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Development of a vaccine based on bacteria-mimicking tumor cells coated with novel engineered toll-like receptor 2 ligands

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For a successful tumor vaccine, it is necessary to develop effective immuno-adjuvants and identify specific tumor antigens. Tumor cells obtained from surgical or biopsy tissues are a good source of tumor antigens but, unlike bacteria, they do not induce strong immune responses. Here, we designed 2 novel lipopeptides that coat tumor cell surfaces and mimic bacterial components. Tumor cells coated with these lipopeptides (called bacteria-mimicking tumor cells [BMTC]) were prepared and their efficacy as a tumor vaccine examined. Natural bacterial lipopeptides act as ligands for toll-like receptor 2 (TLR2) and activate dendritic cells (DC). To increase the affinity of the developed lipopeptides for the negatively charged plasma membrane, a cationic polypeptide was connected to Pam2Cys (P2C), which is the basic structure of the TLR2 ligand. This increased the non-specific binding affinity of the peptides for the cell surface. Two such lipopeptides, P2CSK11 (containing 1 serine and 11 lysine residues) and P2CSR11 (containing 1 serine and 11 arginine residues) bound to irradiated tumor cells via the long cationic polypeptides more efficiently than the natural lipopeptide MALP2 (P2C-GNNDESNISFKEK) or a synthetic lipopeptide P2CSK4 (a short cationic polypeptide containing 1 serine and 4 lysines). BMTC coated with P2CSR11 or P2CSK11 were efficiently phagocytosed by DC and induced antigen cross-presentation in vitro. They also induced effective tumor-specific cytotoxic T cell responses and inhibited tumor growth in in vivo mouse models. P2CSR11 activated DC but induced less inflammation-inducing cytokines/interferons than other lipopeptides. Thus, P2CSR11 is a strong candidate antigen-specific immuno-adjuvant, with few adverse effects.

KEYWORDS
adjuvant, bacterial lipopeptide, drug design, toll-like receptor ligand, tumor vaccine

1 | INTRODUCTION

Immunotherapy using immune checkpoint inhibitors is an effective anti-tumor treatment.[1,2] Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are the most critical effectors against tumor cells; however, during tumorigenesis, tumor cells acquire multiple mechanisms that allow then to attenuate/escape the cytotoxic activity of these effector cells. Accumulation of effector cells at tumor sites is often prevented, and their efficacy is suppressed by negative regulator cells such as myeloid-derived suppressor cells,[3] M2-like macrophages[4,5] and regulatory T cells.[6] The interaction between cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and CD80/CD86, and between programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1), suppresses T cell proliferation, cytokine production and cytotoxic function.[7,8] To overcome such tumor-mediated immunosuppression, it is necessary to develop effective strategies to stimulate tumor-specific effector cells.

We previously developed effective and useful immuno-adjuvants that activate effector cells.[9,10] We constructed new anti-tumor drugs by altering the structure of a mycoplasma lipopeptide, MALP2, which is the basic component of immuno-adjuvants. MALP2, a natural toll-like receptor 2 (TLR2) ligand, consists of a mycoplasma peptide containing 13 amino acids and an N-terminal cysteine conjugated to 2 palmitate moieties (S-2,3-bis(palmitoyloxy)-propyl-cysteine [Pam2Cys/P2C]).[11,12] Although natural lipopeptides/TLR2 ligands harbor some peptide sequence motifs that are necessary for biosynthetic processes,[13,14] these are unnecessary if we generate the lipopeptides by chemical synthesis. However, synthetic lipopeptides must meet essential conditions for TLR2/TLR6 ligand activity[15,16] (a small amino acid at the 2nd position[17] and a highly hydrophilic peptide sequence).[18,19] Therefore, to increase anti-tumor immuno-adjuvant activity, we exchanged bacterial peptides for other functional peptide sequences that meet the conditions essential for TLR ligand activity. In a previous report, we named this process of designing and developing synthetic adjuvants "adjuvant engineering."[10]

Tumor cells and tissues obtained by surgical operation or biopsy are the most suitable antigen sources for constructing a personalized tumor vaccine because they include individual tumor antigens, even if they have not yet been formally identified. This strategy has been developed to generate autologous tumor vaccines.[20,21] However, most therapies based on tumor antigens, including peptide vaccines, do not show marked therapeutic effects.[22,23] Bacteria express both TLR ligands and specific antigens; therefore, bacteria activate dendritic cells (DC) directly via TLR; DC then present bacteria-derived peptide antigens to effector cells to induce strong immune responses. Tumor cells alone cannot induce strong immune responses because although tumors express many specific antigens (such as cancer-testis antigens and tumor-specific neoantigens) they do not express TLR ligands. To induce strong immune responses, simple mixtures of TLR ligands and antigens[24-28] or TLR ligands conjugated with antigen peptide[29,30] are widely used as vaccines. Another study shows that gram-negative bacteria, such as *Salmonella* species, expressing identified tumor antigens are useful for tumor immunotherapy.[31] Thus, mimicking bacterial cells/materials may induce strong immune responses to antigens present on the original cells/materials.

Here, we developed cationic lipopeptides that bound electrostatically to negatively charged tumor cell membranes and used them to prepare tumor cells coated with lipopeptides/TLR2 ligands acting as immuno-adjuvants. We then examined the effects of these bacteria-mimicking tumor cells (BMTC) as vaccines to initiate anti-tumor immune responses.

2 | MATERIALS AND METHODS

2.1 | Mice, cells and reagents

Wild-type and *Tlr2*$^{-/-}$ C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and Oriental BioService (Kyoto, Japan), respectively. OT-I mice were kindly provided by Dr Hiroshi Kosaka (Osaka University). Mice were maintained under specific pathogen-free conditions in the animal facility of Osaka International Cancer Institute. All animal experiments were performed in accordance with institutional guidelines and approved by the Animal Care and Use Committee of our institute (approval number: 16062409).

Ovalbumin (OVA)-expressing EL4 thymoma cells (EG7-OVA cells) were obtained from the American Tissue Culture Collection (Manassas, VA, USA).[32] A murine Wilms tumor 1 (WT1)-expressing acute myeloid leukemia cell line (mWT1-C1498) was kindly provided by Dr H. Sugiyama (Osaka University, Osaka, Japan).[33] YAC-1, a mouse NK target cell line, was kindly provided by Dr T. Seya (Hokkaido University, Sapporo, Japan).[34] RMA-S, a TAP2-deficient subline of RMA (Rauscher leukemia virus-induced lymphoma cell line), was kindly provided by Dr K. Kärre (Karolinska Institute, Sweden).[35] Culture conditions were as previously described.[9,10] Ovalbumin-expressing RMA-S cells (RMA-S-OVA) were established by co-transfecting cells with a pPB-EGFP-neo PiggyBac vector containing FLAG-tagged OVA cDNA (XM 015282166, c.3484-4357) and pCMV-hyPBase (both plasmids were kindly provided by Dr K. Yusa).[36,37] Bone marrow-derived DC (BMDC), inguinal lymph node cells and splenocytes were prepared as previously described.[9,10]

The following peptides and lipopeptides (purity >90%) were customized by Bio-Synthesis (Lewisville, TX, USA) via Biologica (Nagoya, Aichi, Japan):[9,10] OVA peptide, SIINFEKL; murine WT1 peptide, RMFPNAPYL; bio-SK4 peptide, biotinylated-SKKKK; bio-MALP2 peptide, biotinylated-GNNDESNISFKEK; bio-SR11 peptide, biotinylated-SRRRRRRRRRR; bio-SK11 peptide, biotinylated-SKKKKKKKKKK; P2CSK4 lipopeptide, P2C-SKKKK; MALP2 lipopeptide, P2C-GNNDESNISFKEK; P2CSR11 lipopeptide, P2C-SRRRRRRRRRR; and P2CSK11 lipopeptide, P2C-SKKKKKKKKKK.

2.2 | Preparation of bacteria-mimicking tumor cells

Tumor cells were irradiated with X-rays (100 Gy) to prevent proliferation. Prior to use in the phagocytosis assay, tumor cells were cultured for 30 minutes at 37°C with 10 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE). One million tumor cells were treated with 100 μmol/L lipopeptide in 100 μL of PBS for 2 hour at 4°C. For the in vivo experiments, mixtures of lipopeptide and irradiated tumor cells were used as the anti-tumor vaccine (the mixture included both cell-bound and free lipopeptides); this is because BMTC coated with a non-adherent lipopeptide such as P2CSK4 would lose vaccine activity after the washing process. To examine the effects of vaccines that do not contain free lipopeptide, BMTC were then washed twice in culture medium to remove free lipopeptide.

2.3 | In vitro assays

Bone marrow-derived DC and splenocytes were stimulated with lipopeptides as previously described.[9,10] The phagocytosis assay was performed using a Phagocytosis Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, BMDC were stimulated with

each lipopeptide for 24 hours and then cultured with Latex Beads-Rabbit IgG-FITC Complex for 6 hours. For the phagocytosis and cross-presentation assays with BMTC, BMDC were cultured with BMTC (BMDC:BMTC ratio = 1:5) for 12 h (phagocytosis) or 24 hours (cross-presentation) and then analyzed by flow cytometry. Flow cytometry and the ELISA were performed as previously described[9,10] using the following antibodies and kits: FITC-conjugated anti-mouse CD80; phycoerythrin (PE)-conjugated anti-mouse MHC class I molecule K^b bound to the peptide SIINFEKL (Kb-SIINFEKL; BioLegend, San Diego, CA, USA); allophycocyanin (APC)-conjugated anti-CD11c (eBioscience, San Diego, CA, USA); and ELISA kits specific for IFN-γ and IL-12p40 (R&D Systems, Minneapolis, MN, USA).

2.4 | In vivo and ex vivo mouse models

The in vivo experiments with EG7-OVA and mWT1-C1498 cells were performed as previously described.[9,10] On Day 0, EG7-OVA cells (1-2 × 10^6 cells) and WT1-C1498 (3 × 10^5 cells) were transplanted subcutaneously into the back or flank, respectively, of the mice. When the calculated tumor mass reached approximately 0.4-1.0 cm^3, mice were grouped and subjected to the first treatment. The BMTC (50 μL of a mixture containing 10 nmol lipopeptide and 2 × 10^5 irradiated tumor cells) were then administered intradermally 3 times over a period of 1 week (BMTC were injected into the skin around the transplanted tumors between Days 9 and 25). When we examine the effects of vaccines after removal of free lipopeptide, the BMTC were administered twice intradermally. A lipopeptide dose of 10 nmol was validated previously using P2CSK4 as a positive control.[9] An anti-mouse PD-L1 antibody (InVivoMab; 10F.9G2, Bio X Cell, West Lebanon, NH, USA; 200 μg per mouse) was injected intraperitoneally on the same day as the BMTC. Tumor volume was measured as previously described.[9,10] To measure cytotoxicity, mice harboring EG7-OVA or mWT1-C1498 tumors were treated as described above and, 24 hours after additional administration of the vaccine (for the EG7-OVA model, Day 24; for the WT1-C1498 model, Day 28), splenocytes were harvested. After in vitro culture for 6 days with OVA peptide and IL-2, cytotoxic activity against RMA-S cells pulsed with OVA or WT1 peptide or YAC-1 cells was measured in a Calcein AM (Molecular Probes, Eugene, OR, USA) assay. Target cells were cultured with each effector cell (effector: target ratio, 30:1) for 6 h at 37°C, and specific lysis (expressed as a percentage) was calculated as previously described.[9,10]

2.5 | Statistical analyses

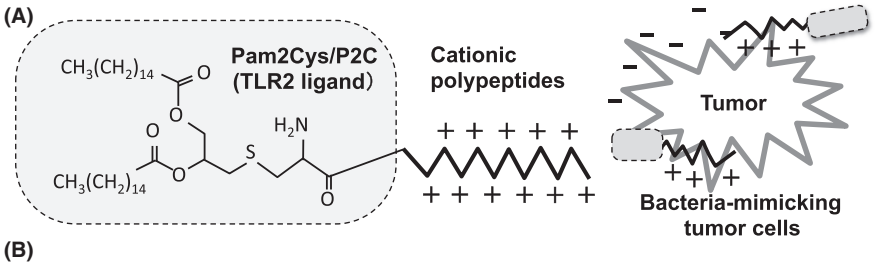
Data are expressed as the mean ± SD (for cytokine measurement) or the mean ± standard error (SE) (for tumor volume). The significance of differences between groups was analyzed using Student's *t* test (comparison of 2 groups) or 1-way ANOVA with Dunnett's test (for multiple comparisons [more than 3 groups]). One-sided *P*-values <.05 were considered statistically significant.

3 | RESULTS

To develop novel artificial immuno-adjuvants that can increase the efficacy of personalized vaccines comprising tumor cells as a source of cancer antigens, we linked P2C (a ligand for TLR2) to polypeptides comprising 11 cationic amino acids. As positively charged peptides are attracted electrostatically to the negatively charged plasma membrane of the tumor cell, the lipopeptides coated the tumor cells to yield BMTC, which could then be used as a candidate tumor vaccine (Figure 1A). We prepared 2 novel lipopeptides containing a long cationic sequence: P2CSK11 (P2C-SKKKKKKKKKK) and P2CSR11 (P2CSRRRRRRRRRR) (Figure 1B). A natural lipopeptide MALP2 (P2C-GNNDESNISFKEK) and a synthetic lipopeptide P2CSK4 (P2C-SKKKK), which has strong anti-tumor activity, were used as controls.[9,10] First, we examined the affinity of the peptide components of the lipopeptides for RMA-S tumor cells. Irradiated RMA-S cells were incubated with biotinylated peptides and then stained with APC-conjugated streptavidin. The bio-SK4 and bio-MALP2 peptides did not bind to RMA-S cells, whereas the bio-SR11 peptide (at 1, 10 and 100 μmol/L) and bio-SK11 peptides (at 10 and 100 μmol/L) bound to cells in a dose-dependent manner (Figure 1c).

Next, to examine the ability of the lipopeptides to activate DC and splenocytes, we monitored expression of CD80/CD86 (a marker of DC maturation) (Figure 2A,B). We also used the Latex Beads-Rabbit IgG-FITC Complex to measure the phagocytic activity of BMDC stimulated with each lipopeptide (Figure 2C). In contrast to P2CSK4, both P2CSK11 and P2CSR11 activated DC to a similar degree in terms of expression of CD80/CD86 and phagocytic activity. BMDC derived from *Tlr2*$^{-/-}$ mice did not respond to the lipopeptides (Figure 2A-C). Furthermore, we examined the ability of BMDC and splenocytes stimulated with the lipopeptides to secrete cytokines. P2CSK11 and P2CSK4 induced similar levels of IL-12p40 production by BMDC, whereas P2CSK11 induced less IFN-γ production by splenocytes than P2CSK4; however, P2CSR11 induced weak production of both cytokines (Figure 2D). No increase in IL-12p40 and IFN-γ production in response to lipopeptides was detected in cells from *Tlr2*$^{-/-}$ mice (data not shown).

To examine the effectiveness of BMTC as a tumor vaccine in vitro, we analyzed their ability to induce phagocytosis and cross-presentation by DC. First, irradiated CFSE-labeled RMA-S-OVA cells were preincubated with the lipopeptides and then cultured with BMDC. Increased phagocytosis was detected only in RMA-S-OVA cells coated with P2CSK11 or P2CSR11. However, no increase was detected in BMDC derived from *Tlr2*$^{-/-}$ or *Myd88*$^{-/-}$ mice (Figure 3A). Furthermore, to examine antigen presentation, we detected expression of MHC class I/OVA peptide complexes on the DC surface using a SIINFEKL/H-$2K^b$-specific antibody. Both P2CSK11-coated and P2CSR11-coated tumor cells induced antigen cross-presentation by BMDC (Figure 3B). To evaluate cross-presentation, we irradiated RMA-S-OVA cells, preincubated them with the lipopeptides, and then co-cultured them with OT-I splenocytes. Because RMA-S cells lack transporter associated protein 2 (TAP2), they cannot present OVA peptides on MHC class I molecules. Antigen-



(B)

Lipopeptide	Name	pI	Hydro-phobicity	Molecular Weight	P2C	1	2	3	4	5	6	7	8	9	10	11	12	13
Natural	MALP2	4.68	−1.50	2135.5	P2C	G	N	N	D	E	S	N	I	S	F	K	E	K
Artificial	P2CSK4	10.04	−2.32	1271.8	P2C	S	K	K	K	K								
Original	P2CSK11	10.66	−3.17	2169.1	P2C	S	K	K	K	K	K	K	K	K	K	K	K	
Original	P2CSR11	12.65	−3.68	2477.5	P2C	S	R	R	R	R	R	R	R	R	R	R	R	

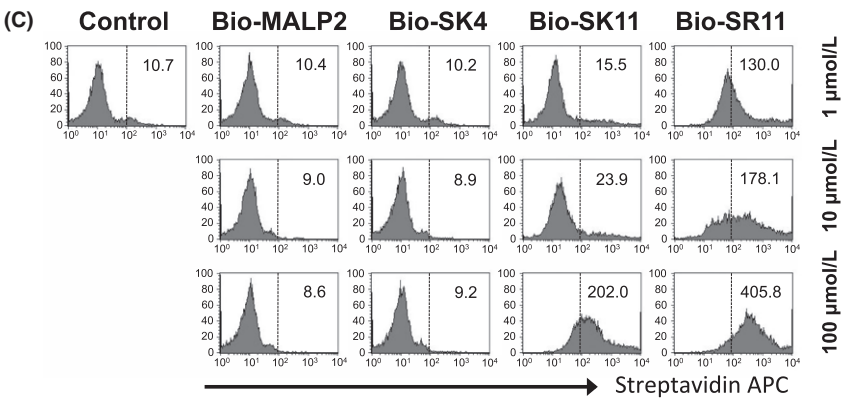


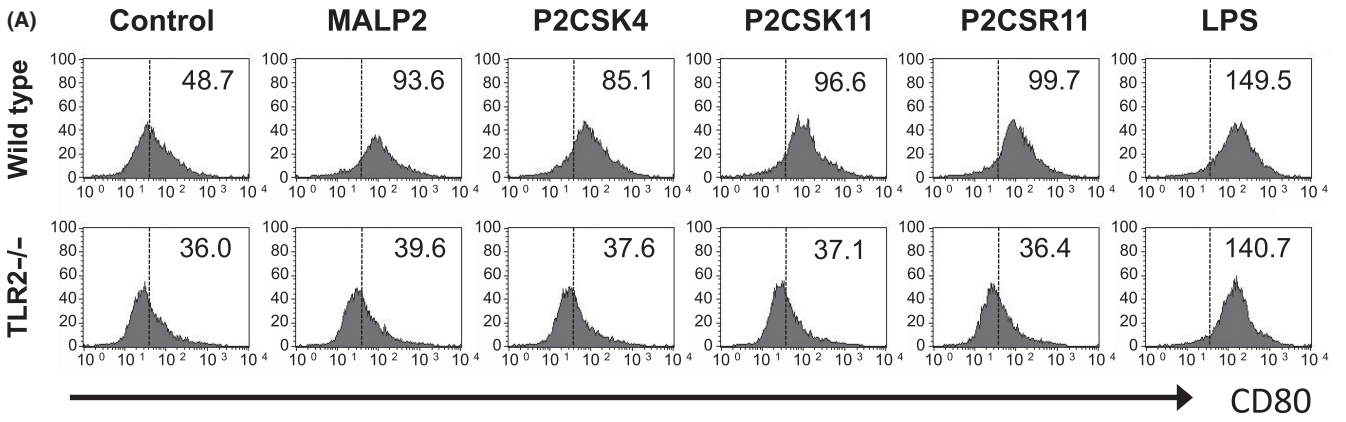
FIGURE 1 The importance of peptide sequences within lipopeptides used for tumor cell modification. A, Strategy used to generate tumor cells coated with a synthetic TLR2 ligand. A synthetic lipopeptide containing positively charged polypeptide sequences binds non-specifically to the negatively charged tumor cell plasma membrane. Tumor cells coated with these lipopeptides, called bacteria-mimicking tumor cells (BMTC), are expected to be recognized by antigen-presenting cells. B, The characteristics, molecular weight, isoelectric point (pI) and hydrophobicity of the natural (MALP2) and synthetic (P2CSK4, P2CSK11 and P2CSR11) lipopeptides used in the study. The pI and hydrophobicity of the peptide sequences was calculated using the ProtParam tool (http://br.expasy.org/tools/protparam.html). C, Affinity of biotinylated peptides for RMA-S cells. Binding of the peptides was detected using streptavidin-APC and flow cytometry. The numbers in the panels represent the mean fluorescence intensities

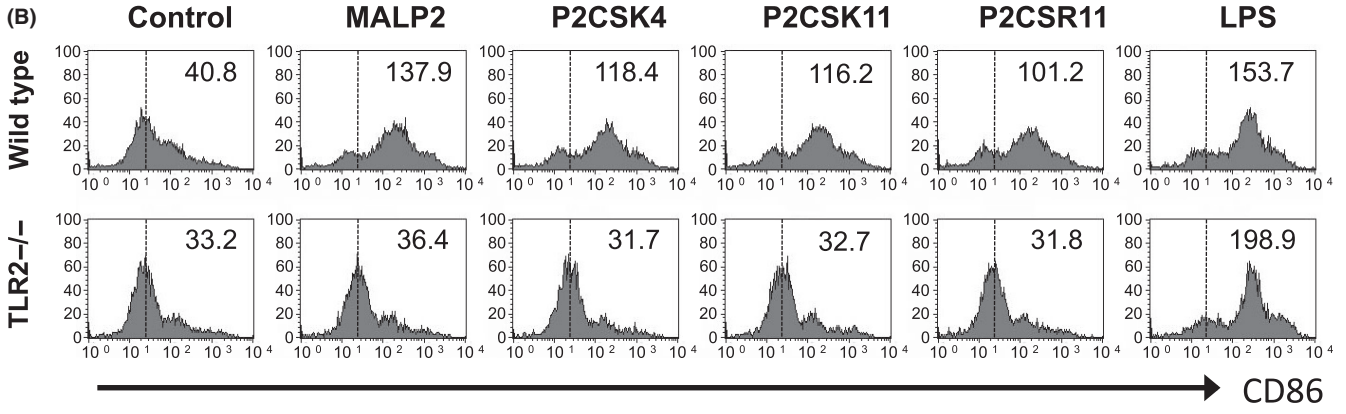
presenting cells derived from OT-I splenocytes should engulf OVA-transfected RMA-S cells and present OVA peptides on MHC class I molecules, thereby inducing IFN-γ production by OT-I T cells. The results showed that OT-I splenocytes stimulated with tumor cells coated with P2CSK11 or P2CSR11 produced IFN-γ (Figure 3C).

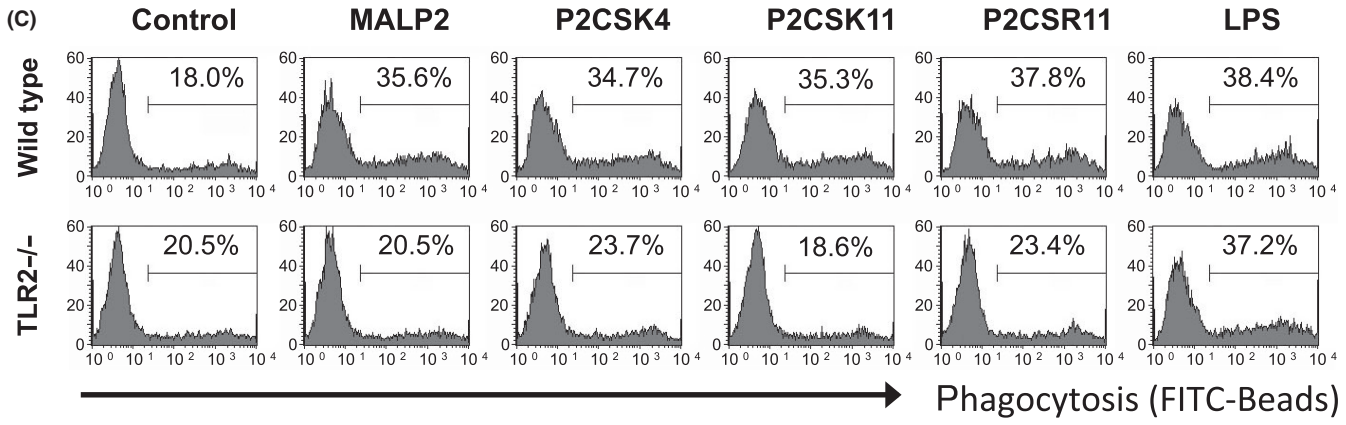
Next, we examined anti-tumor effects and CTL-induction in in vivo mouse models. Mice transplanted with EG7-OVA or mWT1-C1498 cells were treated with the tumor vaccine. In this experiment, a mixture of lipopeptide and irradiated tumor cells were used as the tumor vaccine (which included free lipopeptides). Although P2CSK11 and P2CSR11 showed anti-tumor effects similar to those of P2CSK4 (Figure 4A), all induced skin erosions and inflammation at the site of vaccination (in 20%, 60% and 100% of mice treated with P2CSR11, P2CSK11 and P2CSK4, respectively) (Figure 4B). Next, we examined the effects of vaccines that did not contain free lipopeptides (P2CSK4; Figure 4C, left panel; and P2CSR11; Figure 4C, right panel). BMTC prepared with P2CSK4, but not those prepared with P2CSR11, showed less anti-tumor activity after free peptide was removed; this is because P2CSK4 binds tumor cell membranes more weakly than P2CSR11 (Figure 4C). Furthermore, we examined the cytotoxic activity of lipopeptides against different tumor cells using splenocytes from treated mice. P2CSR11 induced slightly higher levels of specific CTL activity, but lower levels of NK activity, than the other lipopeptides (Figure 4D).

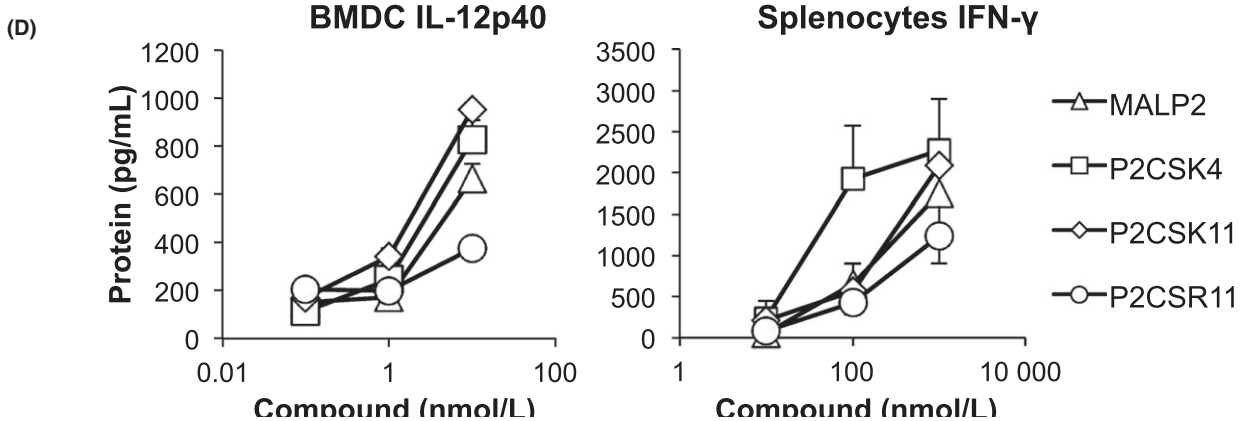
To ascertain the method most suitable for preparation of tumor cells for use as tumor antigens, we compared the in vivo anti-tumor effects of irradiated tumor cells (which are similar to previously reported antigen inclusion bodies comprising liposomes with adjuvant[38]) with those of tumor cell debris prepared by freeze/thaw methods (the simple mixture of antigens and adjuvant). In the presence of tumor cell debris, P2CSR11 generated only weak anti-tumor effects (Figure 5A). However, P2CSK4 showed similar levels of anti-tumor activity when bound to irradiated tumor cells or in the presence of tumor cell debris (Figure 5B). The anti-tumor effects of

FIGURE 2 Exposure of bone marrow-derived dendritic cells (BMDC) to lipopeptides in vitro. Expression of CD80 (A) and CD86 (B) by BMDC stimulated for 24 h with 10 nmol/L lipopeptide or 100 ng/mL LPS was examined by flow cytometry. The numbers in the panels represent the mean fluorescence intensities. C, Phagocytic activity of BMDC stimulated with lipopeptides or LPS. At 24 h post-stimulation, BMDC were cultured for 6 h with Latex Beads-Rabbit IgG-FITC Complex and analyzed by flow cytometry. The numbers in the panels represent the percentage of gated cells. Representative images from 2 repeat experiments with similar results are shown. D, IL-12p40 production by BMDC (left) and IFN-γ production by splenocytes (right) stimulated with each lipopeptide for 48 h (BMDC) or 72 h (splenocytes) was measured in an ELISA


(A)
Control
MALP2
P2CSK4
P2CSK11
P2CSR11
LPS
Wild type
TLR2−/−
48.7
93.6
85.1
96.6
99.7
149.5
36.0
39.6
37.6
37.1
36.4
140.7
CD80


(B)
Control
MALP2
P2CSK4
P2CSK11
P2CSR11
LPS
Wild type
TLR2−/−
40.8
137.9
118.4
116.2
101.2
153.7
33.2
36.4
31.7
32.7
31.8
198.9
CD86


(C)
Control
MALP2
P2CSK4
P2CSK11
P2CSR11
LPS
Wild type
TLR2−/−
18.0%
35.6%
34.7%
35.3%
37.8%
38.4%
20.5%
20.5%
23.7%
18.6%
23.4%
37.2%
Phagocytosis (FITC-Beads)


(D)
BMDC IL-12p40
Splenocytes IFN-γ
Protein (pg/mL)
Compound (nmol/L)
Compound (nmol/L)
MALP2
P2CSK4
P2CSK11
P2CSR11

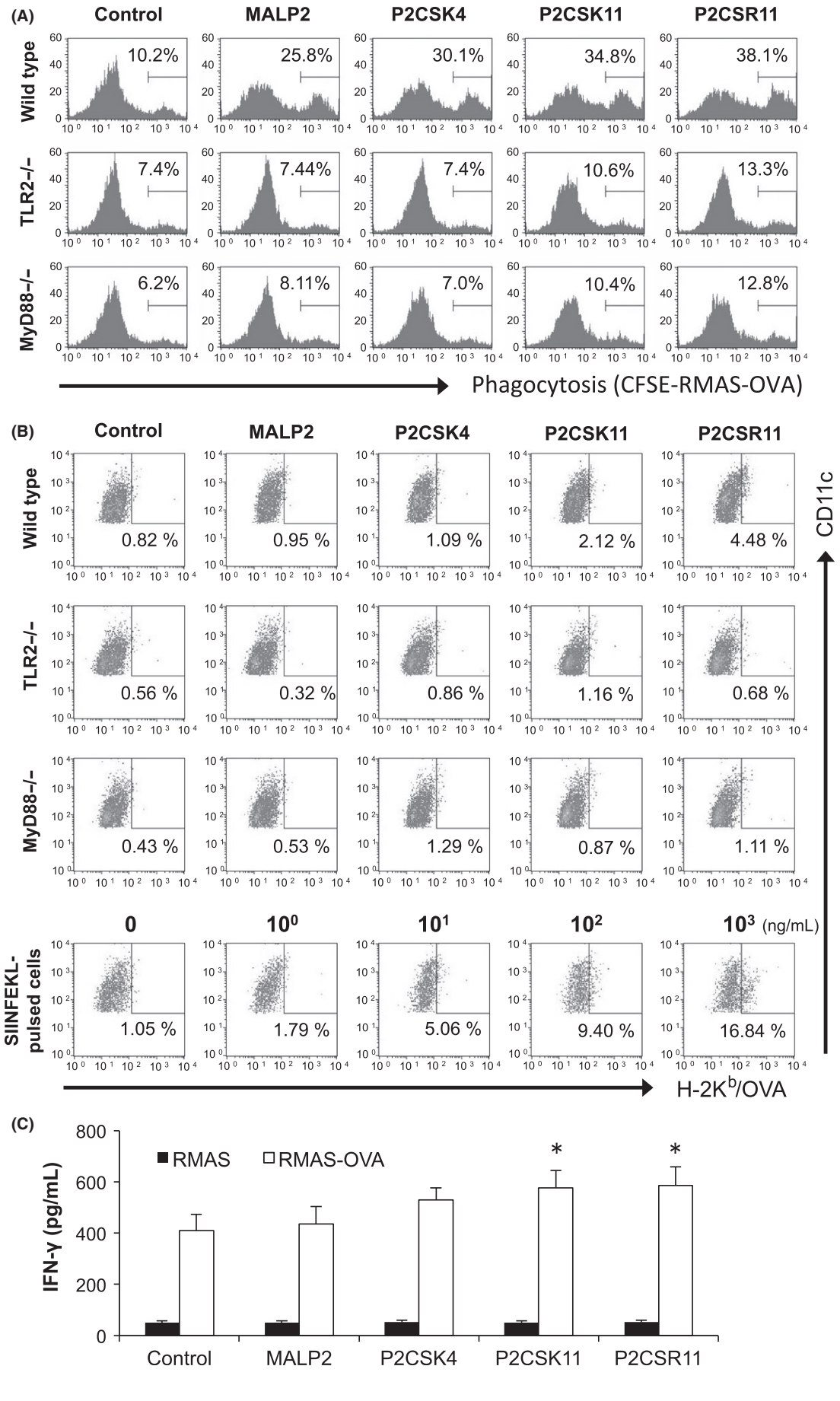

(A)
Control
MALP2
P2CSK4
P2CSK11
P2CSR11
Wild type
TLR2−/−
MyD88−/−
10.2%
25.8%
30.1%
34.8%
38.1%
7.4%
7.44%
7.4%
10.6%
13.3%
6.2%
8.11%
7.0%
10.4%
12.8%
Phagocytosis (CFSE-RMAS-OVA)
(B)
Control
MALP2
P2CSK4
P2CSK11
P2CSR11
Wild type
TLR2−/−
MyD88−/−
SIINFEKL-pulsed cells
0.82 %
0.95 %
1.09 %
2.12 %
4.48 %
0.56 %
0.32 %
0.86 %
1.16 %
0.68 %
0.43 %
0.53 %
1.29 %
0.87 %
1.11 %
1.05 %
1.79 %
5.06 %
9.40 %
16.84 %
0
10^0
10^1
10^2
10^3 (ng/mL)
CD11c
H-2K^b/OVA
(C)
RMAS
RMAS-OVA
IFN-γ (pg/mL)
800
600
400
200
0
*
*
Control
MALP2
P2CSK4
P2CSK11
P2CSR11

FIGURE 3 Responses of bone marrow-derived dendritic cells (BMDC) to tumor cells coated with lipopeptides. A, The efficacy of BMDC-mediated phagocytosis of OVA-transfected RMA-S cells (RMA-S-OVA) coated with lipopeptides. BMDC were prepared from mice lacking TLR2 (*Tlr2*$^{-/-}$) and TLR adaptor molecules (*Myd88*$^{-/-}$). BMDC were cultured for 12 h with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled bacteria-mimicking RMA-S-OVA cells, and the rate of tumor cell uptake was measured by flow cytometry. B, Expression of H-2K^{b}/SIINFEKL by CD11c-positive BMDC was analyzed as a measure of cross-presentation activity. The indicated BMDC were cultured for 24 h with bacteria-mimicking RMA-S-OVA cells. SIINFEKL-pulsed cells were prepared from wild-type BMDC cultured with the indicated dose of SIINFEKL peptide (positive control). The numbers in the panels represent percentage of gated cells (A and B). Representative images from 2 independent experiments with similar results are shown. C, IFN-γ production by OT-I splenocytes cultured for 24 h with RMA-S-OVA cells coated with the indicated lipopeptides. OT-I splenocytes produce IFN-γ after presentation of OVA antigen. Data are representative of 2 experiments. Each point represents the mean + SD (n = 3). *$P < .05$ (1-way ANOVA with Dunnett's test [vs control untreated RMA-S-OVA cells]). These experiments were performed using RMA-S-OVA coated with lipopeptide after removal of free lipopeptide

BMTC coated with P2CSR11 were abolished when tested against cells from *Tlr2*$^{-/-}$ mice (Figure 5C). Finally, we examined combination therapy with BMTC plus an anti-PD-L1 antibody (a known immune checkpoint inhibitor). Because TLR2 ligands stimulate IFN-γ production as shown in Figure 2D and IFN-γ induce PD-L1 expression on tumors,[39] anti-PD-L1 antibody may effectively inhibit tumor growth when tumor-bearing mice are treated with TLR2 ligands. Furthermore, anti-PD-L1 antibody is reported to increase the anti-tumor effect of TLR3 ligand on an in vivo EG7-OVA transplanted model.[40] We showed that anti-PD-L1 antibody increased the anti-tumor effects of BMTC coated with P2CSR11 (Figure 5D).

4 | DISCUSSION

Here, we showed that 2 novel synthetic lipopeptides adjuvants (P2CSR11 and P2CSK11) could be used to prepare a BMTC-based vaccine comprising tumor cells derived from a patient (a source of tumor antigens) and a TLR2 ligand. We showed that BMTC based on P2CSR11/P2CSK11: (i) stimulated DC by acting as both a tumor antigen and an immuno-adjuvant; (ii) promoted phagocytosis and cross-presentation of tumor antigen; (iii) could be purified by removing free lipopeptide; and (iv) showed a strong anti-tumor effect. Moreover, BMTC based on P2CSR11 induced less skin erosion/inflammation at the injection site, activated CTL but not NK cells, and induced a synergistic increase in the effects of immune checkpoint blockade therapy.

Although P2CSR11 induced less cytokine production than other lipopeptides in vitro (Figure 2D), BMTC coated with P2CSR11 induced efficient phagocytosis and cross-presentation (Figure 3), leading to CTL activation and a significant anti-tumor effect in an in vivo model (Figure 4). Overproduction of some cytokines can cause several adverse effects, including skin inflammation, as well as promoting anti-tumor effects. Therefore, P2CSR11 is a good candidate adjuvant for generating tumor antigen-specific immune responses with potentially fewer side effects.

Although some researchers have investigated suitable peptide sequences that may confer TLR2 ligand activity on lipopeptides,[41-43] we succeeded in providing additional specific functions.[9,10] Indeed, it is possible to incorporate several functions by connecting different functional peptides in tandem.[44] Therefore, the selectivity and functionality of lipopeptide-based adjuvants can be improved by rearranging the peptide sequences.

Jackson et al report the use of various modified lipopeptides as anti-tumor immuno-adjuvants.[45,46] A lipopeptide containing a branched cationic peptide (lysine/arginine) had affinity for negatively charged proteins and showed efficient adjuvant activity.[45] To develop new tumor vaccines comprising tumor cells coated with a TLR2 ligand, we focused on cell-penetrating peptides (CPP) containing cationic sequences, such as poly-arginine and poly-lysine. The CPP, which was originally identified in the transactivator of protein of human immunodeficiency virus, has an affinity for negatively charged plasma membranes comprising proteoglycans and phospholipids and is internalized via both passive and active processes.[47,48] When tumor cells are used as a source of tumor antigens, cells derived from biopsy samples must first be killed by irradiation or with anti-tumor chemotherapeutic drugs to inhibit growth. In the case of killed tumor cells, active incorporation is impaired, meaning that CPP binds to the plasma membrane and is retained on the cell surface (Figure 1C). In addition, positively charged peptides like polybrene[49] neutralize the negative charge on the membrane surface, meaning that tumor cells can easily come into contact with the DC membrane. Therefore, P2CSR11 promotes phagocytosis via not only TLR2 signaling but also by neutralizing the electric charge on the cell membrane. Indeed, BMTC coated with P2CSR11 showed slightly higher uptake by BMDC from *Tlr2*$^{-/-}$ mice than control tumor cells lacking lipopeptides (Figure 3A). The ability of a cationic polypeptide to bind to the cell surface seems to depend on the number of cationic amino acids such as lysine and arginine.[50] Because the peptide portion of P2CSR11, but not that of P2CSK4, bound efficiently to the cell membrane (Figure 1C), we surmised that the positive charge of P2CSR11 neutralized the negative charge on the cell membrane of BMTC to a greater extent than that on BMTC coated with other lipopeptides, thereby promoting contact with DC.

Although all the tested lipopeptides activated DC to a similar extent (as measured by expression of maturation markers and phagocytic activity), P2CSR11 in the absence of tumor cells induced less cytokine production than the other lipopeptides (Figure 2). Cytokines and cross-presentation are induced by TLR2-dependent

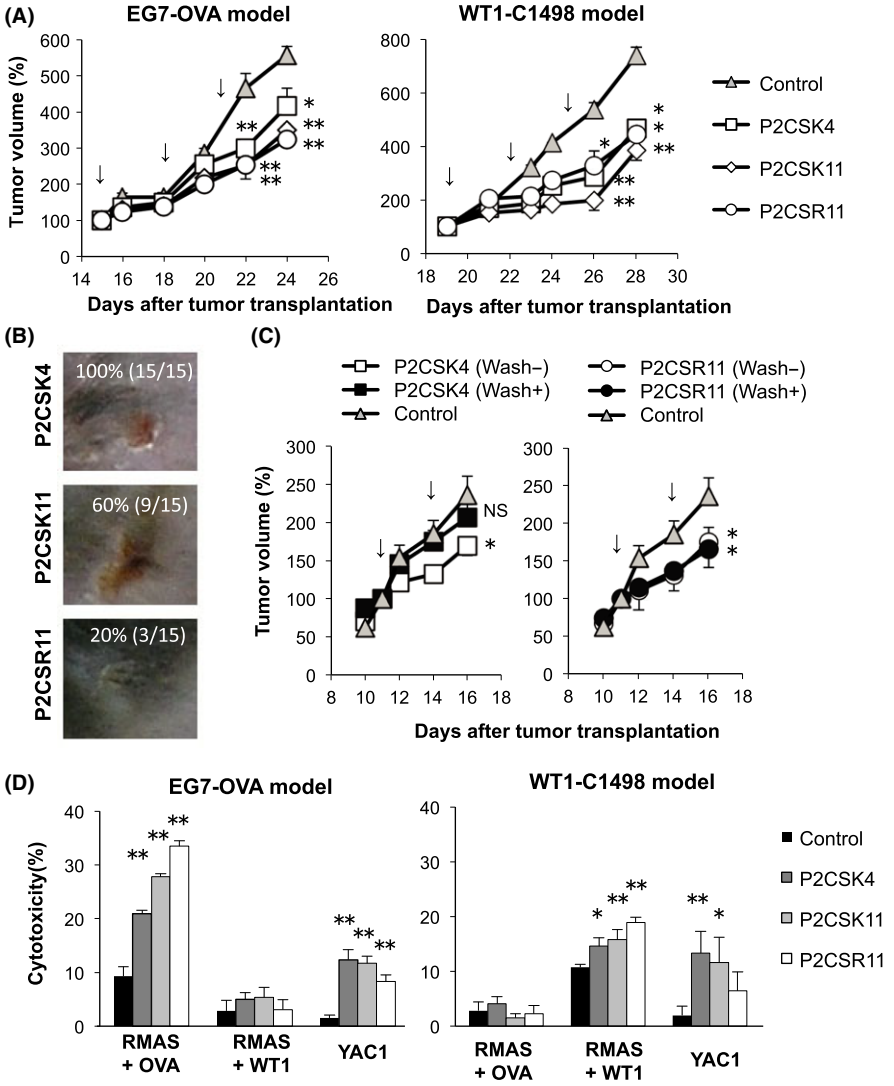


FIGURE 4 The antitumor effects of bacteria-mimicking tumor cells in vivo. A, Bacteria-mimicking tumor cells (BMTC) vaccines were prepared by mixing irradiated tumor cells and each lipopeptide. Vaccination of mice bearing EG7-OVA (left) or mWT1-C1498 tumors (right) was performed on the indicated days (arrows). B, Skin reactions at the vaccination site on EG7-OVA-bearing mice. The percentage of mice suffering skin erosion or inflammation at the vaccination site is shown. Numbers from 3 independent experiments were summed. C, Antitumor effects of BMTC after removal of free lipopeptide. Tumor cells were mixed with P2CSK4 (left) and P2CSR11 (right) for 2 h at 4°C and then washed to remove unbound lipopeptide. The BMTC were administrated intradermally twice. The in vivo data in this figures are representative of 2 (3 in A (left) and B) experiments. EG7-OVA (1×10^6 cells, A (left); 2×10^6 cells, other figures) were transplanted on day 0. Each point represents the mean ± SE (n = 4–5 mice). *$P < .05$, **$P < .01$. NS, not significant (1-way ANOVA with Dunnett's test [vs each control]). D, Cytotoxic T lymphocytes (CTL) and natural killer (NK) activity induced by vaccination of tumor-bearing mice with tumor cells coated with lipopeptides. The cytotoxic activity of CTL against RMA-S cells pulsed with OVA (RMA-S+OVA) or WT1 (RMA-S+WT1) peptides, or the cytotoxic activity of NK (YAC-1) cells, is shown in EG7-OVA (left) and mWT1-C1498 (right) model mice. Data are representative of 2 independent experiments. Each point represents the mean + SD (n = 3). *$P < .05$, **$P < .01$ (1-way ANOVA with Dunnett's test [vs control splenocytes from untreated tumor-bearing mice])

signals; however, although the lipopeptides harboring 2 palmitates (such as MALP2) activate the TLR2-TLR6 heterodimer, other groups report that P2CSK4 can activate TLR6-independent signals via the peptide component (the SK4 peptide).[44] Therefore, we speculated that P2CSR11 might activate other signals that regulate cytokine production in an SR11 peptide-dependent manner. Alternatively, because SR11, but not SK4, binds electrostatically to the cell surface, the SR11 peptide might disturb the membrane dynamics of DC and alter cytokine production signals. However, when BMTC were coated with P2CSK11 or P2CSR11, they induced phagocytosis of tumor cells and cross-presentation of tumor antigens by DC. Moreover, BMTC coated with P2CSK11 or P2CSR11 induced higher levels of antigen-dependent IFN-γ production by OT-I splenocytes than cells coated with other lipopeptides (Figure 3C). Although BMTC coated with P2CSR11 or P2CSK11 showed stronger antitumor effects than P2CSK4 in tumor-bearing mice (Figure 4A, left

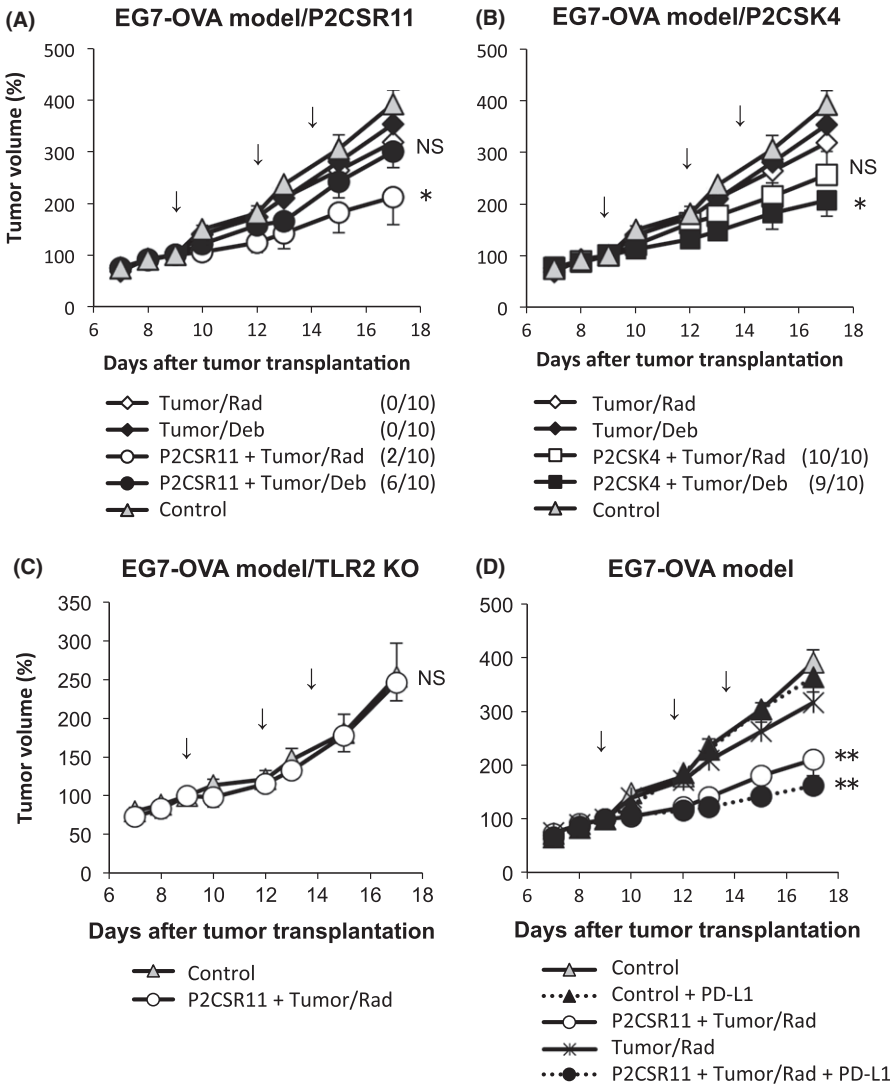


FIGURE 5 Conditions essential for induction of antitumor effects by a bacteria-mimicking tumor cell vaccine based on P2CSR11. A and B, Effects of preparation method on antitumor effects. Vaccines were prepared by mixing P2CSR11 (A) or P2CSK4 (B) with irradiated tumor cells (Rad) or tumor cell debris formed by freeze/thawing cells (Deb). The numbers in parentheses indicate the total number of mice suffering skin erosions or inflammation at the vaccination site (data from 2 independent experiments). C, Antitumor effect of irradiated EG7-OVA coated with P2CSR11 in mice lacking *Tlr2*. D, Anti-tumor effect of combined therapy with an immune checkpoint inhibitor and bacteria-mimicking tumor cells. Anti-PD-L1 antibody (PD-L1, intraperitoneally) and P2CSR11-coated EG7-OVA cells (subcutaneously) were administrated on the indicated days (arrows). Data in this figures are representative of 2 experiments. On Day 0, EG7-OVA cells (2×10^6 cell) were transplanted subcutaneously into the backs of mice. Each point represents the mean ± SE (n = 5 mice). *$P < .05$, **$P < .01$. NS, not significant (C, Student *t* test; all others, 1-way ANOVA with Dunnett's test [vs each control group])

panel), P2CSR11 induced higher antigen-dependent cytotoxic activity (but less NK-dependent cytotoxic activity) than P2CSK11 (Figure 4D). These results suggest that P2CSR11 works as a CTL-orienting adjuvant; therefore, it is a suitable candidate vaccine against CTL-sensitive tumors expressing MHC class I.

We previously reported that TLR2 ligands induce inflammation at the site of administration when used as immuno-adjuvants.[9,10] Such adverse effects of new adjuvants must be considered from a clinical perspective. Although P2CSR11, P2CSK11 and P2CSK4 inhibited tumor growth in in vivo mouse models to a similar extent (Figure 4), P2CSK4 and P2CSK11 induced more severe inflammation at the injection site. Because P2CSK4 is retained for long periods around the injection site,[9] we predict that it is necessary to remove free lipopeptides from the vaccine to avoid such adverse effects. BMTC coated with P2CSR11 maintained anti-tumor effects after removing free lipopeptide (Figure 4C). Thus, P2CSR11 is a strong candidate as it can induce anti-tumor activity with fewer (or less severe) adverse effects.

Lipopeptides bind to TLR2 and induce DC responses. A recent study suggests that lipopeptides are incorporated into endosomes and activate the TLR2/Myd88 signaling pathway in endolysosomes, but not on the cell surface.[51] Because lipopeptides stimulate TLR2 inside DC after phagocytosis, TLR2 might act as a capture receptor, and lipopeptides on the tumor cell surface might act as a marker for phagocytosis via TLR2. Unfortunately, we were unable to clarify whether TLR2 acts as a capture receptor or as a TLR2/Myd88 signaling receptor. However, TLR2 ligands increased the phagocytic activity of DC (Figure 2C), and BMDC derived from *Tlr2*$^{-/-}$ or *Myd88*$^{-/-}$ mice failed to engulf BMTC coated with P2CSR11 (Figure 3A) and failed to present antigen (Figure 3B). These findings suggest that activation of TLR2/Myd88 signaling pathway is essential for phagocytosis and antigen cross-presentation. BMTC coated with lipopeptides can activate DC via the TLR2 signaling pathway and present tumor antigen to DC at the same time; these responses might increase the total amount of antigen engulfed and promote cross-presentation.

The experiments in tumor-bearing mouse models showed that the lipopeptides (administered as a vaccine) inhibited tumor growth. However, the anti-tumor effects are weaker than those observed for nucleic acid-based adjuvants such as poly I:C.[26,27,52] A previous

study showed that induction of the regulatory T cells by TLR2 ligands attenuates the effectiveness of a vaccine;[53] it may be, therefore, that we have to remove such a regulatory mechanism from the immune system to improve the effectiveness of lipopeptide vaccines.[53,54]

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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