adjuvant.		adjuvant. Seattle, Washington, USA		
口頭・ポスター「Antigen free systemic monotherapy by nano-particulate TLR9 agonist induces CTL responses for the tumor regression.」	Kobiyama K, Kitahata Y, Ishii KJ.	第43回 日本免疫学会学術集会. 京都.	2014年12月10日~12月12日.	国内
ポスター「Particulate-induced systemic IgE production is mediated by DAMPs released from alveolar macrophages.」	Kuroda E, Ozasa K, Kobiyama K, Ishii KJ.	第43回 日本免疫学会学術集会. 京都.	2014年12月10日~12月12日.	国内
口頭・ポスター「Investigation of Potential and Mechanism of Hemozoin as Vaccine adjuvant.」	Ozkan M, Onishi M, Ishii KJ, Coban C.	第43回 日本免疫学会学術集会. 京都.	2014年12月10日~12月12日.	国内
口頭・ポスター「Synergistic activity of TLR9- and STING-agonists in innate and adaptive Type-II IFN production.」	Temizoz B, Kuroda E, Kobiyama K, Ishii KJ.	第43回 日本免疫学会学術集会. 京都.	2014年12月10日~12月12日.	国内

2．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
RNA Polymerase III Regulates Cytosolic RNA:DNA Hybrids and Intracellular MicroRNA Expression.	Koo CX, Kobiyama K, Shen YJ, LeBert N, Ahmad S, Khatoo M, Aoshi T, Gasser S, Ishii KJ.	J Biol Chem.	2015 Jan 26	国外
TLR9 and STING agonists synergistically induce innate and adaptive type II IFN.	Temizoz B, Kuroda E, Ohata K, Jonai N, Ozasa K, Kobiyama K, Aoshi T, Ishii KJ.	Eur J Immunol.	2014 Dec 22.	国外
Perivascular leukocyte clusters are essential for efficient activation of effector T cells in the skin.	Natsuaki Y, Egawa G, Nakamizo S, Ono S, Hanakawa S, Okada T, Kusuba N, Otsuka A, Kitoh A, Honda T, Nakajima S, Tsuchiya S, Sugimoto Y, Ishii KJ, Tsutsui H, Yagita H, Iwakura	Nat Immunol.	2014 Nov	国外

	Y, Kubo M, Ng Lg, Hashimoto T, Fuentes J, Guttman-Yassky E, Miyachi Y, Kabashima K.			
Protective properties of a fusion pneumococcal surface protein A (PspA) vaccine against pneumococcal challenge by five different PspA clades in mice.	Piao Z, Akeda Y, Takeuchi D, Ishii KJ, Ubukata K, Briles DE, Tomono K, Oishi K.	Vaccine.	2014 Sep	国外
Hemozoin as a novel adjuvant for inactivated whole virion influenza vaccine.	Uraki R, Das SC, Hatta M, Kiso M, Iwatsuki-Horimoto K, Ozawa M, Coban C, Ishii KJ, Kawaoka Y.	Vaccine	2014 Sep	国外
System vaccinology for the evaluation of influenza vaccine safety by multiplex gene detection of novel biomarkers in a preclinical study and batch release test.	Mizukami T, Momose H, Kuramitsu M, Takizawa K, Araki K, Furuhata K, Ishii KJ, Hamaguchi I, Yamaguchi K.	PLoS One	2014 Jul	国外
The early activation of CD8+ T cells is dependent on type I IFN signaling following intramuscular vaccination of adenovirus vector.	Hemmi M, Tachibana M, Tsuzuki S, Shoji M, Sakurai F, Kawabata K, Kobiyama K, Ishii KJ, Akira S, Mizuguchi H.	Biomed Res Int	2014	国外
Protective epitopes of the Plasmodium falciparum SERA5 malaria vaccine reside in intrinsically unstructured N-terminal repetitive sequences.	Yagi M, Bang G, Tougan T, Palacpac NM, Arisue N, Aoshi T, Matsumoto Y, Ishii KJ, Egwang TG, Druilhe P, Horii T.	LoS One	2014 Jun	国外
Olfactory plays a key role in spatiotemporal pathogenesis of cerebral malaria.	Zhao H, Aoshi T, Kawai S, Mori Y, Konishi A, Ozkan M, Fujita Y, Haseda Y, Shimizu M, Kohyama M,	Cell Host Microbe.	2014 May	国外

	Kobiyama K, Eto K, Nabekura J, Horii T, Ishino T, Yuda M, Hemmi H, Kaisho T, Akira S, Kinoshita M, Tohyama K, Yoshioka Y, Ishii KJ, Coban C.			
Hemozoin is a potent adjuvant for hemagglutinin split vaccine without pyrogenicity in ferrets.	Onishi M, Kitano M, Taniguchi K, Homma T, Kobayashi M, Sato A, Coban C, Ishii KJ.	Vaccine	2014 May	国外
Nucleic acid sensing by T cells initiates Th2 cell differentiation.	Imanishi T, Ishihara C, Badr Mel S, Hashimoto-Tane A, Kimura Y, Kawai T, Takeuchi O, Ishii KJ, Taniguchi S, Noda T, Hirano H, Brombacher F, Barber GN, Akira S, Saito T.	Nat Commun.	2014 Apr	国外
RAE1 ligands for the NKG2D receptor are regulated by STING-dependent DNA sensor pathways in lymphoma.	Lam AR, Le Bert N, Ho SS, Shen YJ, Tang ML, Xiong GM, Croxford JL, Koo CX, Ishii KJ, Akira S, Raulet DH, Gasser S.	Cancer Res.	2014 Apr	国外

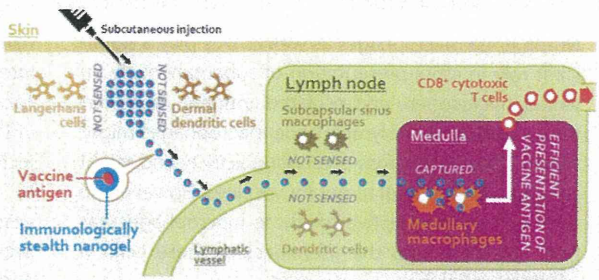
Ⅳ. 研究成果の刊行物・別刷

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

Daisuke Muraoka,[†,∥] Naozumi Harada,[†,‡,∥,*] Tae Hayashi,[†] Yoshiro Tahara,[§,⊥] Fumiyasu Momose,[†] Shin-ichi Sawada,[§,⊥] Sada-atsu Mukai,[§,⊥] Kazunari Akiyoshi,[§,⊥] and Hiroshi Shiku[†,*]

[†]Department of Immuno-Gene Therapy, Mie University Graduate School of Medicine, Mie 514-8507, Japan, [‡]Basic and Preclinical Research, ImmunoFrontier, Inc., Tokyo 101-0021, Japan, [§]Department of Polymer Chemistry, Kyoto University Graduate School of Engineering, Kyoto 615-8510, Japan, and [⊥]ERATO, Japan Science and Technology Agency (JST), Tokyo 102-0076, Japan. [∥]These authors contributed equally to this work.

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

* Address correspondence to nharada@clin.medic.mie-u.ac.jp, shiku@clin.medic.mie-u.ac.jp.

Received for review June 2, 2014 and accepted August 27, 2014.

Published online September 02, 2014
10.1021/nn502975r

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

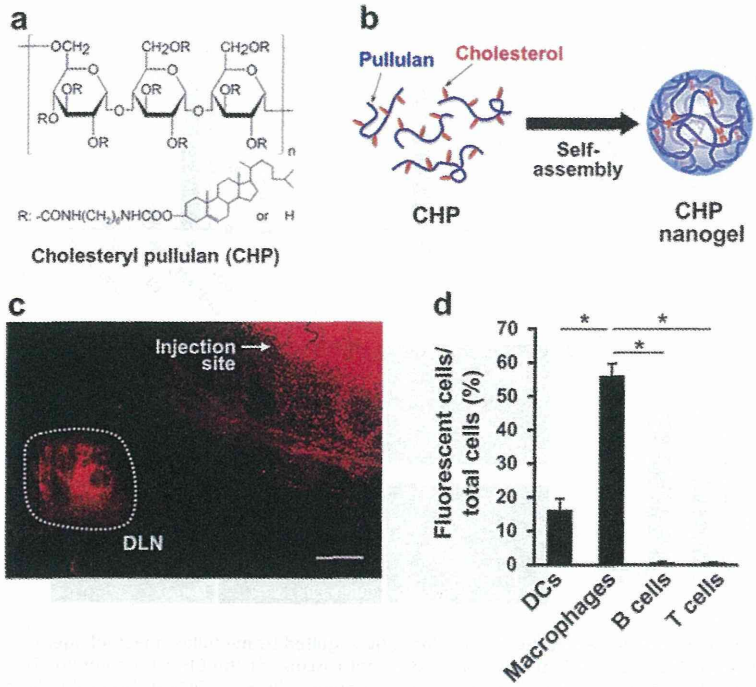


Figure 1. The CHP nanogel is selectively incorporated into macrophages in the DLN after subcutaneous injection to BALB/c mice. (a) Chemical structure of CHP. (b) Schematic representation of nanogel formation *via* self-assembly of CHP. CHP forms a nanogel by hydrophobic interaction between cholesteryl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

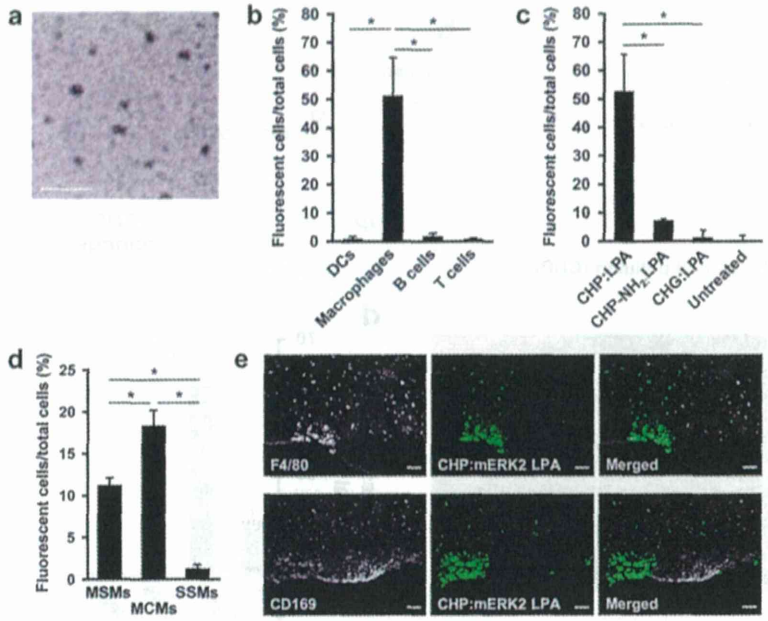


Figure 2. The CHP nanogel:LPA complex is selectively and efficiently engulfed by medullary macrophages in the DLN after subcutaneous injection to BALB/c mice. (a) Transmission electron microscopy of the CHP:LPA complex. The scale bar is 100 nm. (b) Uptake of the CHP:FAM-labeled mERK2 LPA complex in immune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

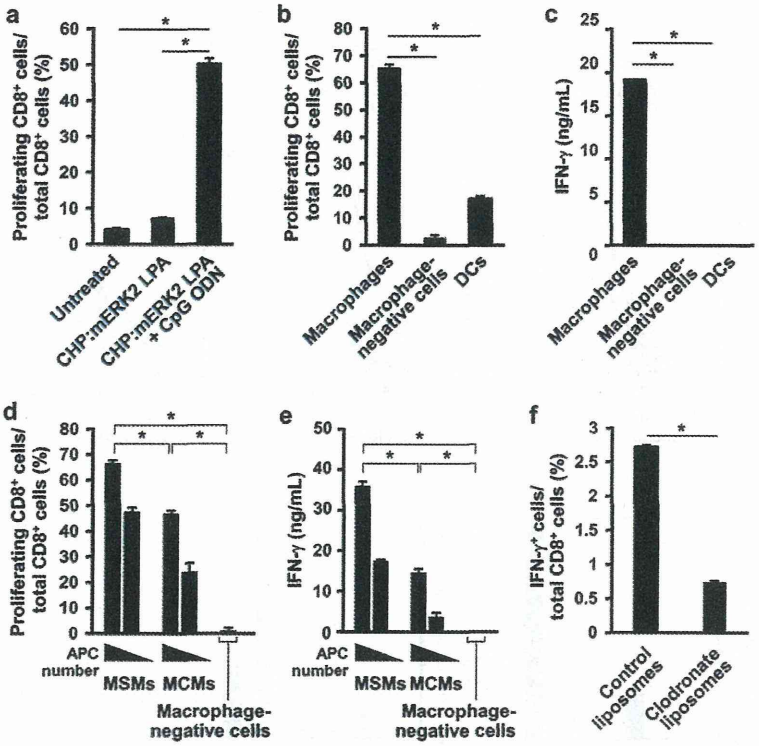


Figure 3. Medullary macrophages directly and efficiently cross-present antigens to specific CD8$^+$ T cells. (a–e) The CHP:mERK2 LPA complex with or without CpG ODN was injected into the footpad of BALB/c mice. Eighteen hours later, the whole lymph node cells (a) or the fractions containing macrophages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

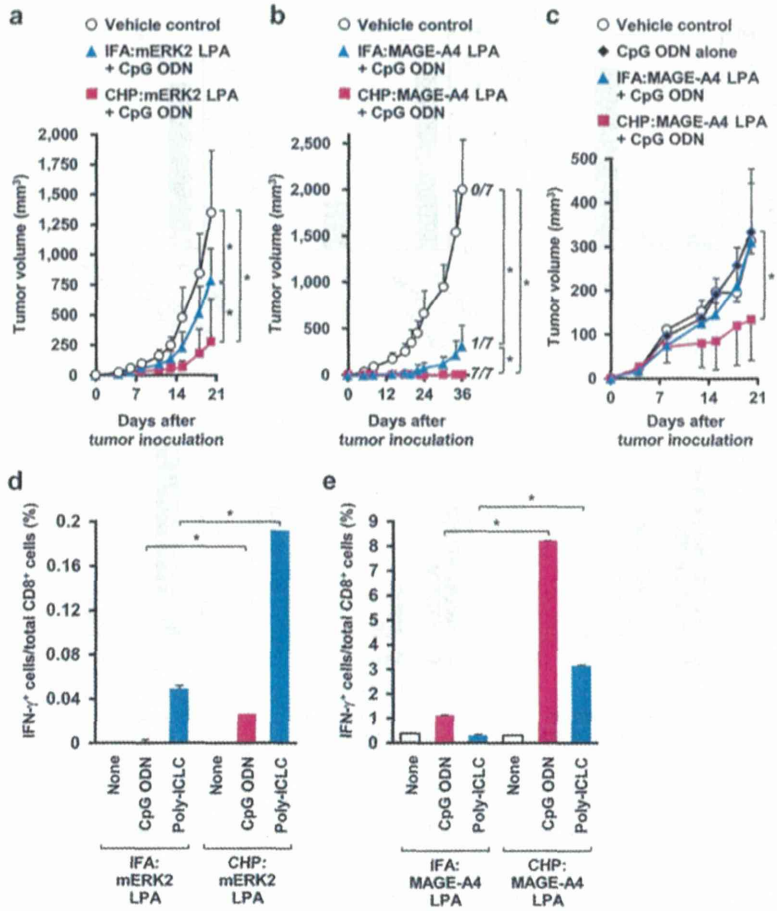


Figure 4. The macrophage-selective nanogel-based vaccine shows strong antitumor efficacy and $CD8^+$ T cell-inducing activity in the presence of Toll-like receptor TLR agonists as an adjuvant. (a and b) Effects of vaccination on *in vivo* tumor growth in the prophylactic setting. The LPA (50 μg) complexed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