Induction of eEF2-specific antitumor CTL responses *in vivo* by vaccination with eEF2-derived 9mer-peptides

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Abstract. Eukaryotic elongation factor 2 (eEF2) is an essential factor for protein synthesis. Previous studies have shown that the *eEF2* gene was overexpressed and plays an oncogenic role in various types of cancers and that eEF2 gene product elicited both humoral immune responses to produce eEF2-specific IgG autoantibody in cancer-bearing individuals and cellular immune responses to induce eEF2 peptide-specific cytotoxic T lymphocytes (CTLs) *in vitro*. The purpose of the present study was to induce eEF2-specific, antitumor CTL responses *in vivo* by vaccination with MHC class I-binding eEF2-derived peptide. First, two mouse MHC class I-restricted eEF2-derived, 9-mer peptides, EF17 (17-25 aa, ANIRNMSVI) and EF180 (180-188 aa, RIVENVNVI) were identified as eEF2-specific CTL peptides, and mice were vaccinated intradermally eight times with either EF17 or EF180 peptide emulsified with Montanide ISA51 adjuvant. Cytotoxicity assay showed that eEF2-specific CTLs were induced in both EF17-and EF180-vaccinated mice, and histological study showed no detectable damage in the organs of these mice. Next, to examine *in vivo* antitumor effects of eEF2 peptide vaccination in a therapeutic model, mice were vaccinated four times with one each of the two eEF2 peptides at weekly intervals after implantation of eEF2-expressing leukemia cells. The vaccination with eEF2 peptides induced eEF2-specific CTLs and suppressed tumor growth, and disease-free survival was significantly longer in EF180-vaccinated mice compared to control mice. The survival was associated with the robustness of eEF2-specific CTL induction. These results indicate that vaccination with MHC class I-binding eEF2 peptide induced eEF2-targeting, antitumor CTL responses *in vivo* without damage to normal organs, which provided us a rationale for eEF2 peptide-based cancer immunotherapy.

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Abbreviations: BFA, brefeldin A; CFU-GM, colony-forming-unit granulocyte-macrophage; CTLs, cytotoxic T lymphocytes; eEF2, eukaryotic elongation factor 2; eEF2K, eukaryotic elongation factor 2 kinase; EPO, erythropoietin; IFA, incomplete Freund's adjuvant; irAE, immune related adverse events; SPF, specific pathogen-free; TAAs, tumor-associated-antigens; TAP, a transporter associated with antigen processing; rIL-2, recombinant interleukin-2

Key words: eEF2, CTL, cancer immunotherapy, peptide vaccine, tumor associate antigen, eukaryotic elongation factor 2

Introduction

After decades of development, immunotherapy has arrived as a potent cancer therapeutic option (1). This is best demonstrated by the clinical efficacy of immune checkpoint inhibitors targeting CTLA-4/CD80/CD86 and PD-1/PD-L1 in cutaneous melanoma and other malignancies such as lung cancer (reviewed in refs. 2-7). Cancer immunotherapy is based on the ability of immune system to specifically distinguish and target non-self from self (8). The robustness and specificity of antitumor immune responses are like two wheels of a cart in the success of cancer immunotherapy. The checkpoint inhibitors can induce robust antitumor immune responses through activation of systemic immunity and enhancement of T cell activity by blocking negative signals, enhancing positive signals or altering the cytokine milieu (9). However, a significant population of patients experienced immune-related adverse events (irAE) in the treatment with checkpoint inhibitors although the majority of them were low-grade. Most commonly reported irAEs were gastrointestinal and dermatological, and less commonly endocrine, hepatic and neurological toxicities (10). In addition, it is likely that any successful immunotherapy strategy will rely to some extent on adaptive immunity, since any sustained antitumor immune responses will depend on the development of immunological memory (8). Therefore, immunotherapies targeting tumor-associated-antigens (TAAs) that induce tumor-specific

immune responses are all the more needed in the new era of immune checkpoint inhibitors.

Protein synthesis is a fundamental metabolic process of cells and its deregulation critically affects cellular functions. Protein synthesis (mRNA translation) consists of three steps: initiation, elongation and termination. One of important regulatory mechanisms of protein synthesis is eukaryotic elongation factor 2 kinase (eEF2K), eukaryotic elongation factor 2 (eEF2) pathway. eEF2 is a gene which catalyzes the translocation of the elongated peptidyl-tRNA from A to P sites of the ribosome and plays an essential role in elongation step of protein synthesis (11,12). eEF2K inhibits eEF2 activity through phosphorylation of eEF2 and slows down the rate of protein synthesis (13-15). Growing evidence has shown the importance of eEF2K-eEF2 pathway in physiological and pathological settings (16). In the nervous system, eEF2K-eEF2 pathway is involved in processes such as learning and memory (17-19). In malignancies, eEF2K mRNA levels were increased in glioblastoma and medulloblastoma and the increased eEF2K mRNA expression was associated with poor prognosis, which could be explained by eEF2K-induced resistance to nutrient deprivation (20). In addition, mTORC1 pathway and the oncogenic Ras/Raf/MEK/ERK pathway cooperate to inhibit eEF2K activity through regulation of its phosphorylation (21).

In previous studies, we showed that eEF2 was overexpressed in various types of solid tumors such as intestinal, lung, pancreatic and breast cancers, glioblastoma multiforme and non-Hodkin's lymphoma (22,23). Knockdown of eEF2 by eEF2-specific shRNA inhibited cell growth of these tumor cells and eEF2 promoted progression of G2/M of the cell cycle in association with activation of Akt and a G2/M regulator, cdc2 proteins, resulting in promotion of *in vivo* cancer cell growth (22). Moreover, we identified HLA-A*02:01- or HLA-A*24:02-restricted 9-mer eEF2 peptides and demonstrated that these eEF2 peptides could induce eEF2-specific cytotoxic T lymphocytes (CTLs) from peripheral blood mononuclear cells of healthy volunteers (23). These results indicated that eEF2-targeting, peptide-based cancer immunotherapy should be worth exploring.

In the present study, we identified two mouse MHC class I-restricted eEF2-derived 9-mer peptides, EF17 (17-25 aa) and EF180 (180-188 aa) and examined the safety and efficacy of eEF2-targeting, peptide-based cancer immunotherapy using a mouse model.

Materials and methods

Mice. Male C57BL/6 (H-2D^b) mice were obtained from Clea Japan, Inc. (Tokyo, Japan), maintained in a specific pathogen-free (SPF) containment facility in accordance with the guidelines of the Regulations on Animal Experimentation at Osaka University, and were used for experiments at 6-8 weeks of age. Animal experiments were approved by Osaka University Gene Modification Experiments Safety Committee and Animal Experimentation Committee.

Peptide synthesis and adjuvant. The primary amino acid sequence of mouse eEF2 was analyzed for consensus motifs for 9-mer peptides capable of binding to mice MHC class I molecule (H-2D^b) using peptide binding prediction programs; NetMHC 3.0 (http://www.cbs.dtu.dk/services/NetMHC/), SYFPEITHI (http://www.syfpeithi.de), HLA peptide motif search (http://www-bimas.cit.nih.gov/molbio/hla_bind/) and RANKPEP (http://imed.med.ucm.es/Tools/rankpep.html). Then, the top 2 candidate peptides for H-2D^b, EF17 (17-25 a.a.ANIRNMSVI) and EF180 (a.a.180-188 RIVENVNVI) (Table I) were synthesized in immunological purity (Sigma-Genosys, Hokkaido, Japan). Synthesized peptide was dissolved in distilled water and stored at -20˚C until use. An incomplete Freund's adjuvant (IFA) Montanide ISA 51 was obtained from Seppic S.A. (Orsay, France).

Table I. Binding of mouse eEF2 peptide to H-2D^b molecules.

Peptide	Position		Amino acid sequence
EF17	17-25 aa		ANIRNMSVI
EF180	180-188 aa		RIVENVNVI
Program	**Rank**	**Peptide**	**Affinity (nM)**
NetMHC3.0	1	EF17	130
	2	EF180	414
Program	**Rank**	**Peptide**	**Binding score**
SYFPEITHI	1	EF17	26
	2	EF180	22
HLA peptide	1	EF180	720
Motif search	2	EF17	660
RANKPEP	1	EF17	21.6
	4	EF180	16.2

eEF2, eukaryotic elongation factor 2.

Cells. RMAS, a transporter associated with antigen processing (TAP)-deficient subline of Rauscher leukemia virus-induced lymphoma cell line of C57BL/6 origin (RMA) was kindly provided by Dr K. Kärre (Karolinska Institute, Sweden) through Dr H.-G. Rammensee (University of Tubingen, Germany) (24). C1498, a WT1-non-expressing murine leukemia cell line of C57BL/6 origin, was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). WT1-expressing murine WT1-C1498 (mWT1-C1498) was generated by transduction of C1498 cells with murine WT1 17AA(+)KTS(+) isoform cDNA that was inserted into pcDNA3.1(+) mammalian expression vector (Invitrogen, Tokyo, Japan) (25).

Vaccination schedule and in vivo tumor challenge. For experiments to evaluate the elicitation of eEF2-specific CTL responses and the damage in normal organs by vaccination with mouse eEF2 peptide, either EF17 or EF180 peptide (100 μg) emulsified with Montanide ISA51 was intradermally administered eight times into flank region of mice at one week interval.

To evaluate *in vivo* antitumor effects of mouse eEF2 peptide vaccine, 3x10^5 eEF2 expressing mWT1-C1498 cells in 100 μl of phosphate-buffered saline (PBS) were intradermally implan-

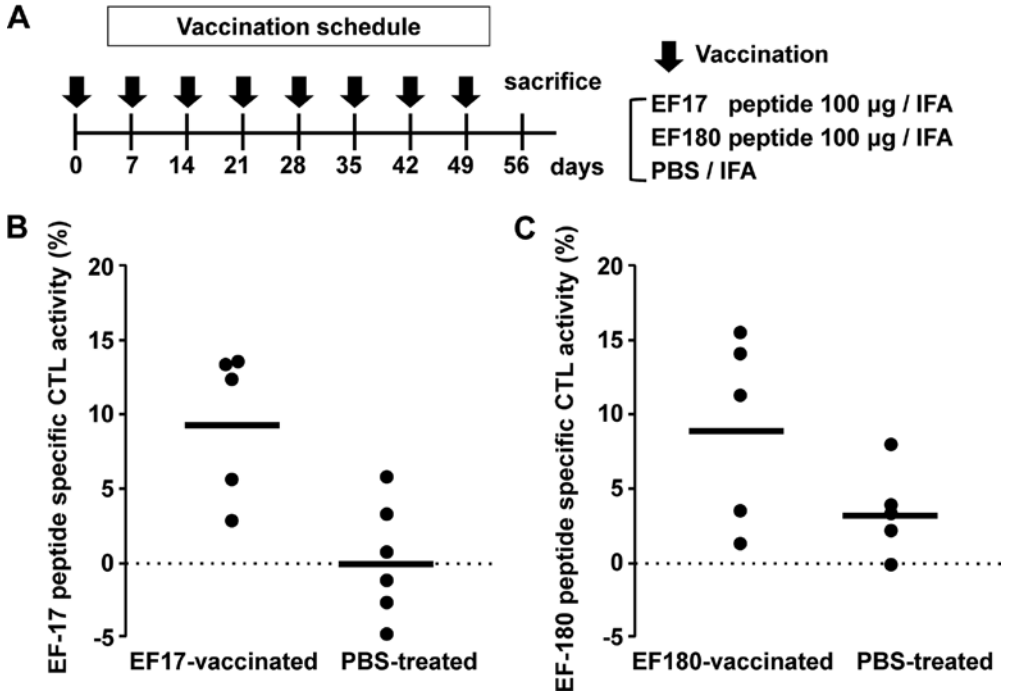

Figure 1. Induction of eEF2-specific CTL responses *in vivo*. Seven days after the last vaccination, splenocytes from Montanide ISA51-adjuvanted EF17- or EF180-vaccinated mice and Montanide ISA51-adjuvanted PBS-treated mice were stimulated *in vitro* with respective eEF2 peptide. Each test group consisted of five mice. (A) Vaccination schedule is shown. (B and C) eEF2-specific cytotoxic activities were assayed in triplicate as cytotoxicties against eEF2 peptide-pulsed and -unpulsed RMAS cells. Cytotoxicty against peptide-pulsed RMAS cells subtracted by that against unpulsed RMAS cells is shown at E/T=45. Bars represent average. The percentage of specific lysis (% specific lysis) was calculated as follows: percentage of specific lysis = (cpm of experimental release - cpm of spontaneous release)/(cpm of maximal release - cpm of spontaneous release) x 100. Radioactivity of the supernatant of the target cell cultures without effector cells and that of the target cells that were completely lysed by the treatment with 1% Triton X-100 was used for spontaneous and maximal release, respectively.

tated to abdominal region of mice on day 0, and then one each of EF17 and EF180 (100 μg) emulsified with Montanide ISA51 or PBS was intradermally administered into flank region of mice on days 1, 8, 15 and 22. Tumor growth was assessed by measuring the longest diameter of the palpable mass.

^{51}Cr release cytotoxicity and flow cytometric cytokine assays. Seven or 8 days after the last vaccination, splenocytes were collected from the vaccinated or PBS-treated mice. The splenocytes were then stimulated with their respective peptides and cultured in complete medium containing 10% heat-inactivated FCS, 45% RPMI-1640 medium, 45% AIM-V medium, 1 x non-essential amino acid (Gibco), 50 μM 2-mercaptoethanol, 50 IU/ml penicillin and 50 μg/ml streptomycin. Two and four days later, recombinant interleukin-2 (rIL-2; Shionogi Biomedical Laboratories, Osaka, Japan) was added to the culture medium at the concentration of 20 IU/ml. After six days of culture, a ^{51}Cr release cytotoxicity assay was performed against eEF2 peptide-pulsed or -unpulsed RMAS cells for eEF2-specific CTL activity, as previously described (25).

For cytokine assay, after 10 days of culture, splenocytes ($2x10^5$ cells) were stimulated with 20 μg/ml of their respective vaccinated peptide and brefeldin A (BFA; Sigma) was added to the concentration of 10 μg/ml for 5 h in 96-well U-bottom plates. After 5 h of culture, the cells were stained with anti-mouse CD3 (17A2) and anti-mouse CD8 (53-6.7) (BD Biosciences, San Jose, CA, USA). Then, the cells were fixed and permeabilized by fixation and permeabilization solution, and stained with anti-mouse IFN-γ (XMG1.2) and anti-mouse TNF-α (MP6-XT22) (both from BD Biosciences). Stained cells were gated for $CD3^+$ $CD8^+$ cells and analyzed by FACSAria (BD Biosciences).

Colony assay. For colony assay of colony-forming-unit granulocyte-macrophage (CFU-GM), bone marrow cells were collected from mouse limbs seven days after the last vaccination, plated at $1x10^4$ cells/plate in methylcellulose medium containing 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml SCF and 3 U/ml erythropoietin (EPO) (MethoCult M3434; Stem Cell Technologies, Vancouver, BC, Canada), and cultured at 37˚C in a humidified incubator under 5% CO_2. Colonies were counted on day 8.

Histological analysis. Formalin-fixed tissue sections of brain, heart, lung, liver, pancreas, kidney, urogenital organs, stomach, intestines, bone marrow and spleen of the EF2 peptide-vaccinated or PBS-treated mice were cut from each paraffin-block. After dewaxing and rehydration, the sections were stained with hematoxylin and eosin. The tissues from EF2-peptide vaccinated mice were pathologically evaluated in comparison with those from PBS-treated mice by a pathologist.

Statistical analysis. The statistical significance in a difference of organ weights and colony numbers among test groups was assessed by Kruskal-Wallis and Student's t-tests, respectively. Significant differences in disease-free survival among test groups were evaluated with the log-rank test.

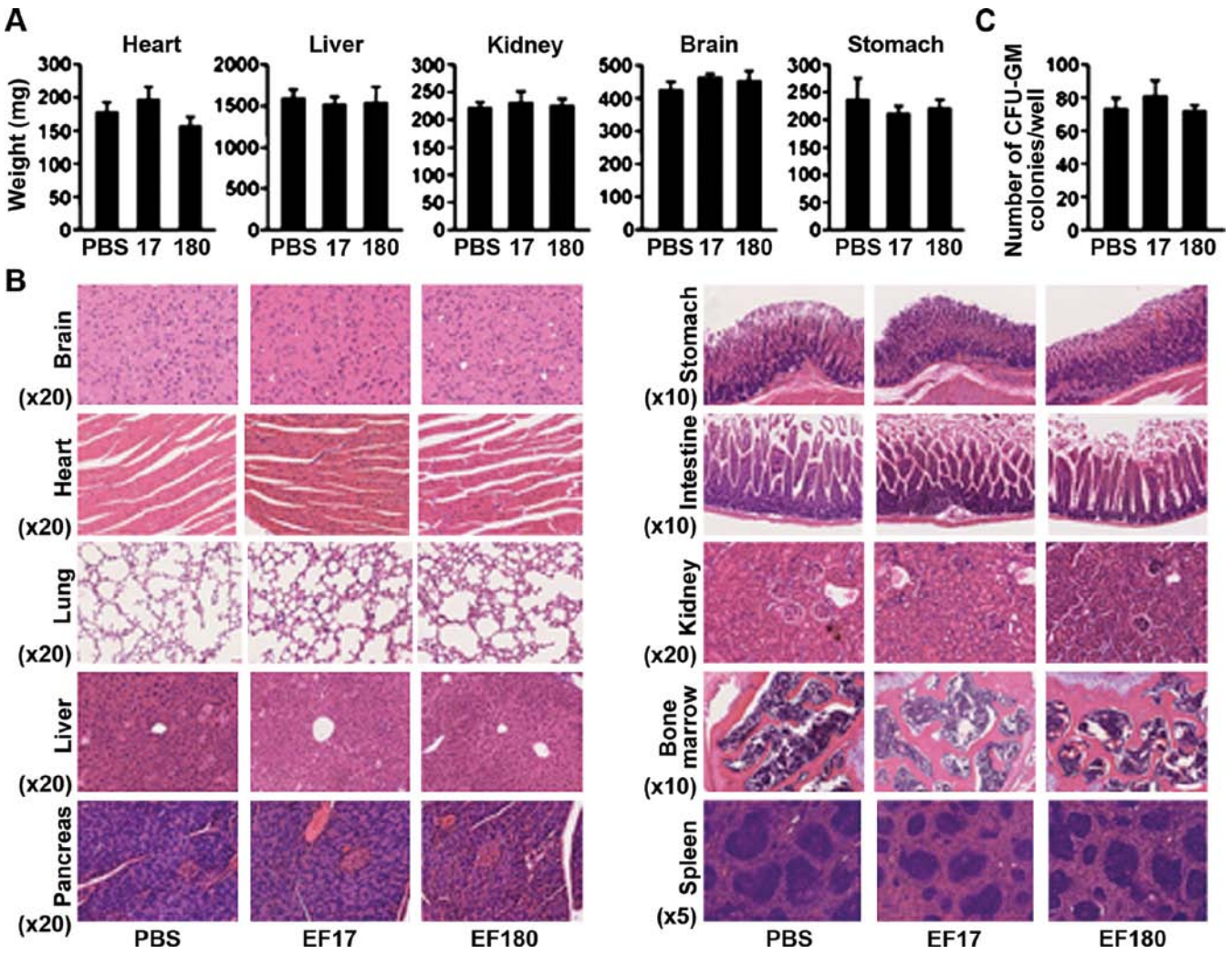

Figure 2. No damage in normal tissues after eEF2 peptide vaccination. To evaluate the safety of EF2 peptide vaccine, the effects of EF2 peptide vaccination on normal organs were examined. (A) The weight of organs. Column, average weight of organs from three mice in each test group; bar, standard error. (B) Representative results of hematoxylin and eosin staining of tissues are shown. No pathological changes such as inflammation, mononuclear cells (lymphocyte) infiltration and tissue destruction and repair were observed. (C) No inhibition of colony-forming ability of bone marrow cells from mice vaccinated with eEF2 peptide. No significant differences in numbers of CFU-GM colonies were found among the three test groups. Bars represent standard errors. (A-C) PBS, PBS-treated; 17 or EF17, EF17 peptide-vaccinated; and 180 or EF180, EF180 peptide-vaccinated.

Results

In vivo induction of eEF2-specific CTL responses. Primary sequence of mouse eEF2 protein was analyzed for 9-mer peptides with consensus motifs that were essential for binding to the mouse MHC class I molecule (H-2D^b) using peptide binding prediction programs and two candidate peptides, EF17 (17-25 aa ANIRNMSVI) and EF180 (180-188 aa RIVENVNVI) (Table I) were selected.

To investigate whether vaccination with these two eEF2 protein-derived 9-mer peptides induced *in vivo* eEF2 peptide-specific CTL responses, mice were eight times vaccinated with one each of Montanide ISA51-adjuvanted EF17 and EF180 at weekly intervals (Fig. 1A). Splenocytes were collected from these mice 7 days after the last vaccination, stimulated *in vitro* with respective eEF2 peptide, and assayed for eEF2 peptide-specific cytotoxic activity against eEF2 peptide-pulsed RMAS cells. The splenocytes from EF17-vaccinated mice showed higher EF17 peptide-specific cytotoxic activity than those from Montanide ISA51-adjuvanted PBS-treated mice (Fig. 1B). Similarly, the splenocytes from EF180-vaccinated mice showed higher EF180 peptide-specific cytotoxic activity than those from Montanide ISA51-adjuvanted PBS-treated mice (Fig. 1C).

These results indicated that vaccination with EF2-derived 9-mer peptides, EF17 and EF180, induced *in vivo* eEF2-specific CTL responses.

No damage of normal tissues by eEF2 peptide vaccination. To evaluate the safety of EF2 peptide vaccination, adverse effects of EF2 peptide vaccination on normal organs were examined (Fig. 2). First, the weights of organs such as heart, liver, kidney, brain and stomach were examined. There were no significant differences in the weights of such organs among PBS-treated, EF17- and EF180-vaccinated mice (Fig. 2A). Next, specimen from formalin-fixed paraffin-embedded blocks of brain, heart, lung, liver, pancreas, stomach, intestine, kidney, bone marrow and spleen were pathologically examined. Representative results are shown in a Fig. 2B. All organs showed normal structure and cellularity in all mice

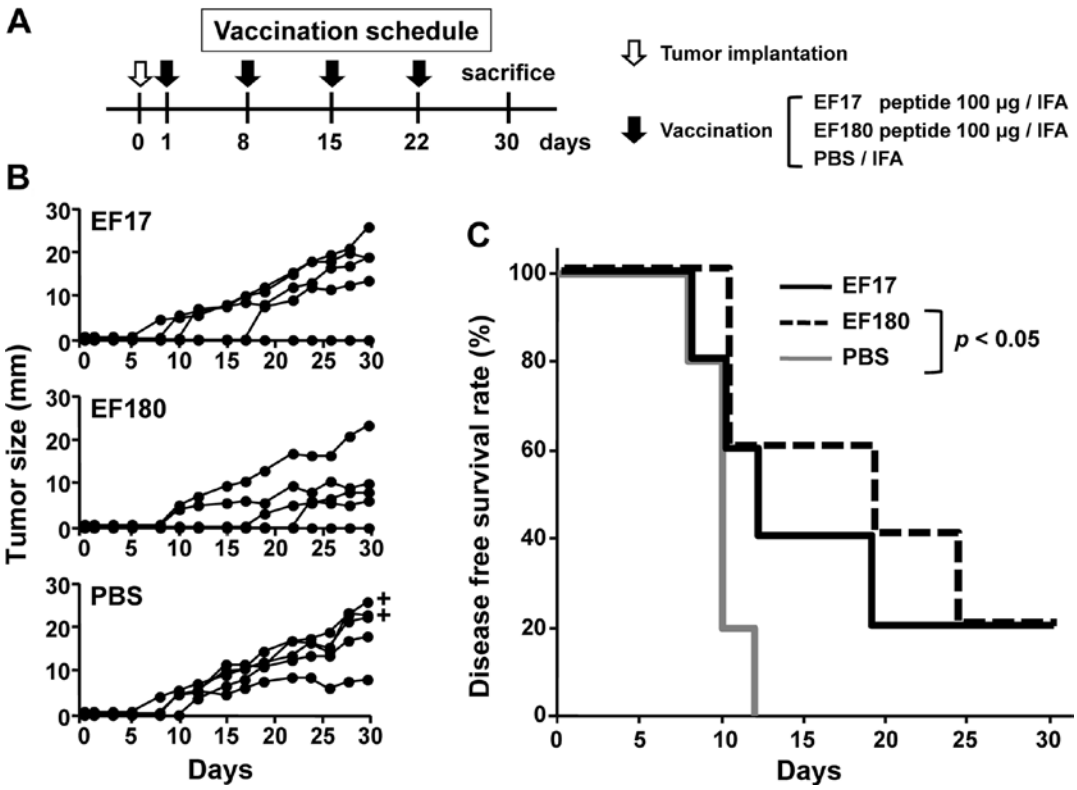

Figure 3. Vaccination with eEF2 peptides induces antitumor effects against eEF2-expressing tumor *in vivo*. (A) *In vivo* tumor cell challenge and vaccination schedule. Mice were abdominally and intradermally implanted with eEF2-expressing mWT1-C1498 cells ($3x10^5$ cells) on day 0, followed by vaccination with one each of Montanide ISA51-adjuvanted EF17 and EF180 peptide or by treatment with Montanide ISA51-adjuvanted PBS on the indicated days. Each test group consists of five mice. (B) Time course of tumor development. Tumor development was observed for 30 days after implantation of eEF2-expressing mWT1-C1498 cells. The longest diameter of tumor was measured and presented as tumor size. (C) Disease-free survival. Solid black, broken and solid gray lines represent disease-free survival curves of EF17- or EF180-vaccinated and PBS-treated mice, respectively. $^*p<0.05$.

examined, and no pathological changes that would be caused by immune response such as mononuclear cells (lymphocytes) infiltration and tissue destruction were observed. Furthermore, the colony-forming ability of bone marrow cells was examined. No differences in numbers of CFU-GM colonies were found among the PBS-treated, EF17- and EF180-vaccinated mice (Fig. 2C).

These results showed that eEF2 peptide vaccination did not damage normal tissues.

Induction of in vivo antitumor effects against eEF2-expressing tumor by eEF2 peptide vaccination. Whether or not vaccination with eEF2-derived 9-mer peptides EF17 or EF180 induced *in vivo* antitumor effects against eEF2-expressing tumor was investigated in a mouse therapeutic model (Fig. 3A). Mice were implanted with eEF2-expressing mWT1-C1498 cells on day 0 and then vaccinated four times with either Montanide ISA51-adjuvanted EF17 or EF180, or treated with Montanide ISA51-adjuvanted PBS at weekly intervals and tumor size (Fig. 3B) and survival rates (Fig. 3C) were assessed. All of the five PBS-treated mice developed tumors until day 15 and two of the five died on day 30. In EF17-vaccinated mice, two did not develop tumors until day 15 and one of the two rejected a tumor. All EF17-vaccinated mice were alive on day 30. In EF180-vaccinated mice, three did not develop tumors until day 15 and one of the three rejected a tumor. All EF180-vaccinated mice were alive on day 30 (Fig. 3C). Disease-free survival rates of PBS-treated, EF17- and EF180-vaccinated mice were 0, 20 and 20%, respectively, on day 30. The disease-free survival rates of EF180-vaccinated mice were significantly higher than those of PBS-treated mice ($p<0.05$) (Fig. 3C).

These results indicated that vaccination with eEF2 peptides, EF17 and EF180 induced *in vivo* antitumor effects against eEF2-expressing tumor.

Correlation between eEF2-specific CTL responses and antitumor effects. To analyze eEF2-specific CTL responses in the therapeutic model, splenocytes were collected from eEF2 peptide-vaccinated mice on day 30 and examined for frequency of $CD8^+$ T cells producing Th1-type cytokines such as IFN-γ and TNF-α, and for eEF2 peptide-specific cytotoxic activity. Flow cytometric analysis showed that EF17-specific IFN-γ- and TNF-α-producing (IFN-γ^+ TNF-α^+) $CD8^+$ T cells were detected in splenocytes from EF17-vaccinated mice with frequency ranging from 2.5 to 6.5%, which was significantly higher than that in splenocytes from Montanide ISA51-adjuvanted PBS-treated mice (Fig. 4A). Similarly, EF180-specific IFN-γ^+ TNF-α^+ $CD8^+$ T cells were detected in splenocytes from EF180-vaccinated mice with frequency ranging from 1.0 to 12.1%, which was higher than that in splenocytes from Montanide ISA51-adjuvanted PBS-treated mice although the difference was not statistically significant (Fig. 4A). When responders and non-responders were defined to be mice that did not and did develop tumors until day 15, respectively, the frequency of eEF2 peptide-specific

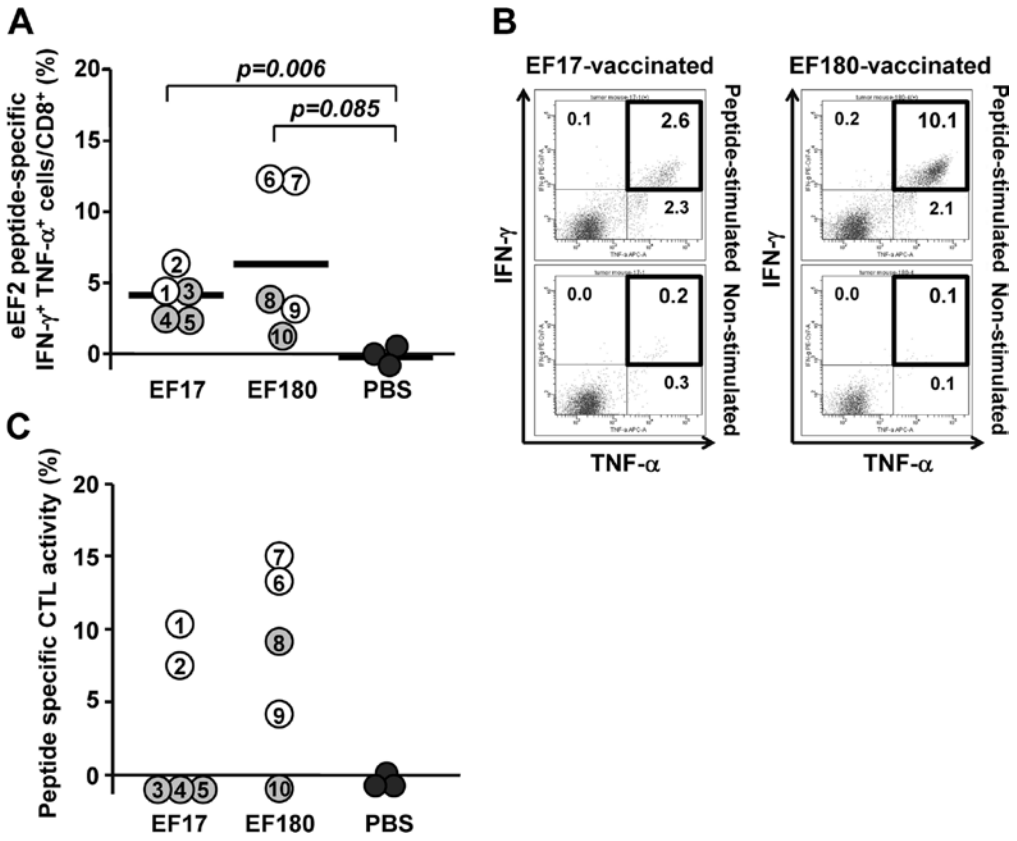

Figure 4. Correlation between eEF2-specific CTL responses and antitumor effects of eEF2 peptide vaccine. Eight days after the last vaccination, splenocytes were collected and stimulated *in vitro* with their respective peptide. Frequency of peptide-specific cytokine production and cytotoxic activities were analyzed. (A) Frequency of eEF2 peptide-specific IFN-γ- and TNF-α-producing cells is shown as frequency of IFN-γ and TNF-α-producing cells in peptide-stimulated $CD8^+$ T cells subtracted by those in unstimulated $CD8^+$ T cells are shown. Horizontal lines represent average value of each group. (B) Frequency of eEF2 peptide-specific IFN-γ- and TNF-α-producing cells in $CD8^+$ cells from EF17- or EF180-vaccinated mice that rejected implanted tumors. Representative results are shown. (C) eEF2 peptide-specific cytotoxic activity of splenocytes from eEF2 peptide-vaccinated mice. Cytotoxicity against peptide-pulsed RMAS cells subtracted by that against unpulsed RMAS cells is shown at E/T=30. (A and C) White and gray circles represent responder and non-responder, respectively. The number of individual mice is indicated in the circles.

IFN-γ^+ TNF-α^+ $CD8^+$ T cells was higher in responders than non-responders in both EF17 and EF180 peptide-vaccinated mice (Fig. 4A). Representative results of flow cytometric analysis of IFN-γ^+ TNF-α^+ $CD8^+$ T cells in splenocyes from mice that rejected the implanted tumors are shown in Fig. 4B.

To examine a correlation between eEF2-specific CTL responses and antitumor effects by eEF2 peptide vaccination, eEF2 peptide-specific cytotoxic activity was compared between responders and non-responders (Fig. 4C). In EF17-vaccinated mice, the two responders induced EF17-specific CTLs whereas all the non-responders could not induce EF17-specific CTLs. In EF180-vaccinated mice, all the three responders induced EF180-specific CTLs whereas one of the two non-responder could not induce EF180-specific CTLs.

These results indicated that vaccination with EF17 or EF180 elicited respective eEF2 peptide-specific CTL responses and that antitumor effect correlated with the robustness of the eEF2 peptide-specific CTL responses.

Discussion

In a previous study, we showed that eEF2 was overexpressed in various types of cancers, that eEF2 gene product elicited spontaneous humoral IgG immune responses in patients with eEF2-expressing tumors, and that HLA-A*02:01- or HLA-A*24:02-restriced 9-mer eEF2 peptides induced eEF2-specific cytotoxic T lymphocytes (CTLs) from peripheral blood mononuclear cells (PBMCs) of healthy volunteers (23). Importantly, induction of eEF2-specific CTLs from PBMCs of healthy volunteers indicated that precursors of eEF2-specific CTLs naturally existed in healthy individuals without cancer. These results strongly indicated that eEF2-expressing tumor-bearing patients should have eEF2-specific CTLs and/or the CTL precursors and thus the eEF2 peptide-based immunotherapy should be effective for such patients. In the present study, two mouse eEF2-derived 9-mer peptides, EF17 (17-25 aa) and EF180 (180-188 aa) were identified as eEF2-specific CTL epitopes, and a mouse model of eEF2 peptide-based immunotherapy was established.

eEF2 is an essential translational elongation factor and is ubiquitously expressed in normal cells. However, eEF2-specific CTLs that were induced by repeated eEF2 peptide vaccination did not give rise to damage in normal cells. This can be explained by the low expression of the target molecule eEF2 in normal cells. Our previous immunohistochemical study showed that eEF2 protein was undetectable in normal cells, whereas, it was overexpressed in tumor cells in the majority of the cases examined. Reportedly, the expression levels of target antigen proteins in tumor cells were associated with recognition of tumor cells by CTLs (26). A threshold level

in antigen expression in tumor cells could be determined for their lysis by CTLs (27). Therefore, normal cells with low or undetectable eEF2 expression should escape from the attack by eEF2-specific CTLs. This is supported by the findings that breast cancer MCF7 cells with undetectable eEF2 protein expression escaped from the attack by eEF2-specific CTLs in an *in vitro* killing assay (23).

eEF2 was overexpressed in 94.0% of non-Hodgkin's lymphoma, 80.4% of lung cancer, 75.0% of glioblastoma multiforme, 75.0% of prostate cancer, 73.3% of esophageal squamous cell carcinoma, 60.7% of pancreatic cancer, 52.4% of head and neck squamous cell carcinoma and 50.0% of breast cancer. Particularly, eEF2 was overexpressed in 100% (10/10) of follicular type of non-Hodgkin's lymphoma (23). Therefore, eEF2 peptide immunotherapy should be applicable to various types of cancers, especially to non-Hodgkin's lymphoma.

The loss of target molecules can be a mechanism of tumor escape from immune surveillance (28). We previously demonstrated that knockdown of eEF2 molecule by eEF2-specific shRNA significantly inhibited tumor cell growth. Furthermore, we showed that eEF2 promoted progression of G2/M in the cell cycle and enhanced *in vitro* and *in vivo* tumor cell growth (22). These results indicated that it was unlikely that loss of eEF2 antigen-mediated tumor cell escape from eEF2-specific cellular immune responses since its loss should be disadvantageous for growth and survival of the tumor cells.

It is well known that $CD4^+$ T as well as $CD8^+$ T cells are critical components of antitumor cellular immune responses (29-32). $CD4^+$ T cells can be involved in generation and activation of $CD8^+$ T cells (33-35) and maintenance of $CD8^+$ T cell memory functions (36,37). Moreover, recent studies have shown that $CD4^+$ T cells could develop cytotoxic activity against tumor cells (38,39). We have previously reported that eEF2-specific IgG autoantibody was detectable in eEF2-expressing tumor-bearing patients (23), indicating that eEF2-specific $CD4^+$ helper T cells had been spontaneously elicited in such patients. Therefore, eEF2 peptide vaccination in clinical settings will induce eEF2-specific $CD8^+$ CTLs and the CTLs could eradicate eEF2-expressing tumor cells in cooperation with spontaneously induced $CD4^+$ helper T cells. Therefore, eEF2-targeting immunotherapy could be a promising treatment strategy for cancer.

Acknowledgements

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