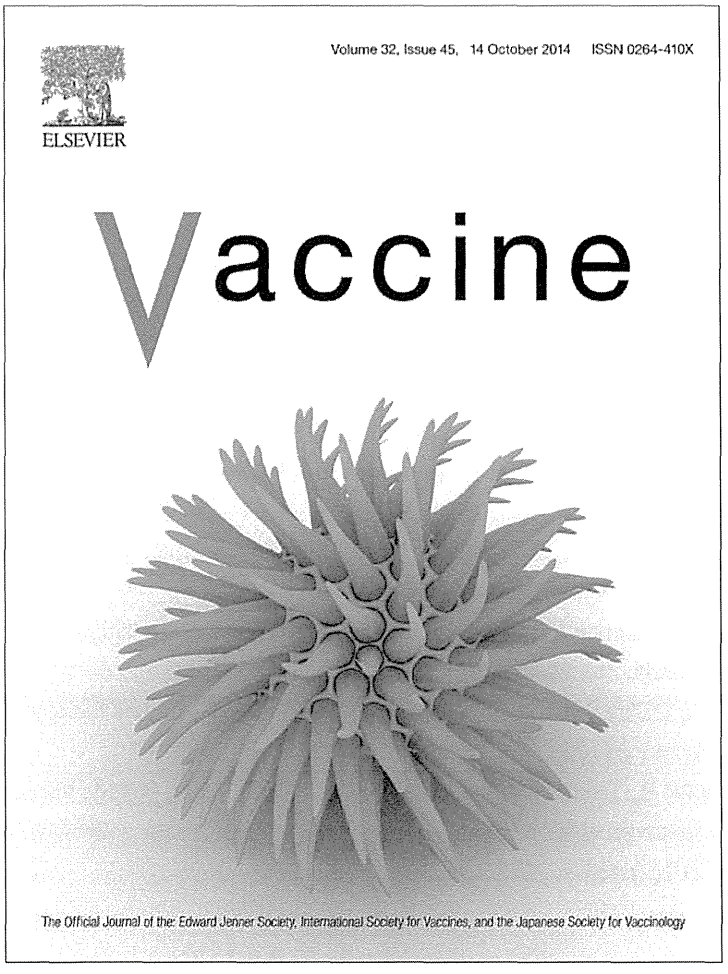

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High expression of MAGE-A4 and MHC class I antigens in tumor cells and induction of MAGE-A4 immune responses are prognostic markers of CHP-MAGE-A4 cancer vaccine☆

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ABSTRACT

Purpose: We conducted a cancer vaccine clinical trial with MAGE-A4 protein. Safety, clinical response, and antigen-specific immune responses were analyzed and the prognostic factors by vaccination were investigated.
Experimental design: Twenty patients with advanced esophageal, stomach or lung cancer were administered MAGE-A4 vaccine containing 300 μg protein subcutaneously once every 2 weeks in six doses. Primary endpoints of this study were safety and MAGE-A4 immune responses.
Results: The vaccine was well tolerated. Fifteen of 20 patients completed one cycle of vaccination and two patients showed SD. A MAGE-A4-specific humoral immune response was observed in four patients who had high expression of MAGE-A4 and MHC class I on tumor cells. These four patients showed significantly longer overall survival than patients without an antibody response after vaccination ($p = 0.009$). Patients with tumor cells expressing high MAGE-A4 or MHC class I antigen showed significantly longer overall survival than those with low expression. Induction of CD4 and CD8T cell responses was observed in three and six patients, respectively, and patients with induction of MAGE-A4-specific IFNγ-producing CD8T cells, but not CD4T cells, lived longer than those without induction.
Conclusions: The CHP-MAGE-A4 vaccine was safe. Expression of MAGE-A4 and MHC class I in tumor tissue and the induction of a MAGE-A4-specific immune response after vaccination would be feasible prognostic markers for patients vaccinated with MAGE-A4.

1. Introduction

The expression of cancer/testis (CT) antigens is normally limited to human germ line cells in the testis and to various types of human cancers [1,2]. Among CT antigens, the melanoma-associated antigen gene (MAGE) family is also known to show such unique expression and to induce spontaneous humoral and cellular immune responses in MAGE-expressing cancer patients [3,4], with the result that they are feasible targets for tumor immunotherapy.

Numerous cancer vaccine strategies are under development and some patients have experienced clinical benefits after vaccination. Among the MAGE family, a phase II cancer vaccine trial with MAGE-A3 protein in non-small-cell lung cancer patients showed 8% reduction of the recurrence rate [5]. Based on the outcome of this phase II study, a randomized double-blind phase III study (MAGRIT trial) with MAGE-A3 protein vaccination was performed [6].

MAGE-A4 is also reported to be expressed in a wide variety of tumors, e.g., 60% esophageal cancer, 50% head and neck cancer, 24% non-small-cell lung cancer, 33% gastric tumor, and 21% Hodgkin's

☆ The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000003188) on February 15, 2010 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

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disease but not in normal tissues besides the testis. MAGE-A4 elicits spontaneous humoral or cellular immune responses in patients with MAGE-A4-expressing non-small-cell lung cancer, head and neck cancer and adult T cell leukemia/lymphoma [3,4,7,8]. High expression of MAGE-A4, as well as other CT antigens, in tumors was correlated with the poor prognosis of patients with bladder cancer, ovarian cancer, non-small-cell lung cancer and head and neck cancer [9–14]. Many MAGE-A4 epitope peptides recognized by CD4 and CD8 T cells in the context of human leukocyte antigen (HLA) class I and class II have been identified, e.g., HLA-A0201 [15,16], HLA-A2401 [17], HLA-B3701 [18], HLA-DP0501, and HLA-DR1403 [19].

Because tumor-specific T cells are considered to be a direct effector of tumor immunity, the expression level of MHC class I on cancer cells is crucial for the prognosis of cancer patients, especially in the case of an immune therapy such as a cancer vaccine. It is reported that deficient MHC class I surface expression is associated with reduced patient survival in colon cancer, gastric cancer and non-small-cell lung cancer [20–23], and is considered to be one of the causes of the immune escape of tumor cells [24,25]. In patients vaccinated with tumor antigens, some papers reported the effect of the expression level of MHC class I on cancer cells on the clinical effect of vaccinated patients, but there are few reports on their prognosis after vaccination [26,27].

In this study, we conducted a cancer vaccine clinical trial with a complex of MAGE-A4 protein and cholesteryl pullulan (CHP) nanoparticles in advanced cancer patients. We monitored and analyzed the safety, clinical effect, humoral and cellular immune responses and expression of antigens in these patients.

2. Materials and methods

2.1. CHP-MAGE-A4 vaccine

The complex of cholesterol-bearing hydrophobized pullulan (CHP) and MAGE-A4 protein (CHP-MAGE-A4) was provided by ImmunoFrontier, Inc. (Tokyo, Japan) [28]. The synthesis, production, formulation and packaging of the investigational agent were performed in accordance with current Good Manufacturing Practices (cGMP) and met the applicable criteria for use in humans. The toxicity of the drug products was assessed using animal models, and stability was monitored during the clinical trial using representative samples of the investigational drug product.

2.2. Study design

A phase I, open-label, single-institutional clinical trial of the CHP-MAGE-A4 vaccine was designed to evaluate the safety, immune response and clinical response. Patients eligible for entry were those who had advanced cancers that were refractory to standard therapy and expressed MAGE-A4 antigen as assessed by immunohistochemistry (IHC). The CHP-MAGE-A4 vaccine containing 300 μg MAGE-A4 protein was administered subcutaneously once every 2 weeks in six doses. Two weeks after the last administration, the safety, immune response and clinical response were evaluated. Thereafter, the vaccine was administered additionally. Clinical response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST ver1.1) [29]. Safety was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) [30]. The protocol was approved by the Ethics Committee of Osaka Universities according to the Declaration of Helsinki. Written informed consent was obtained from each patient before enrolling in the study. The study was conducted in compliance with Good Clinical Practice and was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000003188) on February 15, 2010 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

2.3. MAGE-A4 protein and peptides

For ELISA, recombinant N-His-tagged MAGE-A4 protein was given by Mie University. For Western blot analysis, the MAGE-A4 open reading frame was given by Hokkaido University and was cloned into pGEX-HT plasmid given by Dr. J. Takagi (Osaka University, Osaka, Japan). N-GST-His-tagged MAGE-A4 protein was expressed in M15 *Escherichia coli* cells and purified by Glutathione Sepharose 4B. Finally, recombinant MAGE-A4 protein without a His-tag was purified by TEV protease [31]. For in vitro stimulation of T cells, the following series of 31 MAGE-A4 overlapping peptides spanning the protein was synthesized: 1–20, 11–30, 21–40, 31–50, 41–60, 51–70, 61–80, 71–90, 81–100, 91–110, 101–120, 111–130, 121–140, 131–150, 141–160, 151–170, 161–180, 171–190, 181–200, 191–210, 201–220, 211–230, 221–240, 231–250, 241–260, 251–270, 261–280, 271–290, 281–300, 291–310, and 300–317.

2.4. ELISA

Recombinant protein (0.4 μg/ml) in coating buffer was adsorbed onto 96-well plates and incubated overnight at 4 °C. Plates were washed with phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA). 100 μl of serially diluted serum was added to each well and incubated for 2 h at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells. Ovalbumin (OVA, albumin from chicken egg white; Sigma, St. Louis, MO) was used as the control protein in each assay. The cut-off value of the antibody reaction was 0.47 O.D., calculated from the results of 47 healthy donors with the average + 2 SD.

2.5. Immunohistochemistry (IHC)

IHC was performed using formalin-fixed paraffin-embedded cancer specimens obtained from all patients enrolled in this trial and 57 esophageal cancer patients who had received surgical treatment. Monoclonal antibodies were anti-MAGE-A4 protein (57B), anti-HLA class I (EMR 8–5) and anti-CD8 (clone C8/144B). The reaction was evaluated as +++ (>50% stained cells), ++ (25–50%), + (5–25%), ± (1–5%) and − (<1%) for MAGE-A4 and HLA class I expression.

2.6. In vitro stimulation of CD4 and CD8 T cells

CD8 and CD4 T cells were purified from peripheral blood mononuclear cells (PBMCs) using CD8 Microbeads and a CD4+ T Cell Isolation Kit (Miltenyi Biotec). The remaining cells were used as antigen-presenting cells (APCs) after pulsing with a mixture of 31 MAGE-A4 overlapping peptides. Then, 5×10^5 CD4 or CD8 T cells were cultured with 10×10^5 APCs after irradiation with IL-2 (10 U/mL; Roche Diagnostics) and IL-7 (20 ng/mL; R&D Systems) for 21 days or 8 days, respectively. CD4 or CD8 T cells harvested were re-stimulated with T-APCs pulsed with a mixture of 31 MAGE-A4 overlapping peptides or HIV (p17, 39–51) peptide as the control for 6 h [32].

2.7. IFNγ intracellular staining (ICS)

ICS was performed with an ICS kit (BD Biosciences) according to the manufacturer's instructions followed by treatment with GolgiStop reagent containing monensin (BD Biosciences) for 1 h. Cells

Table 1
Immune responses and clinical responses following CHP-MAGE-A4 vaccination.

Patient ID	Immunization	MAGE-A4-specific immune response							
		Antibody[a]		CD4[b]		CD8[b]		Clinical response	OS (days)
		Pre	Post	Pre	Post	Pre	Post		
P-1	16	–	+	–	+	–	+	PD	218
P-2	13	–	–	–	–	–	–	PD	254
P-3	5	+	nd	nd		nd		NE	(74)
P-4	6	–	–	–	–	–	–	PD	82
P-5	7	+	+	–	–	–	+	PD	206
P-6	15	–	–	–	–	–	–	SD	228
P-7	31	–	++	–	+	–	+	PD	436
P-8	2	–	nd	nd		nd		NE	(42)
P-9	16	–	–	–	–	–	–	PD	340
P-10	7	–	–	–	–	–	–	PD	90
P-11	5	+	nd	nd		nd		NE	(81)
P-12	35	–	+	–	+	–	+	SD	767
P-13	7	–	–	–	–	–	–	PD	129
P-14	9	–	–	–	–	–	–	PD	179
P-15	7	–	–	–	–	–	+	PD	96
P-16	40	–	++	–	–	–	+	PD	1029
P-17	4	–	nd	nd		nd		NE	(63)
P-18	4	–	nd	nd		nd		NE	(66)
P-19	6	–	–	–	–	–	–	PD	92
P-20	7	+	+	–	–	–	–	PD	116

OS: overall survival; PD: progressive disease; SD: stable disease; NE: not evaluated; nd: not done.

[a] Antibody response was determined by ELISA. Antibody response shown here represents O.D. for MAGE-A4 protein: ++ ≥1.0; + >1.0 to ≥0.47; – >0.47.

[b] CD4 and CD8 T cell responses were determined by IFNγ intracellular cytokine staining with those cells stimulated in vitro once. IFNγ-positive cells: +++ >10%; ++ >5% to ≤10%; + >1% to ≤5%; – ≤1%.

were stained with CD8-V450 (clone RPA-T8; BD Biosciences), CD4-V450 (clone RPA-T4; BD Biosciences), CD3-Alexafluor 700 (clone UCHT1; BD Biosciences), eFluor 780-fixable viability dye (eBioscience, San Diego, CA) and IFNγ-FITC (clone 4S.B3; BD Biosciences).

2.8. Western blot

Recombinant protein (20 ng) in sample buffer was boiled for 5 min and subjected to SDS-PAGE with 10–20% polyacrylamide Bio-Rad Ready-Gels (Bio-Rad). After electrophoresis, the membrane was blocked with 5% FCS/PBS and then incubated with patients' sera diluted 1:100 for 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (MBL) was added to the membrane. Signals were developed with a 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chromogenic substrate kit (Bio-Rad). Anti-MAGE-A4 monoclonal antibody (57B) used as the positive control at 1:200 dilution was given by Dr G.C. Spagnoli (University Hospital Basel, Basel, Switzerland).

2.9. Activated regulatory T cells in PBMC

Activated regulatory T cells (Treg) were analyzed by a flow cytometer using CD3-PerCPCy5.5 (clone OKT3; eBioscience), CD4-Alexafluor 700 (clone RPA-T4; eBioscience), CD8-V500 (clone RPA-T8; BD Biosciences), CD45RA-FITC (clone HI100; BD Biosciences), eFluor 780-fixable viability dye (eBioscience) and FoxP3-PE (clone 236A/E7; eBioscience). The details of the assay and the definition of activated Tregs were described previously [33].

2.10. Statistics analysis

Rates of the immune responses 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. All analyses were performed using the SPSS statistical package, version 15.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Patient characteristics

Twenty advanced cancer patients were enrolled: 18 patients with esophageal cancer, a patient with lung cancer and a patient with gastric cancer expressing MAGE-A4 antigen (Supplementary Table). They received 2–40 immunizations and 15 patients completed a cycle of vaccination (Table 1).

3.2. Safety

Grade 1 fever and Grade 1 injection site reactions, e.g., skin redness or pruritus, were observed in 4 and 13 patients, respectively, after vaccination, and improved without any treatment (Supplementary Table). No severe adverse event was observed.

3.3. Clinical response

All patients underwent image analysis and routine physical checks during and after vaccination. An SD response was observed in two esophageal cancer patients, P-6 and P-12, out of 15 patients who completed vaccination (Table 1). In patient P-6, relapsed lymph node metastasis in the right neck after radical esophagectomy showed a 9% increase in its diameter after 6 immunizations with CHP-MAGE-A4. In patient P-12, although the main tumor disappeared after chemotherapy, metastasis in the left lung was observed with a 15% increase in its diameter after a cycle of vaccination. Both patients received additional cycles of CHP-MAGE-A4 vaccination; however, these target lesions showed rapid enlargement after the second cycle.

3.4. Monitoring of humoral immune response

MAGE-A4 antibody in sera obtained from all patients at baseline and 15 vaccine-completed patients two weeks after the final immunization were analyzed by ELISA. Four patients, P-3, P-5, P-11

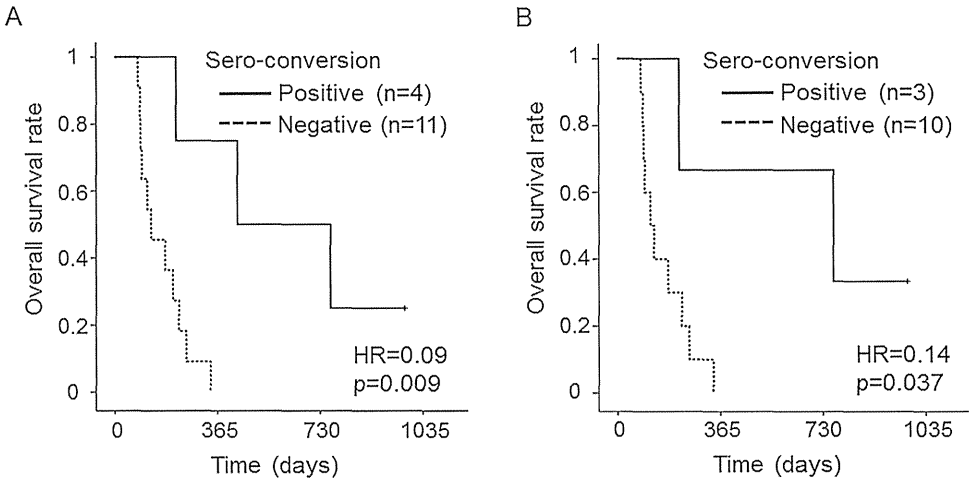

Fig. 1. Antibody production and prognosis. Overall survival of 15 patients and 13 esophageal cancer patients who completed 1 cycle of vaccination and the antibody response determined by ELISA were analyzed. Kaplan–Meier curves illustrate the duration of overall survival of sero-converted patients (solid line) and patients without an antibody response (dotted line) in 15 patients (A) and 13 esophageal cancer patients (B). The hazard ratio (HR) and log-rank *P* value for overall survival comparing patients with positive against negative antibody responses were calculated.

and P-20, showed the production of MAGE-A4 antibody at baseline (sero-positive) while others did not (sero-negative) (Table 1). After vaccination, 4 of 13 sero-negatives among 15 vaccine-completed patients showed increased O.D. values by ELISA and were considered positive serological responses (Supplementary Fig. 1A). No increased response was observed with sera from two sero-positives. These sero-conversions were observed just after a cycle of vaccination in all four patients. Anti-MAGE-A4, but not anti-His-tag, antibody responses in sera from patients P-1, P-12 and P-16 were analyzed by Western blot analysis using recombinant MAGE-A4 protein without any tags (Supplementary Fig. 1B).

Then, the overall survival after the first immunization in sero-conversion positives and negatives was analyzed in 15 vaccine-completed patients. The four sero-converted patients showed prolonged overall survival, significantly longer than that of patients without an antibody response after vaccination (Fig. 1A). When the analysis was limited to esophageal cancer patients, the overall survival of the three sero-converted patients was also significantly longer than that of patients without a MAGE-A4 antibody response (Fig. 1B).

3.5. Immunohistochemical analysis of MAGE-A4, MHC class I and CD8

Expression of MAGE-A4 and MHC Class I antigens on tumor cells was analyzed by IHC using formalin-fixed paraffin-embedded tumor tissues obtained from all enrolled patients (Supplementary Table). Among 15 vaccine-completed patients, high expression of MAGE-A4 (>25% tumor cells) and MHC class I (>5% tumor cells) was observed in tumor tissues from 12 and 12 patients, respectively. Then, we analyzed whether there is any relation between the expression of MAGE-A4 and MHC class I antigens on tumor cells and the induction of immune responses by CHP-MAGE-A4 vaccination. Four of eight patients with high expression of MAGE-A4 or MHC class I antigen on tumor cells showed an antibody response while no patients with low expression of either antigen on tumors showed an antibody response. High expression of both MAGE-A4 and MHC class I antigens was observed on tumor cells from sero-converted patients (Fig. 2A and B). Next, we analyzed whether there is any relation between the expression of those antigens and overall survival by CHP-MAGE-A4 vaccination. Patients with tumor cells expressing high MAGE-A4 or MHC class I antigen showed significantly longer overall survival than those with lower expressions (Fig. 2C and D).

3.6. Induction of MAGE-A4-specific CD4 and CD8 T cell responses

MAGE-A4-specific CD4 and CD8 T cell responses were analyzed by ICS assay using PBMCs obtained from 15 vaccine-completed patients at baseline and 2 weeks after the 6th immunization (Supplementary Fig. 2). MAGE-A4-specific IFNγ-producing CD4 and CD8 T cells were observed in no patient at baseline. After vaccination, induction of a CD4 T cell response was observed in three patients, P-1, P-7, P-12, who showed sero-conversion, and induction of a CD8 T cell response was observed in six patients, P-1, P-5, P-7, P-12, P-15, P-16, who showed antibody production (Table 1). Patients with induction of MAGE-A4-specific IFNγ-producing CD8 T cells, but not CD4 T cells, lived longer than those without induction (Supplementary Fig. 3).

3.7. Impact of CD4+ FoxP3 high+ regulatory T cells on overall survival

The ratio of CD4+ Foxp3 high+ cells in CD3+ T cells was analyzed using PBMCs obtained at baseline from 15 vaccine-completed patients. When the patients were divided by the mean of the ratio, the two SD patients, P-6 and P-12, belonged in the low ratio group (Supplementary Fig. 4A and B). Patients with a low ratio of CD4+ Foxp3 high+ cells in CD3+ T cells showed longer overall survival than patients with a high ratio after vaccination, although it was not significant (Supplementary Fig. 4C).

4. Discussion

We showed that the induction of MAGE-A4-specific immune responses correlated well with the prognosis of patients vaccinated with CHP-MAGE-A4. In our previous study of cancer vaccines with NY-ESO-1 protein [34–39], NY-ESO-1f peptide [40] and NY-ESO-1 overlapping peptide [41], feasible clinical responses were observed in several patients; however, we could not confirm the effects of NY-ESO-1 vaccines on the good prognosis of enrolled patients. There are several reports of successful cancer vaccines which prolonged the overall survival of vaccinated patients [42,43], and some studies revealed that patients with the induction of an antigen-specific CD8 T cell response, but not an antibody response,

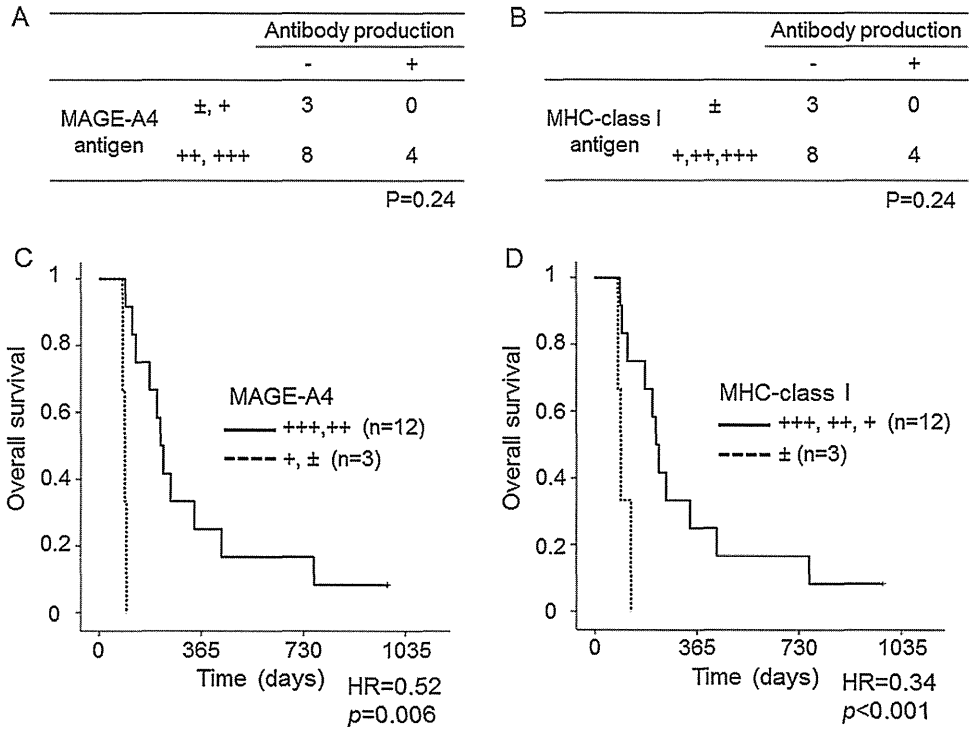

Fig. 2. MAGE-A4 and MHC-class antigen expression and prognosis. MAGE-A4 and MHC-class I were analyzed by immunohistochemical analysis with monoclonal antibodies; anti-pan-MAGE protein (57B), anti-human leukocyte antigen (HLA) class I (EMR 8–5). The reaction was evaluated as +++ (>50% stained cells), ++ (25–50%), + (5–25%), ± (1–5%) and – (<1%) for MAGE-A4 and HLA class I expression. Among 15 vaccine-completed patients, tumor tissues from the four MAGE-A4 sero-converted patients showed higher expression of both MAGE-A4- and MHC-class I-antigens of tumor cells (A and B). The patients with tumor cells expressing higher MAGE-A4 or MHC-class I antigen showed a significantly longer overall survival than those with lower expressions (C and D).

survived longer [44]. Our results of immune monitoring indicated that the induction of not only MAGE-A4 CD8 T cells but also MAGE-A4 antibody responses could be a marker for predicting the good prognosis of patients vaccinated with MAGE-A4 protein. Detection of an antibody response is considered to be a useful tool for monitoring cancer vaccines with protein because it is easy to analyze with sera using ELISA [35]. In our previous studies of NY-ESO-1 antigen, specific humoral and cellular responses were spontaneously induced in patients with NY-ESO-1-expressing tumors, and elicited much more frequently and earlier in patients vaccinated with NY-ESO-1 than MAGE-A4 [39–41]. CT antigens, among tumor antigens, are known to have better immunogenicity because of their unique expression pattern [1]. However, MAGE-A4 antigen might not possess such strong immunogenicity as other CT antigens, for example, NY-ESO-1. Although it is not easy to determine and explain the intent of the immunogenicity of antigenic molecules, one possibility for determining immunogenicity is the immune competition by other molecules. The existence of ubiquitous expressions of other MAGE family members might interfere with the immune response of MAGE-A4 as a tumor antigen [3]. Another possibility is the stability of MHC and antigenic peptide complexes. It is reported that MAGE-A4 epitope peptide combined with HLA-A2 is less stable than Tax10 or influenza matrix epitope peptides but is consistent with common sets of A2-complexes determined by thermal denaturation measurements [15]. Nevertheless, the induction of a MAGE-A4 antibody response was a good marker of the long survival of patients vaccinated with MAGE-A4 protein, indicating that the immunogenicity of MAGE-A4 might be adequate to induce immune responses which can be used for immune monitoring to predict the prognosis of vaccinated patients.

To investigate which factors induce a humoral immune response by MAGE-A4 vaccine, the expression of MAGE-A4 and MHC class I antigens in tumor tissues was analyzed by IHC, and it was shown that the four sero-converted patients had cancers with high expression of both MAGE-A4 and MHC class I. Moreover, overall survival was prolonged in patients with tumors with high expression of MAGE-A4 antigen, suggesting that these patients might have elicited MAGE-A4-specific immune responses to some extent by MAGE-A4 vaccination, resulting in a good prognosis. The weak band observed in sero-negative patient P-16 at baseline by Western blot analysis (Supplementary Fig. 1B) might indicate such an undetectable level of MAGE-A4 immune responses by ELISA, probably due to the property of recombinant MAGE-A4 protein or MAGE-A4 antigen itself.

Next, we tried to find direct immunological activity against tumor cells, resulting in some clinical benefit, e.g., OS, progression-free survival, or tumor shrinkage. In two SD patients, while one showed sero-conversion but not the other, seromics analysis showed the antigen spreading among CT antigens in both patients after vaccination (Supplementary Fig. 5). In addition, activated regulatory T cells were abundantly observed in PBMC from both SD patients, although they did not influence OS. In our previous study of patients vaccinated with NY-ESO-1, antigen spreading was also observed [37], and Tregs were not increased after vaccination [41]. Antigen spreading of CTL against tumor-specific antigens after cancer vaccine with MAGE-1, 3 was also reported, indicating its contribution to tumor regression [45]. P-16 underwent resection of lung metastasis before and after vaccination, and both specimens were available for IHC analysis (Supplementary Fig. 6). Although the expressions of MAGE-A4 and MHC class I were consistent, the number of tumor-infiltrating CD8+ T cells after vaccination was twice as many as at the baseline.

In summary, CHP-MAGE-A4 vaccine was safe and two SD patients were observed. High expression of MAGE-A4 and MHC class I antigens in tumor cells and the induction of MAGE-A4 humoral and cellular immune responses would be feasible prognostic markers for patients vaccinated with MAGE-A4 protein.

Conflict of interest statement

All authors have declared that there are no financial conflicts of interest in regard to this work, but Hiroshi Shiku is a stockholder of ImmunoFrontier, Inc. CHP-MAGE-A4 reagent used in this study was supplied by ImmunoFrontier, Inc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.09.002.

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Nanogel-Based Immunologically Stealth Vaccine Targets Macrophages in the Medulla of Lymph Node and Induces Potent Antitumor Immunity

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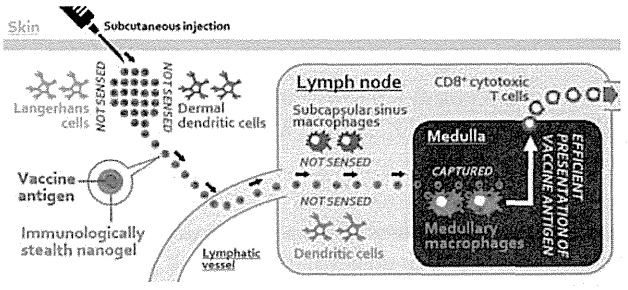
ABSTRACT Because existing therapeutic cancer vaccines provide only a limited clinical benefit, a different vaccination strategy is necessary to improve vaccine efficacy. We developed a nanoparticulate cancer vaccine by encapsulating a synthetic long peptide antigen within an immunologically inert nanoparticulate hydrogel (nanogel) of cholesteryl pullulan (CHP). After subcutaneous injection to mice, the nanogel-based vaccine was efficiently transported to the draining lymph node, and was preferentially engulfed by medullary macrophages but was not sensed by other macrophages and dendritic cells (so-called "immunologically stealth mode"). Although the function of medullary macrophages in T cell immunity has been unexplored so far, these macrophages effectively cross-primed the vaccine-specific $CD8^+$ T cells in the presence of a Toll-like receptor (TLR) agonist as an adjuvant. The nanogel-based vaccine significantly inhibited *in vivo* tumor growth in the prophylactic and therapeutic settings, compared to another vaccine formulation using a conventional delivery system, incomplete Freund's adjuvant. We also revealed that lymph node macrophages were highly responsive to TLR stimulation, which may underlie the potency of the macrophage-oriented, nanogel-based vaccine. These results indicate that targeting medullary macrophages using the immunologically stealth nanoparticulate delivery system is an effective vaccine strategy.



KEYWORDS: cancer vaccine · nanogel · vaccine delivery · macrophages · lymph node · T cells

Although immunotherapy is emerging as a new therapeutic modality for cancer, most of clinical trials of cancer vaccines have failed to prove their clinical activity thus far. A novel approach to improve immunogenicity and efficacy of cancer vaccines is therefore urgently needed. The primary target of vaccines is professional antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs), because these cells efficiently capture, process, and present vaccine antigens to both $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells in a major histocompatibility complex (MHC)-dependent manner. Simultaneously, professional APCs also provide T cells with co-stimulatory signals using a variety of membrane-bound proteins including CD80 and CD86. Professional APCs thus control the quality, extent, and duration of T cell immunity. Vaccine delivery system targeting these professional APCs is therefore vital for the improvement of cancer vaccine efficacy. Recently, the use of synthetic nanoparticulate carriers has emerged as a novel strategy for effective vaccine delivery. When subcutaneously injected, nanoparticulate carriers preferentially enter into the lymphatic vessels, possibly prompted by interstitial fluid flow, and then move *via* lymphatic flow to the draining lymph node (DLN),[1] where various professional APCs survey and engulf particulate antigens by phagocytosis, macropinocytosis, and/or endocytosis, depending on the property of particles including size, surface charge, and presence of ligands for phagocyte

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surface receptors. *Via* this mechanism, nanoparticulate carriers are capable of successfully transporting antigens to professional APCs in the DLN[1−3] and enhance immunogenicity if applied to vaccines.[4,5]

In the efforts to exploit vaccine delivery for the improvement of efficacy, not macrophages but DCs have been considered as the most important target thus far, because antigen presentation by macrophages to T cells is thought to be less efficient than that by DCs.[6] However, in recent years, macrophages localized in lymph nodes are beginning to attract interest, because a recent finding indicates a specific subset of lymph node macrophages also may play a major role as APCs in tumor vaccination; subcutaneously injected dead tumor cells containing particulate antigens induce antigen-specific $CD8^+$ T cell response dependent on $CD169^+$ macrophages in the DLN but not on migratory DCs or lymph node-resident conventional DCs.[7] Another study also shows that lymph node macrophages as well as DCs play a significant role in cross-presentation of subcutaneously injected, microsphere-encapsulated antigen.[8] Thus, certain population(s) of lymph node macrophages might have a remarkable cross-presenting activity and might serve as a preferential target for vaccines. However, no delivery system selective for these macrophages has been available, and the usefulness of these cells in vaccination has been unexplored.

We have developed a series of nanosized hydrogel particles (nanogels) to create novel nanomaterials for biomedical applications.[9] In particular, cholesteryl pullulan (CHP), a pullulan polysaccharide partially hydrophobized by a chemical modification with cholesteryl groups, forms physically cross-linked nanogel particles with a diameter of ~50 nm *via* self-assembly in water.[10,11] CHP efficiently forms a stable complex with a polypeptide through hydrophobic interactions[12] and thereby helps solubilization and long-term stabilization of the polypeptide. Fabrication of the CHP:polypeptide complex is feasible, simple, and reproducible. These features of the CHP nanogel make it an ideal nanoparticulate carrier for the delivery of polypeptide-based therapeutic molecules.[13,14] Besides, when used for *in vitro* antigen delivery, the CHP nanogel enhances cross-presentation of protein antigen.[15,16] This finding led us to evaluate the CHP nanogel as a vaccine delivery system in a series of clinical studies.[17] However, the behavior of CHP nanogel *in vivo* has been unclear so far. In the present study, we investigated *in vivo* vaccine delivery function of the CHP nanogel in detail. When subcutaneously injected, the CHP nanogel efficiently travels to the DLN owing to its small size and uncharged surface. The CHP nanogel is immunologically inert (*i.e.*, the lack of either potential ligands for or stimulatory activity toward immune cells); hence, it evades capture by immune cells including DCs in the DLN. The CHP nanogel then reaches the medulla, a central area of lymph node, where it is vastly engulfed by medullary macrophages. We termed such behavior of the CHP nanogel "immunologically stealth mode". Although the function of medullary macrophages has so far remained elusive, the CHP nanogel-based vaccine elicits a strong antitumor T cell response dependent on these cells. Thus, we simultaneously identified medullary macrophages as a useful cancer vaccine target and the CHP nanogel as a novel delivery system specific for these cells, providing a new strategy to enhance vaccine efficacy.

RESULTS

Immunologically Inert CHP Nanogel Is Selectively Engulfed by Medullary Macrophages in the Lymph Node. CHP was synthesized by grafting 0.9−1.5 cholesterol groups to every 100 glucose units on a hydrophilic pullulan polysaccharide (mean molecular weight 100 000) (Figure 1a). In water, CHP spontaneously forms an uncharged nanogel with a diameter of 40−60 nm *via* hydrophobic interaction among cholesterol groups (Figure 1b and Table 1).[10,11] We supposed that the CHP nanogel may efficiently travel to the DLN when subcutaneously injected, because the nanogel is small enough (<100 nm) to pass through the clefts and pores of lymphatic vessels and its uncharged hydrophilic surface would prevent nonspecific binding to the extracellular matrix and cells. Indeed, the CHP nanogel accumulated in the DLN after subcutaneous administration to mice (Figure 1c). Previous works demonstrated that a potential receptor for pullulan, the backbone of CHP, is not detected in the lymphoid tissues,[18,19] indicating that immune cells in lymph nodes do not express the receptor. In addition, the CHP nanogel possesses no stimulatory activity toward macrophages and DCs (Supporting Information, Figure S1). These facts indicate that the CHP nanogel has neither affinity for nor effect on immune cells, *i.e.*, it is immunologically inert. We therefore anticipated that lymph node cells do not engulf the CHP nanogel, and indeed, uptake by DCs ($CD11c^+F4/80^-$), B cells ($CD45R/B220^+$), or T cells ($CD3\varepsilon^+$) was quite low or absent (Figure 1d). However, intriguingly, massive accumulation was observed in macrophages ($F4/80^+CD11b^+$). This result led us to examine the ability of the CHP nanogel to deliver a vaccine antigen selectively to lymph node macrophages. We prepared a complex of the CHP nanogel with a chemically synthesized long peptide antigen (LPA) (the CHP:LPA complex; Table 1, Figure 2a, and Supporting Information, Figure S2). LPA was designed to include an epitope recognized by mouse $CD8^+$ cytotoxic T cells, *i.e.*, the epitope derived from either a murine tumor-specific antigen mutated ERK2 (mERK2)[20] or a clinically relevant human tumor antigen MAGE-A4.[21] When the CHP:LPA complex was exposed to serum *in vitro*, the LPA still existed as the complex (more than 20% of LPA at least) over 40 h (Supporting

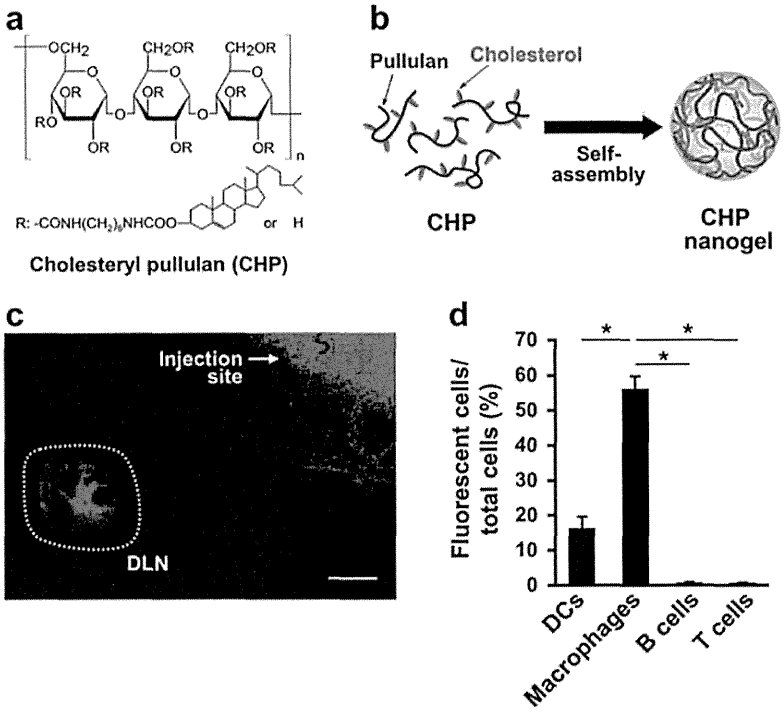

Figure 1. The CHP nanogel is selectively incorporated into macrophages in the DLN after subcutaneous injection to BALB/c mice. (a) Chemical structure of CHP. (b) Schematic representation of nanogel formation *via* self-assembly of CHP. CHP forms a nanogel by hydrophobic interaction between cholesteryl groups in an aqueous solution. (c) *In situ* confocal laser scanning microscopy analysis of a DLN of a mouse that received subcutaneous injection of the rhodamine-labeled CHP nanogel. The scale bar is 1 mm. (d) Incorporation of the subcutaneously injected rhodamine-CHP nanogel (0.5 mg) into immune cells in the DLN of BALB/c mice (three mice per group). Sixteen hours after the injection, uptake of the rhodamine-CHP nanogel was evaluated using flow cytometry in DCs ($CD11c^+F4/80^-$), macrophages ($F4/80^+CD11b^+$), B cells ($CD45R/B220^+$), and T cells ($CD3\varepsilon^+$) isolated from the DLN. Data are mean ± SD; *p*-values were determined by Dunnett's multiple comparison test. $*p < 0.05$. Experiments were performed in triplicate.

TABLE 1. Dynamic Light Scattering Analysis and ζ-Potential Measurement of the Nanogels or the Complex between CHP[a] Nanogel and LPA[b]

nanogel or CHP:LPA complex	amino acid sequence of LPA[c]	size (*d*, nm)	ζ-potential (mV)
CHP	None	42	−4.6
CHP-NH_2	None	86	16.1
CHG[d]	None	183	−1.9
CHP:mERK2 LPA	NDHIAYFLYQILRGLQYIHSANVLHRDLKPSNLLLNT	57	−2.7
CHP:MAGE-A4 LPA	GSNPARYEFLWGPRALAETSYVKVLEHVVRVNARVRIAYP	57	−3.0

[a] Cholesteryl pullulan. [b] Long peptide antigen. [c] Underline indicates the epitope recognized by mouse $CD8^+$ cytotoxic T cells. [d] Cholesteryl glycogen.

Information, Figure S3). After subcutaneous injection into mice, the CHP:mERK2 LPA complex was largely captured by macrophages but not by other immune cells including DCs in the DLN (Figure 2b). The CHP:MAGE-A4 LPA complex also gave a similar result (data not shown). The mERK2 LPA complexed with a cationic (CHP-NH_2) or large-sized (cholesteryl glycogen, CHG; >100 nm) nanogel was not detected in the organ (Table 1 and Figure 2c), indicating that the size and surface charge of nanoparticle is critical for its transportation to lymph node. Further dissection of the macrophage subsets[22] incorporating the CHP:mERK2 LPA revealed that the uptake occurred in medullary sinus macrophages (MSMs, $F4/80^+CD169^+$) and medullary cord macrophages (MCMs, $F4/80^+CD169^-$), but not in subcapsular sinus macrophages (SSMs, $F4/80^-CD169^+$) (Figure 2d,e). A similar result was also obtained with the CHP:MAGE-A4 LPA (data not shown). Histochemical analysis of the DLN showed that the cells incorporating the complex of CHP and fluorescently labeled LPA had a large and round shape and were located in the medullary region of the lymph node (Supporting Information, Figure S4), thus showing characteristics of medullary macrophages. According to these data, we identified the CHP nanogel as a novel nanomaterial suitable for selective vaccine delivery to medullary macrophages in lymph nodes.

LPA Delivered by the CHP Nanogel Is Cross-Presented by Medullary Macrophages to $CD8^+$ Cytotoxic T Cells with High Efficiency. The CHP nanogel allows us to deliver

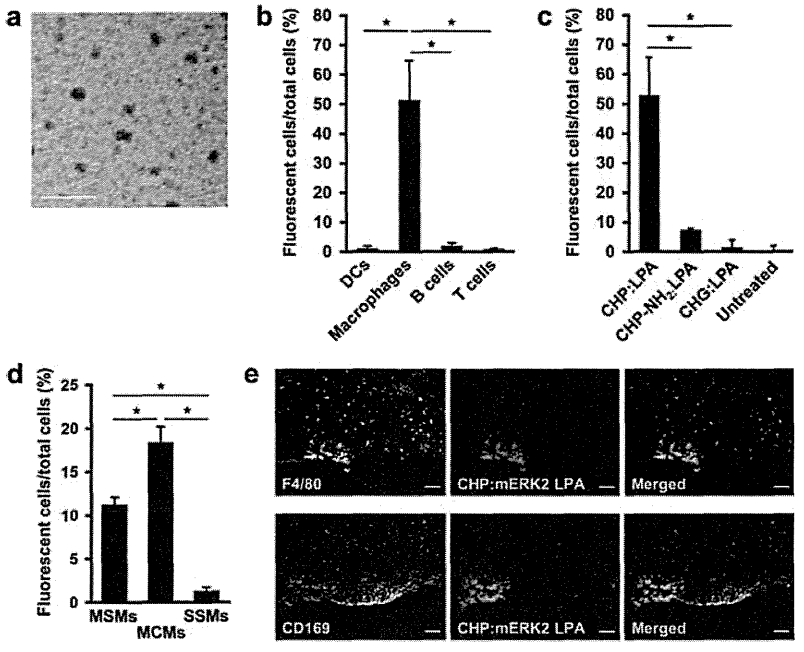

Figure 2. The CHP nanogel:LPA complex is selectively and efficiently engulfed by medullary macrophages in the DLN after subcutaneous injection to BALB/c mice. (a) Transmission electron microscopy of the CHP:LPA complex. The scale bar is 100 nm. (b) Uptake of the CHP:FAM-labeled mERK2 LPA complex in immune cells in the DLN. (c) Uptake of the complexes of indicated nanogels and FAM-mERK2 LPA in macrophages in the DLN. (d) Uptake of the CHP:FAM-mERK2 LPA complex in MSMs (F4/80$^+$CD169$^+$), MCMs (F4/80$^+$CD169$^-$), and SSMs (F4/80$^-$CD169$^+$) in the DLN. (e) Immunohistochemical analysis of incorporation of the CHP:FAM-mERK2 LPA into medullary macrophages. The scale bar is 100 μm. The experiments in panels b–d were performed as in panel d of Figure 1 using the complexes of nanogel and FAM-mERK2 LPA (50 μg). Data are mean ± SD of triplicates. *p*-values were determined by Dunnett's multiple comparison test. *$p < 0.05$. The experiments were repeated thrice with similar results.

a vaccine antigen to medullary macrophages selectively, but the ability of these macrophages to stimulate antigen-specific CD8$^+$ T cells and their usefulness as a vaccine target have not been studied to date. Therefore, we tested whether these macrophages cross-present a vaccine antigen in mice injected with the CHP:mERK2 LPA and a Toll-like receptor (TLR) 9 agonist, CpG oligodeoxynucleotide (CpG ODN), as an adjuvant.[23,24] Whole lymph node cells isolated from the DLN of the mice effectively stimulated DUC18 CD8$^+$ T cells that express a transgene of T cell receptor (TCR) recognizing a mERK2-derived, tumor-specific epitope[25] within the mERK2 LPA (Figure 3a). This stimulation occurred only in the presence of CpG ODN. Macrophages (F4/80$^+$CD11b$^+$) purified from whole lymph node cells also cross-presented the antigen, whereas the macrophage-negative fraction (F4/80$^-$CD11b$^-$) lacked such activity and DCs (CD11c$^+$F4/80$^-$) were evidently inferior to macrophages in our system (Figure 3b,c), in agreement with the uptake of CHP:LPA to these cell populations (Figure 2b). To identify the subset of macrophages responsible for this cross-presentation, we purified MSMs (F4/80$^+$CD169$^+$) and MCMs (F4/80$^+$CD169$^-$) from the immunized mice (Supporting Information, Figure S5). MSMs and MCMs both competently cross-presented the vaccine antigen to DUC18 CD8$^+$ T cells (Figure 3d,e). The activity of MSMs was higher than that of MCMs, indicating that MSM is the cell type most capable of cross-presenting antigens. Subcutaneous injection of a liposomal formulation of clodronate (clodronate liposome) into mice selectively depleted macrophages but not DCs in lymph nodes (Supporting Information, Figure S6).[8,26] After ablation by clodronate liposomes of lymph node macrophages, *in vivo* induction of the LPA-specific CD8$^+$ T cell response was significantly impaired (Figure 3f). Taken together, these data support the notion that medullary macrophages in lymph nodes can effectively cross-prime CD8$^+$ T cells.

Vaccination with the CHP Nanogel:LPA Complex and TLR Agonist as Adjuvant Elicits Vaccine-Specific, Strong CD8$^+$ T Response and Effectively Suppresses *in Vivo* Tumor Growth. On the basis of the finding that the CHP nanogel vaccine selectively targets medullary macrophages possessing high cross-presenting activity, we expected that this vaccine system could induce a potent antitumor CD8$^+$ T cell response. We next evaluated antitumor efficacy of the CHP:LPA complex vaccine in a mouse pharmacological model compared to that of a vaccine containing LPA emulsified in incomplete Freund's adjuvant (IFA), a widely used conventional vaccine delivery system that is known to exert the depot effect at the injection site. The immune response induced by an IFA-based vaccine is reported to be mediated by DCs.[27]

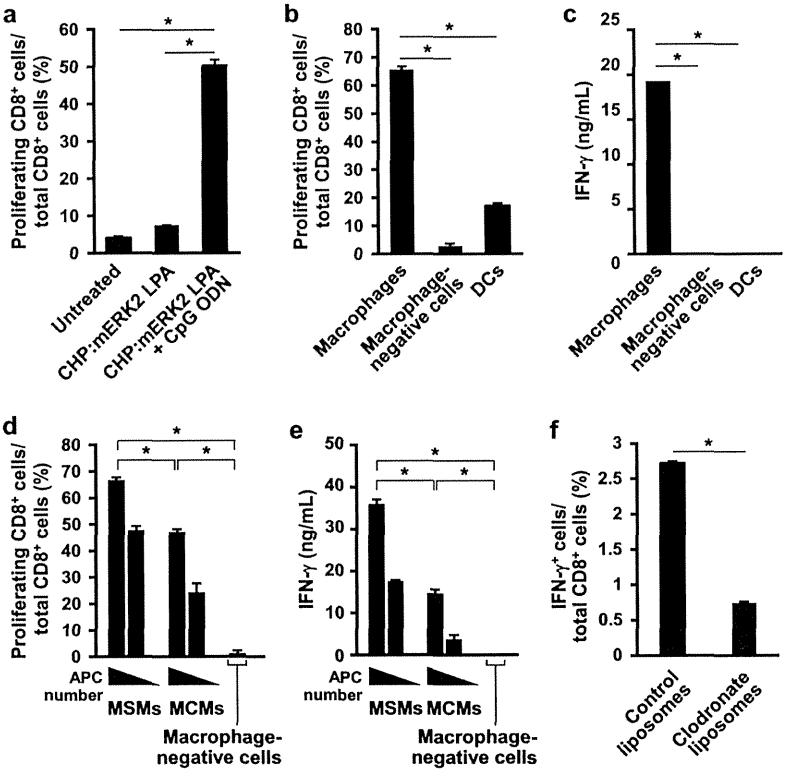

Figure 3. Medullary macrophages directly and efficiently cross-present antigens to specific $CD8^+$ T cells. (a–e) The CHP:mERK2 LPA complex with or without CpG ODN was injected into the footpad of BALB/c mice. Eighteen hours later, the whole lymph node cells (a) or the fractions containing macrophages ($F4/80^+CD11b^+$), macrophage-negative cells ($F4/80^-CD11b^-$), and DCs ($CD11c^+F4/80^-$) (panels b and c) were isolated from the DLN. Macrophages were further separated into MSMs ($CD169^+$) and MCMs ($CD169^-$) (panels d and e, for the gating strategy in cell sorting, see Supporting Information, Figure S5). These cells were cocultured as APCs with mERK2-specific DUC18 $CD8^+$ T cells for 48 h *in vitro*. (a, b, and d) Proliferation of DUC18 $CD8^+$ T cells was measured using a CFSE dilution assay. The numbers shown in histograms indicate the percentages of proliferating cells. (c and e) The concentration of IFN-γ produced by $CD8^+$ T cells into the culture supernatant was determined using ELISA. (f) Clodronate liposomes or control liposomes were subcutaneously injected into the footpad of BALB/c mice, and 6 days later, the CHP:mERK2 LPA was injected at the same site. Seven days after the immunization, splenocytes were isolated and restimulated with mERK2 LPA *in vitro*. Activated specific $CD8^+$ T cells were quantified using intracellular IFN-γ staining followed by flow cytometry (two mice per group). The data are mean ± SD of triplicates. *p*-values were determined using Tukey-Kramer multiple comparison test, Dunnett's multiple comparison test, and Student's *t* test in panels a, d, and e; panels b and c; and panel f, respectively. $*p < 0.05$. The results are representative of one of at least two experiments.

We also observed that the IFA:LPA vaccine is dependent on DCs but not on macrophages (Supporting Information, Figure S7). Using these two vaccine delivery systems, we evaluated the inhibitory effect of vaccination with the mERK2 or MAGE-A4 LPA on tumor growth in mice transplanted with syngeneic tumors. Tumors included murine fibrosarcoma CMS5a cells[28,29] expressing endogenous mERK2 and murine colon carcinoma CT26 cells[30] stably expressing a transgene of human MAGE-A4 (CT26/MAGE-A4).[21] As a result, in the prophylactic setting, growth of both types of tumors was significantly inhibited in the mice vaccinated with the CHP:LPA complex relative to the control group (Figure 4a,b). Vaccination with the IFA:LPA was almost ineffective against the CMS5a tumor, and was less effective than vaccination with the CHP:LPA against the CT26/MAGE-A4 tumor. All of mice rejected the CT26/MAGE-A4 tumor in the CHP:LPA vaccine group ($n = 7$), while only one animal did in the IFA:LPA vaccine group ($n = 7$) (Figure 4b). Efficacy of the vaccines was also evaluated in the therapeutic setting; vaccination with the CHP:MAGE-A4 LPA and CpG ODN significantly suppressed the growth of CT26/MAGE-A4 tumor, while the IFA:LPA vaccine and CpG ODN or CpG ODN alone did not affect the tumor growth (Figure 4c). The ability of the CHP:LPA and IFA:LPA vaccines to induce antigen-specific $CD8^+$ T cell response was also assessed by immunizing mice in a manner similar to the tumor development experiment above. Seven days after the last vaccination, the frequency of LPA-specific $CD8^+$ T cells in the spleen was measured (Figure 4d,e). The CHP:LPA and IFA:LPA vaccines both induced a measurable specific $CD8^+$ T cell response when administered with TLR agonists such as a TLR9 agonist CpG ODN or a TLR3 agonist poly-ICLC RNA, but not in the absence of TLR stimulation. Notably, in accordance with the *in vivo* tumor growth experiment, the frequency of specific $CD8^+$ T cells was much higher in mice immunized with the CHP:LPA than in those immunized with the IFA:LPA. This result indicated that

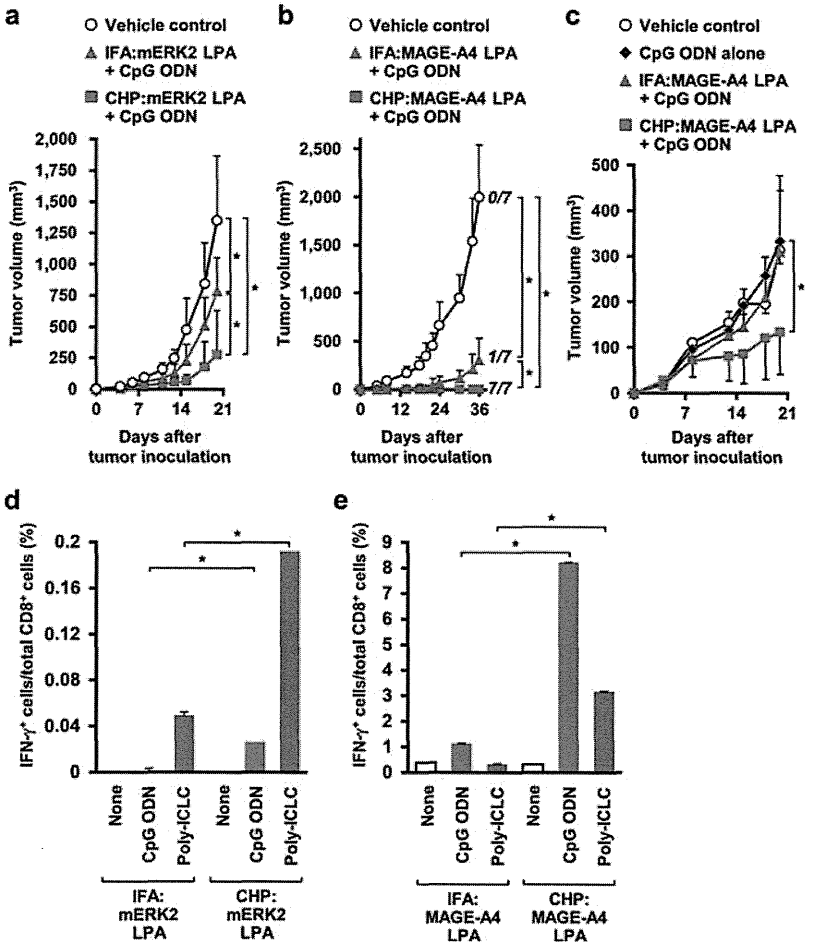

Figure 4. The macrophage-selective nanogel-based vaccine shows strong antitumor efficacy and $CD8^+$ T cell-inducing activity in the presence of Toll-like receptor TLR agonists as an adjuvant. (a and b) Effects of vaccination on *in vivo* tumor growth in the prophylactic setting. The LPA (50 μg) complexed with either CHP nanogel or IFA was subcutaneously injected into BALB/c mice followed by immediate injection of CpG ODN (50 μg) on day −7. On day 0, 10^6 CMS5a cells (a) or CT26/MAGE-A4 cells (b) were subcutaneously transplanted into the mice. Subsequently, the tumor volume was measured three times a week. Numbers in italic in panel b indicate the mice who rejected the tumor. Each group included 4−7 mice. (c) Effects of vaccination on *in vivo* tumor growth in the therapeutic setting. This experiment was performed in a way similar to panel b, but the vaccines were administered at days 4 and 11 and the dose of CpG ODN was 25 μg. (d and e) Antigen-specific $CD8^+$ T cell response induced by the vaccines. BALB/c mice were injected with either the CHP:LPA or IFA:LPA vaccine. Some groups were also injected with CpG ODN (50 μg) or poly-ICLC RNA (50 μg) immediately after administration of vaccine. Seven days after vaccination, splenocytes were isolated and restimulated with mERK2 LPA or MAGE-A4 LPA *in vitro*. Frequency of activated specific $CD8^+$ T cells was quantified by intracellular IFN-γ staining followed by flow cytometry (two mice per group). The data are mean ± SD. *p*-values were determined by Student's *t* test. *$p < 0.05$. These experiments were repeated at least twice with similar results.

TLR agonists had a greater adjuvant effect on the CHP:LPA vaccine than on the IFA:LPA vaccine. We also confirmed that the CHP:LPA vaccine elicited a much greater $CD8^+$ T cell response when compared to a saline-based vaccine (Supporting Information, Figure S8). Altogether, these data demonstrate that the lymph node macrophage-targeted nanogel-based vaccine system showed a remarkable antitumor effect through enhanced induction of tumor-specific $CD8^+$ T cells.

Lymph Node Macrophages Are Highly Sensitive to TLR Stimulation: A Possible Mechanism for the Potency of CHP nanogel-Based Vaccine. Lymph node macrophages efficiently cross-presented nanoparticulate antigen delivered by the CHP nanogel, but only in the presence of TLR stimulation. By analyzing the susceptibility of lymph node macrophages to TLR stimulation, we investigated a possible mechanism that underlies the observed strong cross-presenting activity. Incorporation of subcutaneously injected, fluorescently labeled CpG ODN into macrophages ($F4/80^+CD11b^+$) and DCs ($CD11c^+F4/80^-$) in the DLN was assessed in mice. Higher accumulation of CpG ODN was observed in macrophages than in DCs (Figure 5a). Uptake of another TLR agonist, poly-ICLC RNA, could not be examined because of the lack of an appropriate analytical method. Activation of macrophages and DCs in the DLN by CpG ODN or poly-ICLC RNA was then determined based on the expression of co-stimulatory

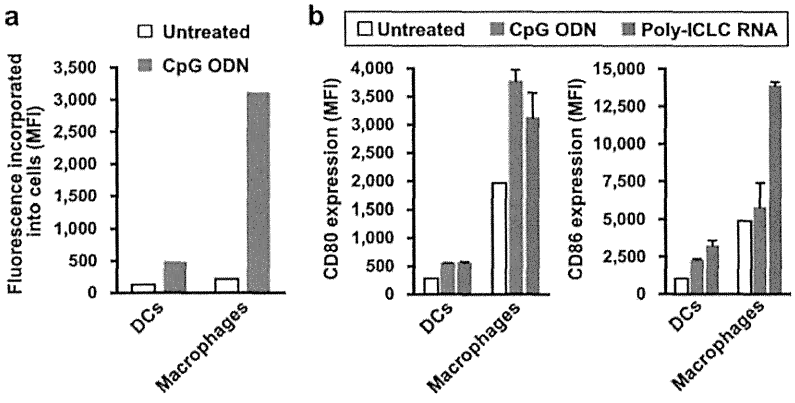

Figure 5. Lymph node macrophages are highly responsive to TLR agonists. (a) Incorporation of subcutaneously injected, FITC-labeled CpG ODN into macrophages and DCs in the DLN. BALB/c mice (two mice per group) were injected with FITC-CpG ODN (50 μg), and 16 h later, macrophages ($F4/80^+CD11b^+$) and DCs ($CD11c^+F4/80^-$) in the DLN were isolated, and uptake of FITC-CpG ODN in these cells was measured using flow cytometry. (b) Activation of macrophages and DCs in the DLN by TLR agonists. BALB/c mice (two mice per group) were subcutaneously injected with CpG ODN (50 μg) or poly-ICLC RNA (50 μg), and 16 h later, macrophages and DCs in the DLN were tested for expression of CD80 and CD86. MFI, mean fluorescence intensity.

molecules CD80 and CD86. In accordance with the uptake of CpG ODN, the up-regulation of CD80 and CD86 by TLR stimulation was prominent in macrophages (Figure 5b). These data revealed that lymph node macrophages are highly responsive to TLR stimulation, and possibly this way they acquire the high cross-presenting activity. Therefore, concurrent, appropriate TLR stimulation would be a prerequisite for the activity of macrophage-dependent CHP nanogel-based vaccine. Conversely, thanks to the high susceptibility of lymph node macrophages to TLR stimulation, the CHP nanogel-based vaccine would acquire its high potency.

DISCUSSION

Thus far, no antigen delivery system targeting macrophages localized in the lymph node has been available, although several vaccine formulations selective for splenic macrophages have been developed including a micropatriculate antigen with a diameter of 0.5 μm for $MARCO^+$ macrophages[31] and an anti-CD169 antibody-fused protein antigen for $CD169^+$ macrophages.[32] The present study showed for the first time that the CHP nanogel functions as a lymph node medullary macrophage-selective vaccine delivery system. A precise mechanism for the preferential incorporation of the CHP nanogel into medullary macrophages has yet to be elucidated, but the unique characteristics of CHP nanogel may help to explain that phenomenon. Because the CHP nanogel is immunologically inert, immune cells including DCs would not be able to sense and engulf the nanogel. Nevertheless, medullary macrophages can, likely due to their very high phagocytic activity.[22] Thus, the CHP nanogel-based vaccine likely acquires its macrophage-selective delivery function as a consequence of avoiding surveillance by other cells (the "immunologically stealth vaccine"). The difference between medullary and subcapsular sinus macrophages also may be explained by a similar reason.[22] The present study also validated a novel approach to give a material a delivery function targeting a certain immune cell population by strictly preventing acquisition by unintended cells.

The functions of medullary macrophages that involve scavenging of pathogens and particulate antigens from the lymph and supporting plasma-cell survival have been reported,[22] but their involvement in the induction of T cell response has been unexplored. To our knowledge, the present study is the first report describing the participation of medullary macrophages in T cell immunity. Cross-presentation by these macrophages required the presence of TLR agonist probably because the TLR stimulation up-regulated the phagocytic, antigen processing, and/or presenting functions of these macrophages. On the other hand, it is also possible that TLR stimulation attenuates cross-presentation, because TLR stimulation is also known to increase the digestive activity of phagosomes that destroys antigens,[33] which can result in destruction of putative epitopes. Medullary macrophages may possess some intrinsic mechanism to avoid this effect, for example, increased escape of antigens from phagosomes to the cytosol.[34] Alternatively, the CHP nanogel may protect antigens from excess degradation in phagosomes. We also confirmed that similar to murine macrophages, human macrophages were also highly capable of cross-presenting antigens but only in the presence of TLR stimulation (Supporting Information, Figure S9). To further evaluate the significance of cross-presentation by lymph node macrophages and to harness it for effective tumor vaccination, the direct and/or indirect mechanisms by which TLR stimulation regulates the quality

and outcome of macrophage-mediated cross-presentation should be clarified in the future.

CONCLUSION

The present study demonstrated that selective vaccine delivery to medullary macrophages localized in the medulla, the core of lymph nodes, can be achieved by using an immunologically stealth nanoparticulate delivery system. We also revealed that medullary macrophages have a potential to effectively induce specific $CD8^+$ T cell response to vaccines. These two key findings build a theoretical basis to design a macrophage-oriented cancer vaccine with high potency, which may overcome the limited clinical efficacy of existing cancer vaccines. This strategy may improve the therapeutic efficacy of vaccines against not only cancers but also infectious diseases reactive to T cell immunity, and may thus have great impact in the field of immunotherapy. Cancer vaccines utilizing the CHP nanogel used in the present study have already been tested in clinical trials and confirmed to be highly safe and immunogenic, also supporting the clinical application of macrophage-oriented vaccines utilizing this nanoparticulate delivery system.

MATERIALS AND METHODS

Fabrication and Analysis of the Complex of CHP Nanogel with LPA. CHP, rhodamine-labeled CHP, CHP-NH_2, and CHG were synthesized as described previously.[35−37] LPAs were chemically synthesized by Bio-Synthesis, Inc. (Lewisville, TX). The sequences of the LPAs used were as follows: human MAGE-A4-derived LPA (GSNPARYEFLWGPRALAETSYVKVLEHVVRVNARVRIAYP), mERK2-derived LPA (NDHIAYFLYQILRGLQYIHSANVLHRDLKPSNLLLNT), and human NMW LPA (**SLLMWITQC**YYYYYY**NYKRCFPVI**YYYYYY**CMTWNQMNL**). The MAGE-A4 LPA and mERK2 LPA contain epitopes for murine $CD8^+$ T cells (underlined), and the human NMW LPA does for HLA-A2-restricted NY-ESO-1, HLA-A24-restricted MAGE-A4, and HLA-A24-restricted WT1 epitopes (bold). For analysis of antigen incorporation, these LPAs were labeled with fluorescein amidite (FAM). LPA and CHP were dissolved in dimethyl sulfoxide and phosphate-buffered saline (PBS) containing 6 M urea, respectively. Both solutions were combined and gently mixed at room temperature, followed by dialysis against PBS to remove urea. The resulting solution of the CHP:LPA complex was stored at 4 °C until use. The peptide concentration in the CHP:LPA complex solution was determined by measuring the absorbance at 280 nm, where CHP does not contribute to absorbance. The final concentration of CHP was approximately 10 mg/mL and that of LPA was 0.2−0.4 mg/mL. Complexation of the CHP nanogel and FAM-labeled LPA was confirmed by means of high performance size exclusion chromatography (HPSEC) using a Superose 12 10/300 GL column (GE Healthcare). An aliquot of the sample was injected into the HPSEC system (Shimadzu), eluted with PBS, and detected by means of ultraviolet absorbance at 495 nm. To determine the size of the CHP nanogel and CHP:LPA complex, dynamic light scattering measurement was performed using a Zetasizer Nano ZS (Malvern Instruments, Ltd.) at 633 nm and a 173° detection angle at 25 °C. The measured autocorrelation function was analyzed using the cumulant method. The hydrodynamic diameter (D_H) of the samples was calculated from the Stokes−Einstein equation. The ζ-potential of CHP:LPA complex was also measured using the Zetasizer Nano ZS at a 90° detection angle at 25 °C in PBS. The mERK2 CHP:LPA complex was also analyzed using transmission electron microscopy (TEM). Briefly, the sample was applied to a carbon-coated grid, and the grid was stained with TI blue (Nisshin EM, Japan), dried, and subjected to TEM (HT7700, Hitachi) at an accelerating voltage of 100 kV.

Mice and Tumors. Female BALB/c mice were obtained from SLC Japan and used at 6−12 weeks of age. DUC18 mice, transgenic for TCRα/β that interacts with a K^d-restricted $mERK2_{136-144}$ peptide epitope, were established as described previously.[25] CD90.1-congenic BALB/c mice were kindly provided by Dr. Sakaguchi of Osaka University, Japan. We mated DUC18 mice and CD90.1-congenic mice at our animal facility, and obtained DUC18/CD90.1 mice. T cells isolated from these mice can be traced in *in vitro* and *in vivo* experiments using anti-CD90.1 antibody. All mice were maintained at the Experimental Animal Facility of Mie University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University.

CT26 is a colon epithelial tumor cell line that was produced by intrarectally injecting *N*-nitroso-*N*-methylurethane in BALB/c mice.[30] CT26 cells stably expressing human cancer/testis antigen MAGE-A4 (CT26/MAGE-A4) were established as described previously.[21] CMS5a is a subclone derived from CMS5, a 3-methylcholanthrene-induced sarcoma cell line of BALB/c origin, and expresses mERK2 as a neoantigen.[20] In *in vivo* tumor growth experiments, mice (n = 4 or more) were inoculated subcutaneously in the right hind flank with 10^6 CT26/MAGE-A4 or CMS5a cells and monitored three times a week. The tumor size was estimated using the following formula: tumor size (mm^3) = 1/2[length (mm) × width (mm) ^{2}].

Tracking of the Subcutaneously Injected CHP or CHP:LPA Complex. For tracking of the injected CHP nanogel, rhodamine-labeled CHP nanogel was subcutaneously injected to the back of BALB/c mice. Six hours later, the skin harboring DLN was harvested and observed using confocal laser scanning microscope (LSM780, Carl Zeiss, Germany). For tracking of the injected CHP:LPA, a fluorescently labeled LPA complexed with the CHP nanogel was subcutaneously injected into the right hind flank of the mice. The inguinal DLN was harvested 16 h after the injection, mashed, and filtered through a nylon mesh. The resulting cell suspension was analyzed using flow cytometry for incorporation of labeled LPA and expression of CD11b, CD11c, CD169, F4/80, CD3ε, and B220. For immunohistochemical analysis, cryosections were prepared from the DLN. Optimum cutting temperature (O.C.T.) compound-embedded cryosections were stained with fluorescent dye-conjugated anti-CD169 or anti-F4/80 monoclonal antibodies (mAbs) and examined under a fluorescence microscope (BX53F, Olympus).

Immunization of Mice. The CHP:LPA complex or LPA emulsified with IFA (Sigma-Aldrich) was subcutaneously injected into the back of mice at the dose of 50 μg as LPA. Phosphorothioate-containing CpG ODN 1668 (Hokkaido System Science, Japan) or poly-ICLC RNA (Oncovir, Inc.) was simultaneously and subcutaneously injected near the site of vaccination. To deplete macrophages, a clodronate liposome solution (FormuMax Scientific) was subcutaneously injected into the footpad of mice 6 days prior to immunization.

Flow Cytometric Analysis. Fluorescent dye-conjugated mAbs including anti-CD8 (53−6.7), anti-CD4 (RM4−5), anti-interferon (IFN)-γ (XMG1.2), anti-CD80 (2D10.4), anti-CD86 (IT2.2), anti-CD11b (M1/70), anti-CD11c (N418), anti-F4/80 (BM8), anti-CD3ε (145−2C11), anti-CD45R/B220 (RA3−6B2), and anti-CD169 (3D6.112) were purchased from Biolegend. The cell suspension prepared from the inguinal DLN or spleen was stained for surface markers using antibodies at the appropriate concentrations in PBS containing 2% fetal bovine serum for 15 min at 4 °C, and analyzed on a FACSCanto II system (BD Biosciences). For intracellular cytokine staining, splenocytes were incubated with mERK2 LPA or MAGE-A4 LPA for 1 h at 37 °C and then incubated for an additional 6 h with GolgiPlug (BD Bioscience). After permeabilization and fixation using the

Cytofix/Cytoperm Kit (BD Bioscience), the cells were stained with allophycocyanin-conjugated anti-IFN-γ mAb and analyzed using flow cytometry.

T Cell Proliferation and IFN-γ Release Assay. For *in vitro* T cell proliferation assay, the CHP:mERK2 LPA and CpG ODN were subcutaneously injected into the footpad of mice. DLN was resected 20 h after the injection. To isolate $CD11b^+$ cells, the total cell suspension prepared from the DLN was mixed with anti-CD11b microbeads (Miltenyi Biotec) and separated by positive selection on a magnetic bead column. For further fractionation of $CD11b^+$ cells, the cells were stained with FAM-labeled anti-CD11b, PE-labeled anti-CD169, PerCP-Cy5.5-labeled anti-F4/80, and allophycocyanin-labeled anti-CD11c mAbs, and sorted using a FACSAria system (BD Biosciences). The isolated cells (0.1, 0.3, or 1×10^5) were then cocultured with 2.5×10^5 DUC18 T cells prelabeled with carboxyfluorescein succinimidyl ester (CFSE) for 48 h. The dilution of CFSE was measured using flow cytometry to determine cell proliferation. IFN-γ release into the culture medium at 48 h was quantified using a mouse IFN-γ ELISA kit (BD Bioscience).

For *in vivo* T cell proliferation assay, either the CHP:mERK2 LPA or IFA:mERK2 LPA was subcutaneously injected into the back of mice. At day 0 or day 4, 2.5×10^5 DUC18 T cells prelabeled with CFSE were intravenously infused into the vaccinated mice. DLNs were collected on day 3 or day 7, and dilution of CFSE was measured using flow cytometry to determine the *in vivo* proliferation of DUC18 T cells.

***In Vitro* Cross-Presentation by Human Macrophages.** Peripheral blood mononuclear cells were isolated from buffy coats prepared using density gradient centrifugation of the blood of HLA-A0201-positive healthy donors over Ficoll. $CD14^+$ cells were purified with anti-CD14 microbeads and then incubated with GM-CSF, 20 ng/mL in the X-VIVO15 medium, for 7 days to induce differentiation to macrophages. The obtained macrophages were pulsed with human CHP:NMW LPA (10 μg/mL) and stimulated with poly-ICLC RNA (10 μg/mL). The human IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed as previously described. Briefly, a 96-well nitrocellulose ELISPOT plate (Millipore) was coated with an anti-human IFN-γ mAb (clone 1-D1K, Mabtech) overnight at 4 °C. The wells were washed with PBS containing 0.01% Tween 20 (PBS-T) and blocked with the RPMI1640 medium containing 10% fetal calf serum for 2 h at 37 °C. HLA-A0201-restricted NY-ESO-1 epitope-specific $CD8^+$ T cells (clone 1G4, 10^5 cells per well) and antigen-pulsed macrophages were seeded into each well. After incubation for 22 h at 37 °C in 5% CO_2, the plate was washed thoroughly with PBS-T. A biotin-conjugated anti-human IFN-γ mAb (clone 7-B6-1, Mabtech) was then added (final concentration 1.25 μg/mL), and the plate was incubated overnight at 4 °C. After a wash step with PBS-T, a streptavidin-alkaline phosphatase conjugate (1 μg/mL; Roche Diagnostics) was added. After incubation for 60 min at room temperature, the wells were washed thrice with PBS-T and stained using an alkaline phosphatase conjugate substrate kit (Life Technologies). The reaction was stopped by intensive washing with distilled water. After the plate dried out, the number of spots in each well was counted using an ELISPOT reader (Cellular Technologies, Ltd.).

Statistical Analysis. The data were analyzed using Student's *t* test, Dunnett's multiple comparison test, or Tukey-Kramer multiple comparison test. Differences with $p < 0.05$ were considered statistically significant.

Conflict of Interest: The authors declare the following competing financial interest(s): Naozumi Harada is an employee of ImmunoFrontier, Inc. The other authors have no conflicts of interest.

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Supporting Information Available: Characterization of the nanogel:LPA complex, associated *in vivo* mouse immunological data, and *in vitro* experiment with human macrophages. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Dose-dependent effects of NY-ESO-1 protein vaccine complexed with cholesteryl pullulan (CHP-NY-ESO-1) on immune responses and survival benefits of esophageal cancer patients

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Abstract

Background: Cholesteryl pullulan (CHP) is a novel antigen delivery system for cancer vaccines. This study evaluated the safety, immune responses and clinical outcomes of patients who received the CHP-NY-ESO-1 complex vaccine, Drug code: IMF-001.

Methods: Patients with advanced/metastatic esophageal cancer were enrolled and subcutaneously vaccinated with either 100 μg or 200 μg of NY-ESO-1 protein complexed with CHP. The primary endpoints were safety and humoral immune responses, and the secondary endpoint was clinical efficacy.

Results: A total of 25 patients were enrolled. Thirteen and twelve patients were repeatedly vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1 with a median of 8 or 9.5 doses, respectively. No serious adverse events related to the vaccine were observed. Three out of 13 patients in the 100-μg cohort and 7 out of 12 patients in the 200-μg cohort were positive for anti-NY-ESO-1 antibodies at baseline. In the 100-μg cohort, an antibody response was observed in 5 out of 10 pre-antibody-negatives patients, and the antibody levels were augmented in 2 pre-antibody-positive patients after vaccination. In the 200-μg cohort, all 5 pre-antibody-negative patients became seropositive, and the antibody level was amplified in all 7 pre-antibody-positive patients. No tumor shrinkage was observed. The patients who received 200 μg of CHP-NY-ESO-1 survived longer than patients receiving 100 μg of CHP-NY-ESO-1, even those who exhibited unresponsiveness to previous therapies or had higher tumor burdens.

Conclusions: The safety and immunogenicity of CHP-NY-ESO-1 vaccine were confirmed. The 200 μg dose more efficiently induced immune responses and suggested better survival benefits. (Clinical trial registration number NCT01003808).

Keywords: Esophageal cancer, Cancer vaccine, NY-ESO-1, Cholesteryl pullulan (CHP)

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Background

Complexes of cholesteryl pullulan (CHP) nano-particles that contain a tumor antigen are a new type of cancer vaccine with a novel antigen delivery system that presents multiple epitope peptides to both the MHC class I and class II pathways [1-4]. We have been developing CHP-protein human cancer vaccines that efficiently induce immune responses against multiple T cell epitopes for various HLA types. Previous clinical studies using CHP-HER2 and CHP-NY-ESO-1 vaccines showed that these vaccines could be administered repeatedly without serious adverse effects, and both vaccines induced antigen-specific $CD4^+$ and $CD8^+$ T cell immunity as well as humoral immunity [5-7].

Because the NY-ESO-1 antigen is a cancer-testis antigen that is exclusively expressed in the tumor tissue, aside from expression in the normal testis and placenta, this antigen is considered an ideal target for cancer immunotherapy [8,9].

The appropriate dose for NY-ESO-1 protein vaccine has not been determined, although doses up to 100 μg have been examined, in which a higher dose was more immunogenic compared to lower doses of 10 μg and 30 μg [10].

We conducted a dose-escalating trial with CHP-NY-ESO-1 vaccine doses of 100 μg and 200 μg for esophageal cancer patients who were resistant to standard therapies. We evaluated the safety and immune responses to the NY-ESO-1 antigen over the vaccination period, and explored the clinical impact on esophageal cancer patients with a poor prognosis.

In this study, we analyzed IgG antibody responses as antigen-specific immune responses. Although T cells that are induced by a cancer vaccine should be evaluated as an immune-monitoring marker, T cells can be difficult to detect directly and quantitatively assess, whereas IgG titers measured by ELISA could act as a suitable immune-monitoring marker. Analyzing antibody responses induced by CHP-NY-ESO-1 vaccine, the 200 μg-dose more efficiently induced immune responses and suggested better survival benefits.

Materials and methods

Preparation of CHP-NY-ESO-1 complex vaccine

CHP-NY-ESO-1 complex vaccine (Drug code: IMF-001) was provided by ImmunoFrontier, Inc. (Tokyo, Japan). All processes were performed following current Good Manufacturing Practices (cGMP) conditions. The toxicity of the drug products was assessed using animal models, and stability was monitored during the clinical trial using representative samples of the investigational drug product.

Study design

This study was a phase 1, open-label, multi-institutional, dose-escalating clinical trial of the CHP-NY-ESO-1 complex vaccine administered subcutaneously to patients with unresectable, advanced, or refractory esophageal tumors that expressed the NY-ESO-1 antigen. The primary objective was to determine the maximum tolerated dose (MTD) and the biological recommended dose, and the secondary objective was to assess clinical efficacy.

Patients were eligible for entry, if they had a performance status of 0, 1, or 2, were at least 20 years old, had a life expectancy of 4 months or more, and did not have impaired organ function. Patients were ineligible if they were positive for HIV antibody, had multiple cancers, autoimmune disease, serious allergy history, or active brain metastasis, or received previous chemotherapy, systemic steroid or immunosuppressive therapy within less than 4 weeks.

The patients were divided into the following two cohorts of 10 patients each: Cohort 1, 100 μg of the NY-ESO-1 protein every two weeks, and Cohort 2, 200 μg of the NY-ESO-1 protein every two weeks. When a patient withdrew from the trial within three vaccinations, they were replaced with an additional patient.

Clinical responses were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST ver1.1) [11] and its modified version. The modified version is based on immune-related Response Criteria (ir-RC) [12] and includes the following: Tumor responses were assessed every 6 weeks. Even if disease progression was observed within the first 12 weeks, PD (progressive disease) was not judged. When disease progression was observed after 18 weeks, PD was determined.

Each patient received 6 administrations. However, the treatment could be continued beyond this period if the patient wished to maintain treatment and met the following criteria: 1) no evidence of tumor progression or worsening of performance status (PS), and 2) an anti-NY-ESO-1 antibody response was confirmed. Safety was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) [13]. All the safety information was collected and evaluated, and dose escalation was judged by the Independent Data and Safety Committee.

The study was performed in accordance with the current version of the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients participating in this study. The protocol was approved by the institutional review board at each site. The clinical trial was sponsored by ImmunoFrontier, Inc. (Tokyo, Japan), and registered as ID: NCT01003808 of ClinicalTrials.Gov.

Expression of NY-ESO-1 antigen

NY-ESO-1 expression was assessed by immunohistochemistry with the monoclonal antibody, E978 (Sigma-Aldrich, Saint Louis, MO), [9] or quantitative RealTime-PCR (qRT-PCR) using specific primers [14].