科大学とシンガポール国立大学、韓国のヨンセイ大学による医師主導国際共同治験として開発がすすめられ、国際的な枠組みでの開発に発展している。

Ⅳ. がんペプチドワクチンアジュバント

癌の微小環境における免疫抑制を打破する為には、適切なアジュバントの開発も重要である。現在のがんペプチドワクチン開発においてはモンタナイドをアジュバントとして用いエマルジョン化してから投与するのが一般的である。一方、近年がんペプチドワクチンの効果を増強する新しいアジュバントの探索が様々に行われている。特に、パターン認識受容体アゴニストとして、Toll-like receptor（TLR）を刺激するアジュバントはがんワクチンの効果を適切に増強すると考えられ、Poly-IC（TLR3 agonist)、MPL（TLR-4 agonist)、イミキモド（TLR-7/8 agonist)、CpG-ODN（TLR-9 agonist）等が、がんワクチンアジュバントとして開発中である。

我々は、in vitro におけるペプチド特異的 CTL の誘導において、CpG-ODN を添加することで効率よく CTL が誘導されることを基礎的検討にて明らかにし[14]、この成果を基に進行食道癌患者に対して複数の Oncoantigen 由来エピトープペプチドと CpG-ODN をアジュバントとして併用する臨床研究を施行した[15]。安全性を示すと共に、CpG-ODN 併用により早期から強力な特異的 CTL が誘導される傾向を認め、本治療法による全生存期間の延長が示唆されたことから CpG-ODN のがんペプチドワクチン療法におけるアジュバントとしての有用性が示唆され、今後の発展が期待される。

また、近年、抗 CTLA-4 抗体である Ipilimumab[16] が転移性悪性黒色腫の患者に対して承認された。Ipilimumab は T 細胞活性化経路を down-regulate する免疫チェックポイント分子である Cytotoxic T-Lymphocyte Antigen 4（CTLA-4）を阻害することで、CTL の抗腫瘍効果を高める作用がある。さらに、同じく免疫チェックポイントを標的とする PD-1（Programmed Cell Death-1）に対する抗体を用いた悪性黒色腫や非小細胞肺癌、腎細胞癌などに対する臨床研究においてもその有用性が示されており[17]、作用メカニズムからがんペプチドワクチンとの併用による効果増強の可能性が期待されている。

Ⅴ. がん治療用ペプチドワクチンガイダンス

さて、これまでの 20 年のがんワクチン開発に関する Translational Research を通して、がんワクチンの効果判定法についての方向性が明らかになってきた。つまり、がんワクチンの作用機序が患者自身の CTL の活性化を介しており、ワクチンによる抗原提示・抗原処理・リンパ球の活性化・がん細胞の死滅といった一連の過程には生体内で相当な時間を要することから、がんワクチン効果は、緩やかに、長期間にわたって現れる。従って、比較的速やかに効果が確認される従来の抗癌剤の評価法では、がんワクチン療法の臨床効果の評価は困難であり、がんワクチン開発における独自の評価方法が必要であると考えられる。

米国の FDA は、2011 年 11 月に「Guidance for Industry: Clinical Considerations for Therapeutic Cancer Vaccines（企業向けガイダンス・がん治療用ワクチンのための臨床学的考察）」

として企業向けのワクチンガイダンスを発行した（http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/UCM278673.pdf）。この中でFDAは、がんワクチンの開発において従来の細胞傷害性薬剤や生物製剤の開発とは異なった臨床試験デザインを考慮する必要がある、と明記している。

一方、FDAによるガイダンスが発行された後においても日本にはがんワクチン治療薬に関するガイダンス・ガイドラインが存在せず、ワクチン新薬開発に関する正当な評価が出来ない状況であった。そこで我々は日本バイオセラピィ学会主導で、日本におけるがんワクチン領域の新薬開発の考え方を示し、その創薬を活性化するべく、わが国独自のガイダンスを作製した。このガイダンスはFDAのガイダンスと異なり、日本において現在創薬化に最も近い段階の研究がおこなわれているがんペプチドワクチンに特化したガイダンスとなっている。2011年11月に原案が作成されパブリックコメントを受けた後、2012年12月に「がん治療用ペプチドワクチンガイダンス」として発行された（http://jsbt.org/guidance）。

バイオセラピィ学会ガイダンスにおいても、FDAのガイダンス同様にがんペプチドワクチンの免疫系を介した遅発性効果を考慮し、適切な対象を選択すること、長期にわたる継続的投与による腫瘍縮小にとらわれない生命予後を主目的とした研究デザインを立案すること、遅発性効果を解析可能な科学的手法で評価することなどを推奨している。

Ⅵ. Harrington-Fleming 検定

バイオセラピィ学会ガイダンスでは、検証試験における有効性の効果判定に用いる統計学的解析について特に踏み込んで記載している。Kaplan-Meier法により算出した生存割合の解析

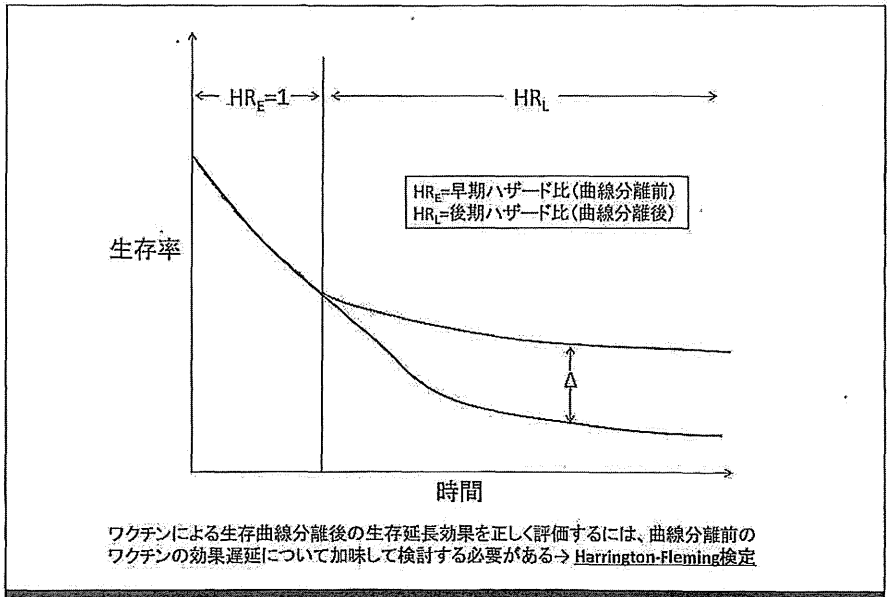

図1. がんワクチン療法における生存曲線分離の効果遅延

Kaplan-Meier法により算出した生存割合の解析において一般に用いられるのは、ハザード比が一定であることを想定したLog-rank検定であるが、がんペプチドワクチンでは遅発性の効果を想定し、観察期間後期に重みを置くHarrington-Fleming検定が必要と考えられる。

において一般に用いられるのは、ハザード比が一定であることを想定したLog-rank検定であるが、がんペプチドワクチンでは遅発性の効果を想定し、観察期間後期に重みを置くHarrington-Fleming検定[18]の必要性について触れられている（図1）。

実際、これまでに報告されているがんワクチンの生存における効果は、ワクチン投与開始の最初の3～6ヵ月では差がなく、その後徐々に生存曲線の分離が起こっているものが多い[3)4)5)]。日本初のがんワクチンにおけるPivotal studyとなったPEGASUS-PC Study（進行膵癌に対するゲムシタビン併用ペプチドワクチン療法の第Ⅱ/Ⅲ相治験）において、Harrington-Fleming法を初めて統計学的解析法に採用したことに引き続き、現在施行中の様々なPivotal studyにおいてPrimary endpointの統計学的解析方法として選択されている。

Ⅶ. irRC（Immune-related Response Criteria）

FDAガイダンスにおいてもバイオセラピィ学会ガイダンスにおいても、前述のようにがんワクチンの遅発性の効果について強調されている。これは、一般に抗癌剤の臨床的有用性を予見する為の代替評価項目として腫瘍縮小の評価に用いられるRECIST（Response evaluation criteria in solid tumours）ガイドラインが、がんワクチンの効果判定法として必ずしも適さないことを示している。つまり、RECISTでは一旦病勢が悪化するとその後に抗腫瘍反応がみられても病勢進行（PD）と評価され、ワクチン治療でしばしば認められる遅発性の効果については評価されないのである。そこで、がんワクチン療法の臨床効果評価におけるPD基準をより緩和した基準としてirRCが提唱された[19]。irRCは、WHOの基準をもとに、がんワクチン投与後の腫瘍特異的なCTLが誘導されるまでの猶予期間などを考慮して修正されている（図2）。ただし、irRCが生存延長のsurrogate markerになるかについては、今後の臨床研究を通してirRCによる効果判定と生存の相関関係に関する評価を継続し、有用性に関するエビデンスを構築していくことが肝要である。

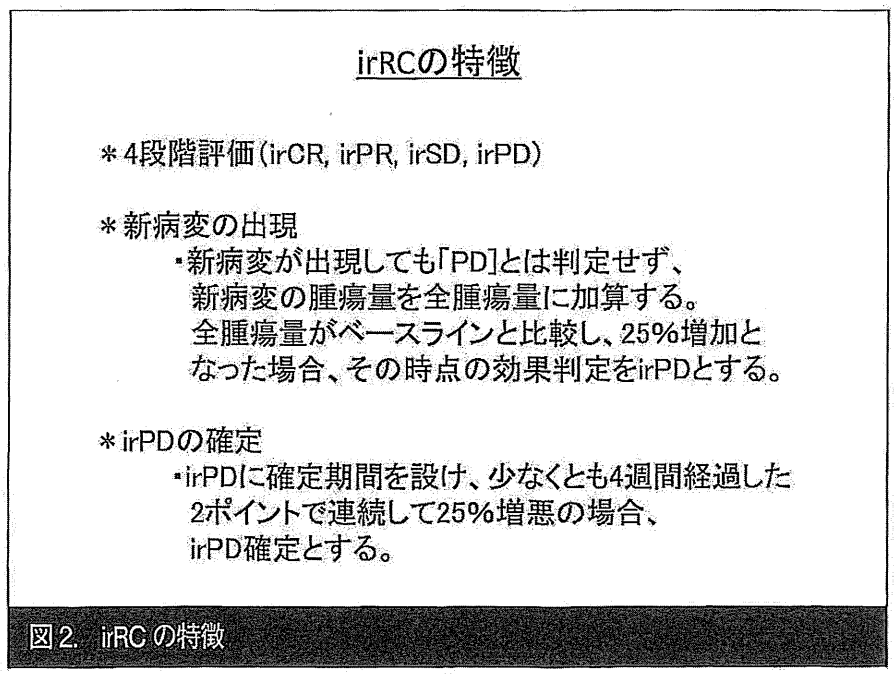

図2. irRCの特徴

Ⅷ. 免疫反応モニタリング

がんワクチンの作用機序として癌抗原特異的免疫反応を惹起することで抗腫瘍活性を引き起こすと考えられることから、がんワクチンの薬力学的解析として免疫反応をモニタリングすることは極めて重要である。バイオセラピィ学会ガイダンスでは、がんペプチドワクチンのモニタリングアッセイ法として、がんペプチドワクチンに対する Delayed type-hypersensitivity(DTH)反応（皮膚反応）、ペプチド特異的細胞傷害試験、ペプチド特異的 IFN-γ ELISPOT アッセイ、ペプチド特異的マルチマーフローサイトメトリー等が推奨される、と明記している。前述の PEGASUS-PC Study は、切除不能進行膵臓癌又は再発膵臓癌の患者 153 例を対象に、VEGFR2 由来のエピトープペプチド：エルパモチドとゲムシタビン併用投与群（実薬群）の安全性及び有効性を確認する目的で、プラセボ（偽薬）とゲムシタビンの投与群（プラセボ群）を対照として行った多施設共同の第 Ⅱ/Ⅲ相二重盲検比較試験である。我々はこの PEGASUS-PC Study を施行する根拠となった第 Ⅰ相試験を施行したが[20]、この試験ではペプチドワクチンの注射部位反応が抗腫瘍効果および生存期間延長と相関することが示され（図３）、皮膚反応と ELSPOT による免疫モニタリングの結果を指標として検証試験のワクチン投与量を決定した。そして、2013 年 1 月の ASCO-GI で報告した検証試験としての PEGASUS-PC Study の結果は、主要評価項目である全生存期間では実薬群とプラセボ群で統計学的有意差は認められなかったものの、注射部位反応によるサブグループ解析を行ったところ、強い皮膚反応（潰瘍）が認められた患者については、生存期間が延長している傾向があることが明らかとなった。特に、注射部位の潰瘍は実薬群においてのみ認められた事象であり、ワクチンにより誘導された特異的 CTL により引き起こされた事象と考えられた。さらに、注射部位反応の有無と生存期間には

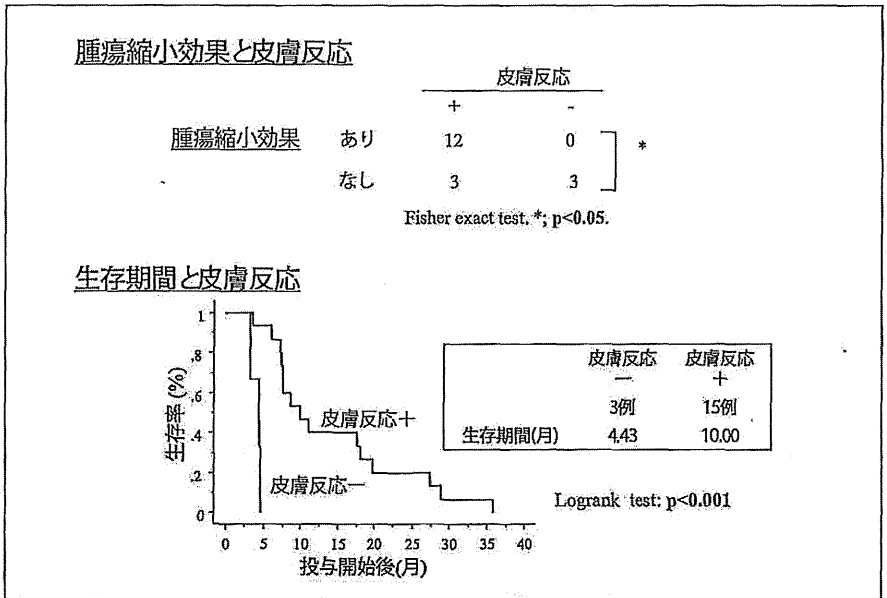

図 3.　VEGFR2 由来エピトープペプチドワクチンの皮膚反応と腫瘍縮小効果および生存期間

ペプチドワクチンによる腫瘍縮小効果および生存期間の延長と投与部位の皮膚反応は、正の相関を認めた。

強い相関が認められ、ワクチン投与部位の皮膚反応は、ワクチン投与による生存期間延長のsurrogate markerとなりうる可能性が示唆された。現在は膵癌術後補助療法として複数のペプチドワクチンのカクテル製剤にGemcitabineを併用した医師主導治験を多施設共同で実施しており（平成23・24・25年度厚労科研【難病・癌等の疾患分野の医療の実用化研究事業】研究代表者：和歌山県立医科大学外科学第2講座山上裕機)、その結果が期待される。また、今後このような臨床研究を進めていく中で、様々なモニタリングアッセイ法の標準化および結果の再現性についてのバリデーションを確実に行い、がんペプチドワクチン療法の効果が最も期待できる患者群を探索することも重要である。

おわりに

近年のがんペプチドワクチン療法開発の現状と、臨床試験の推進により明らかになってきた、がんワクチンの抗癌剤治療とは異なる作用機序を考慮した開発の方向性について概説した。がんペプチドワクチン療法は創薬化に向けた最終段階にあるが、さらなる発展には、適切なペプチドの開発と選択、免疫逃避機構を打破するアジュバント等の開発、サロゲートマーカーの開発による適切な対象の選択などが重要であり、今後の更なる基礎研究、臨床研究の推進が必要である。

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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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0264-410X/

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

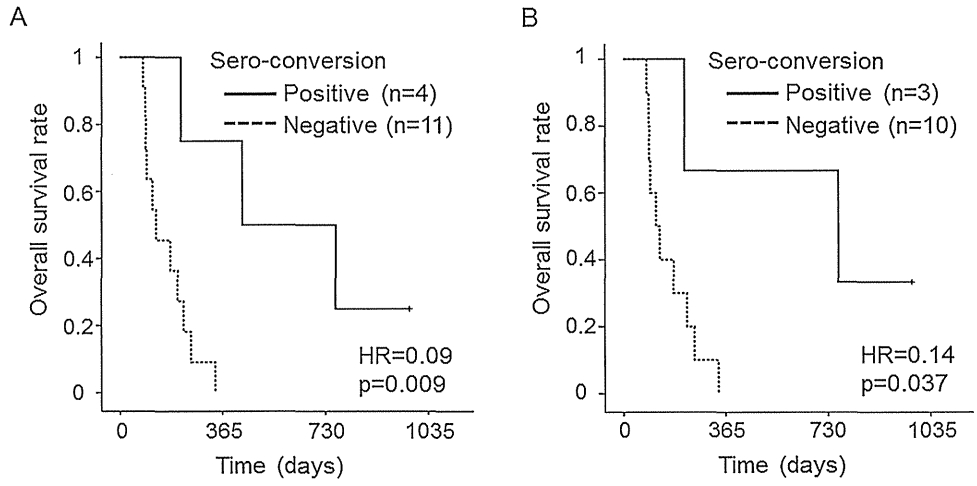

Fig. 1. Antibody production and prognosis. Overall survival of 15 patients and 13 esophageal cancer patients who completed 1 cycle of vaccination and the antibody response determined by ELISA were analyzed. Kaplan–Meier curves illustrate the duration of overall survival of sero-converted patients (solid 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,

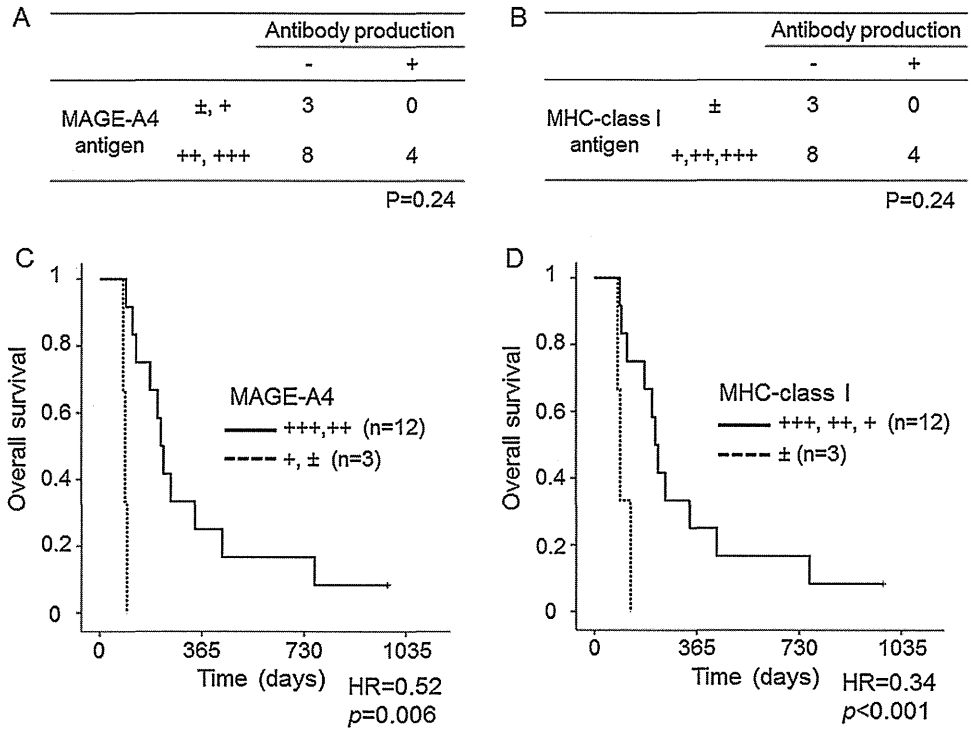

Fig. 2. MAGE-A4 and MHC-class antigen expression and prognosis. MAGE-A4 and MHC-class I were analyzed by immunohistochemical analysis with monoclonal antibodies; anti-pan-MAGE protein (57B), anti-human leukocyte antigen (HLA) class I (EMR 8–5). The reaction was evaluated as +++ (>50% stained cells), ++ (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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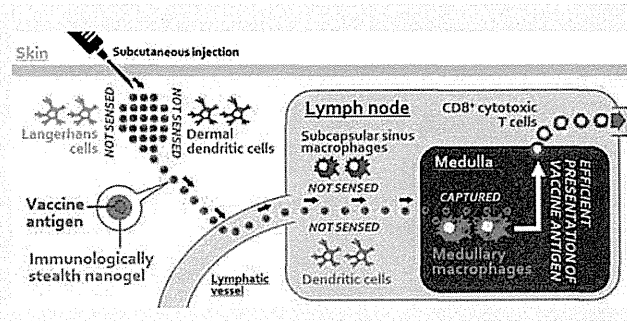
ABSTRACT Because existing therapeutic cancer vaccines provide only a limited clinical benefit, a different vaccination strategy is necessary to improve vaccine efficacy. We developed a nanoparticulate cancer vaccine by encapsulating a synthetic long peptide antigen within an immunologically inert nanoparticulate 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

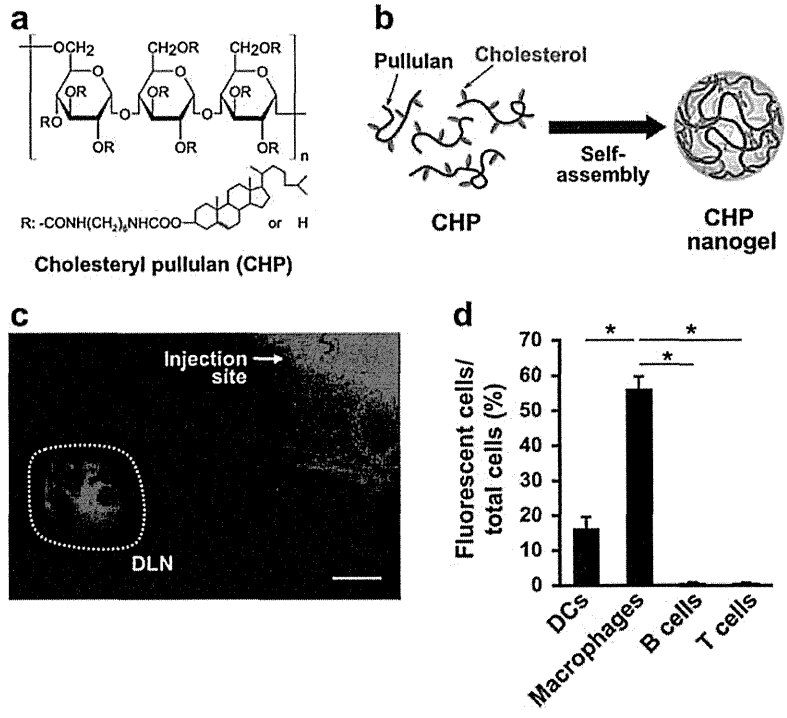

Figure 1. The CHP nanogel is selectively incorporated into macrophages in the DLN after subcutaneous injection to BALB/c mice. (a) Chemical structure of CHP. (b) Schematic representation of nanogel formation *via* self-assembly of CHP. CHP forms a nanogel by hydrophobic interaction between cholesteryl 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

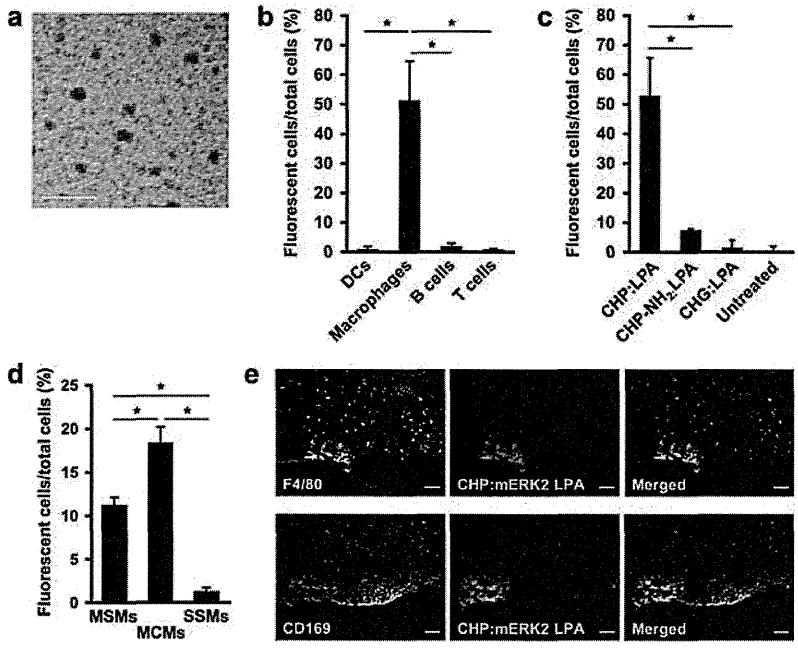

Figure 2. The CHP nanogel:LPA complex is selectively and efficiently engulfed by medullary macrophages in the DLN after subcutaneous injection to BALB/c mice. (a) Transmission electron microscopy of the CHP:LPA complex. The scale bar is 100 nm. (b) Uptake of the CHP:FAM-labeled mERK2 LPA complex in immune 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]

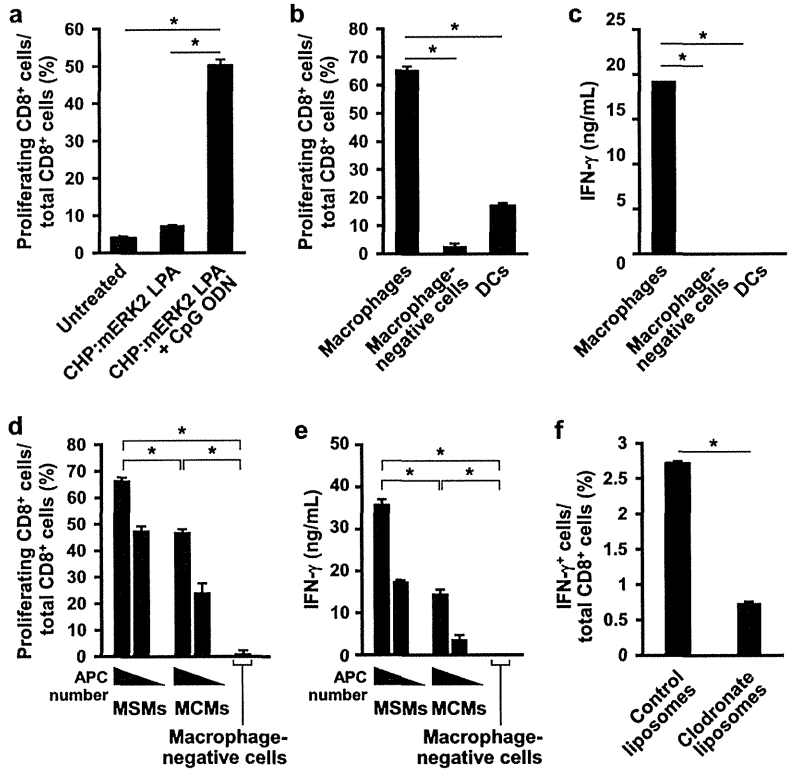

Figure 3. Medullary macrophages directly and efficiently cross-present antigens to specific $CD8^+$ T cells. (a–e) The CHP:mERK2 LPA complex with or without CpG ODN was injected into the footpad of BALB/c mice. Eighteen hours later, the whole lymph node cells (a) or the fractions containing macrophages ($F4/80^+CD11b^+$), 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

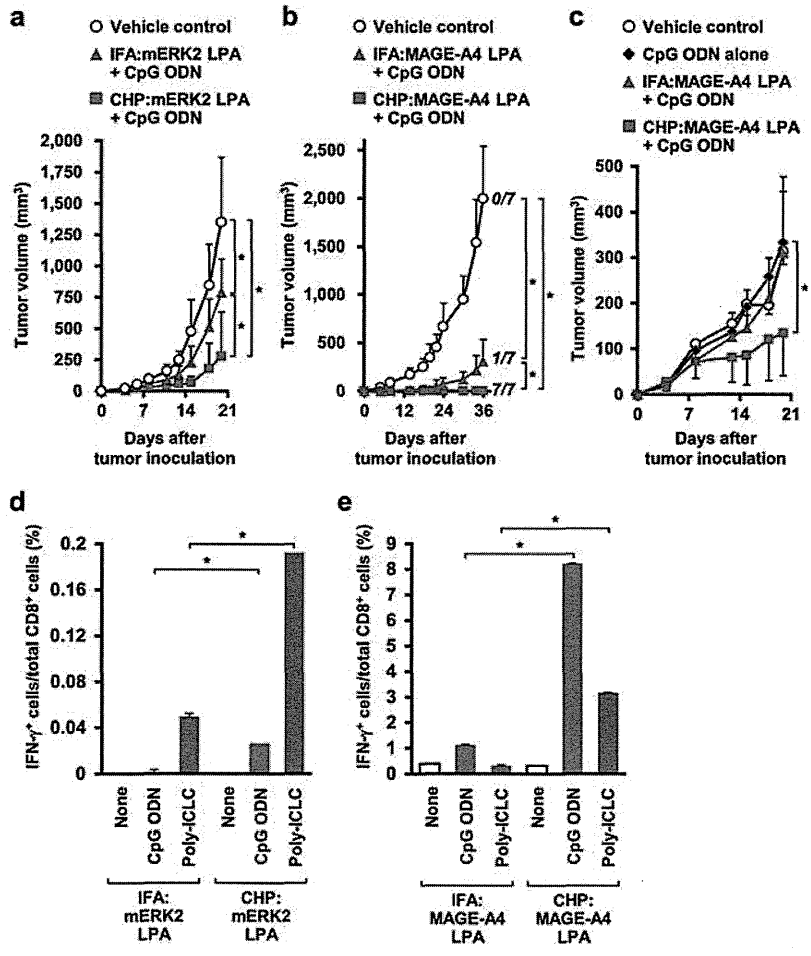

Figure 4. The macrophage-selective nanogel-based vaccine shows strong antitumor efficacy and $CD8^+$ T cell-inducing activity in the presence of Toll-like receptor TLR agonists as an adjuvant. (a and b) Effects of vaccination on *in vivo* tumor growth in the prophylactic setting. The LPA (50 μg) complexed 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

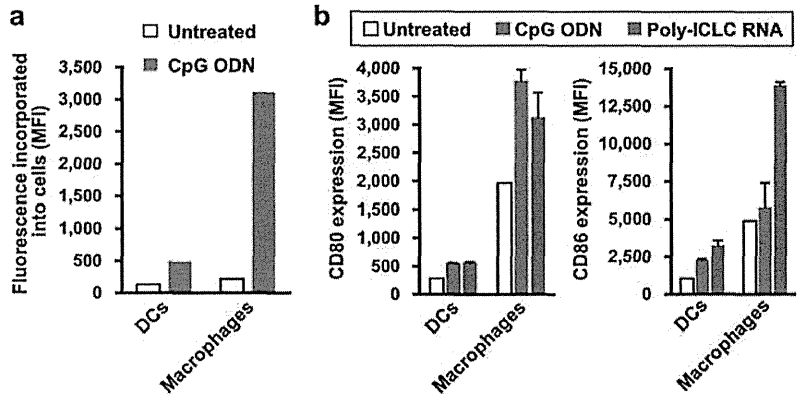

Figure 5. Lymph node macrophages are highly responsive to TLR agonists. (a) Incorporation of subcutaneously injected, FITC-labeled CpG ODN into macrophages and DCs in the DLN. BALB/c mice (two mice per group) were injected with FITC-CpG ODN (50 μg), and 16 h later, macrophages ($F4/80^+CD11b^+$) and 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