive BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequence of NY-ESO-1. $CD8^+$ T cells were obtained from spleens and specific T cell responses were analyzed by ELISPOT assay using peptide pools shown in Supplementary Table 1. A significant number of NY-ESO-1-specific $CD8^+$ T cells was detected against peptide pool #3 (Fig. 1A). To identify the NY-ESO-1 epitope, NY-ESO-1-specific $CD8^+$ T cells were stimulated with each of these peptides. IFN-γ secretion was observed when NY-ESO-1-specific $CD8^+$ T cells were stimulated with 71–90 and 81–100 NY-ESO-1 peptides, suggesting the presence of a minimal epitope within 81–90 residues (Fig. 1B). To determine the minimal epitope, the 81–90 peptide of NY-ESO-1 was further truncated. NY-ESO-1-specific $CD8^+$ T cells recognized the 80–88 and 81–88, but not 80–87 or 82–88 peptides, thus

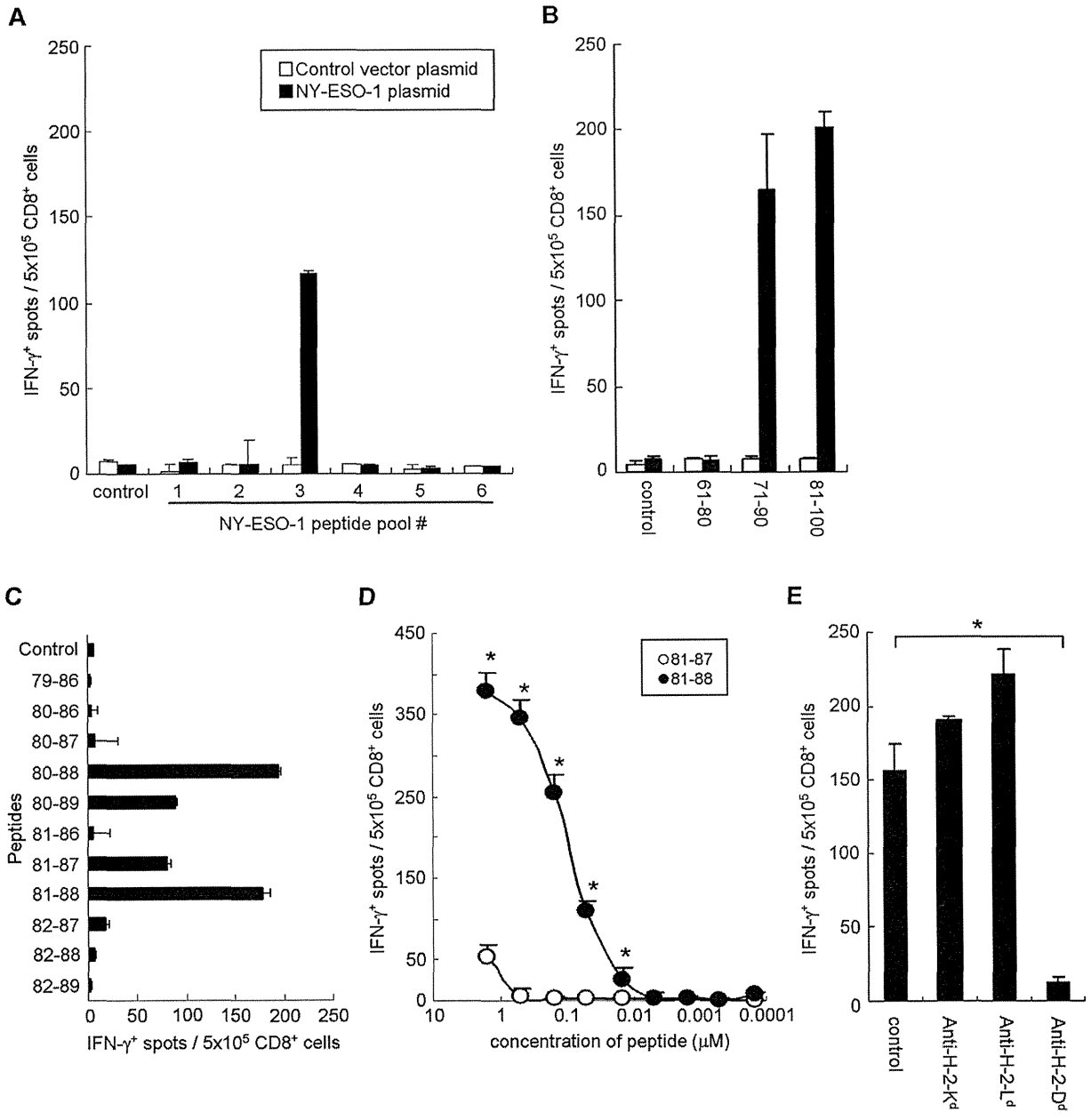

Fig. 1. NY-ESO-1-specific $CD8^+$ T cells recognize D^d-restricted NY-ESO-1_{81-88} peptide. (A–C) BALB/c mice were immunized by gene gun twice at two-week intervals with plasmids encoding the entire sequence of NY-ESO-1. $CD8^+$ T cells were obtained from spleens and specific T cells were analyzed with ELISPOT assay using APCs pulsed with the indicated peptides. (D) Avidity of induced NY-ESO-1-specific $CD8^+$ T cells was analyzed with ELISPOT assay using APCs pulsed with graded doses of peptides ranging from 3 to 0.0001 μM. (E) MHC restriction of induced NY-ESO-1-specific $CD8^+$ T cells was analyzed with ELISPOT assay by the addition of anti-H-2-K^d, anti-H-2-D^d or anti-H-2-L^d mAbs. These experiments were repeated two to four times with similar results. Data are mean ± SD.

the minimal epitope was identified to be NY-ESO-1_{81-88} peptide (Fig. 1C).

To confirm this, graded doses of the peptides were pulsed on antigen presenting cells (APCs) and specific IFN-γ secretion was analyzed by ELISPOT assay. NY-ESO-1-specific $CD8^+$ T cells were high-avidity, and capable to recognize as little as 30 nM of peptide (Fig. 1D), confirming that NY-ESO-1_{81-88} peptide is the minimal epitope. Next, we assessed the restriction of this response using blocking antibodies. NY-ESO-1-specific $CD8^+$ T cell responses were completely blocked by addition of anti-H-2-D^d mAb (Fig. 1E). Taken together, NY-ESO-1-specific $CD8^+$ T cells recognize D^d-restricted NY-ESO-1_{81-88} peptide.

3.2. *MAGE-A4-specific $CD8^+$ T cells recognize D^d-restricted MAGE-$A4_{265-273}$ peptide*

To establish a MAGE-A4 animal model, we determined the minimal epitope of MAGE-A4-specific $CD8^+$ T cells after immunization with MAGE-A4. Naive BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequence of MAGE-A4. Splenic $CD8^+$ T cells were prepared and specific T cell responses were analyzed by ELISPOT assay using peptide pools shown in Supplementary Table 1. MAGE-A4-specific $CD8^+$ T cells were induced in mice immunized with plasmids encoding MAGE-A4 within peptide pool #5 (Fig. 2A). To elucidate the dominant

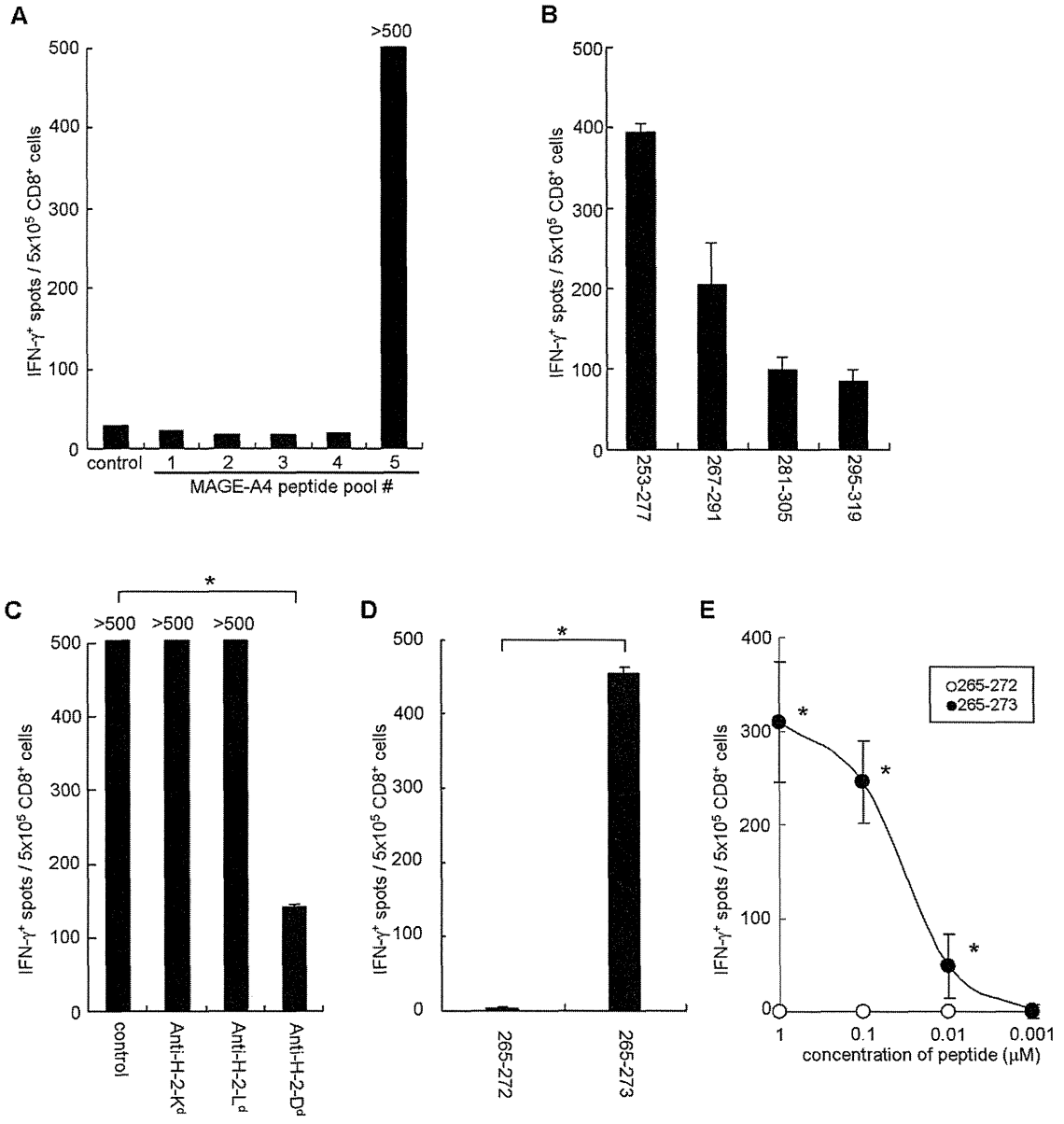

Fig. 2. MAGE-A4-specific CD8+ T cells recognize D^d-restricted MAGE-$A4_{265-273}$ peptide. (A, B) BALB/c mice were immunized by gene gun twice at two-week intervals with plasmids encoding the entire sequence of MAGE-A4. CD8+ T cells were obtained from spleens, and specific T cells were analyzed with ELISPOT assay using APCs pulsed with the indicated peptides. (C) MHC restriction of induced MAGE-A4-specific CD8+ T cells was analyzed with ELISPOT assay by the addition of the indicated mAb. (D) MAGE-$A4_{253-277}$ was subjected to BIMAS program and the highest score within MAGE-$A4_{253-277}$ for a D^d binding motif was predicted in 265–272 and 265–273 of MAGE-A4. These predicted peptides were analyzed with ELISPOT assay for identification of MAGE-A4 epitope peptide. (E) Avidity of MAGE-A4-specific CD8+ T cells was analyzed with ELISPOT assay using APCs pulsed with graded doses of peptides. These experiments were repeated two to four times with similar results. Data are mean ± SD.

MAGE-A4 epitope, MAGE-A4-specific CD8+ T cells were stimulated with each of the peptides from pool #5. The 253–277 peptide was most effective for stimulating MAGE-A4-specific CD8+ T cells (Fig. 2B). We next assessed the restriction of this response using blocking antibodies. MAGE-A4-specific CD8+ T cell responses were completely blocked by anti-H-2 D^d mAb (Fig. 2C). Given the H-2 D^d restriction of this CD8+ T cell response, we employed computer-based BIMAS program to predict optimized MHC class I epitope within the MAGE-$A4_{253-277}$ peptide. This program ranks all the possible MHC class I epitopes within a given polypeptide sequence. MAGE-$A4_{253-277}$ was subjected to this program and the highest score within MAGE-$A4_{253-277}$ for a D^d binding motif was predicted in 265–272 and 265–273 of MAGE-A4 (Supplementary Table 2). MAGE-A4-specific CD8+ T cells recognized the 265–273, but not 265–272 peptides, thus the minimal epitope was considered to be the MAGE-$A4_{265-273}$ peptide (Fig. 2D). To confirm this minimal epitope, graded doses of peptides were pulsed on APC and specific IFN-γ secretion was analyzed by ELISPOT assay. MAGE-A4-specific CD8+ T cells were high avidity, and could recognize as little as 10 nM of the peptide (Fig. 2E). We conclude that MAGE-A4-specific CD8+ T cells recognize D^d-restricted MAGE-$A4_{265-273}$ peptide.

3.3. Kinetics and distribution of NY-ESO-1/MAGE-A4-specific CD8+ T cells after gene gun immunization

Next, we generated MHC/peptide tetramers based on the data of minimal epitope and MHC restriction for NY-ESO-1/MAGE-A4-specific CD8+ T cells. BALB/c mice were immunized with plasmids