Preclinical Development of a WT1 Oral Cancer Vaccine Using a Bacterial Vector to Treat Castration-Resistant Prostate Cancer

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Abstract

Previously, we constructed a recombinant *Bifidobacterium longum* displaying a partial mouse Wilms’ tumor 1 (WT1) protein (*B. longum* 420) as an oral cancer vaccine using a bacterial vector and demonstrated that oral administration of *B. longum* 420 significantly inhibited tumor growth compared with the Db126 WT1 peptide vaccine in the TRAMP-C2, mouse castration-resistant prostate cancer (CRPC) syngeneic tumor model. The present study demonstrated that oral administration of 1.0 × 10⁶ colony-forming units of *B. longum* 420 induced significantly higher cytotoxicity against TRAMP-C2 cells than intraperitoneal injection of 100 μg of Db126, and the *in vivo* antitumor activity of *B. longum* 420 in the TRAMP-C2 tumor model could be augmented by intraperitoneal injections of 250 μg of anti-PD-1 antibody. For the clinical development, we produced the B440 pharmaceutical formulation, which is lyophilized powder of inactivated *B. longum* 440 displaying the partially modified human WT1 protein. We confirmed that *B. longum* 440 could induce cellular immunity specific to multiple WT1 epitopes. In a preclinical dosage study, B440 significantly inhibited growth of the TRAMP-C2 tumors compared with that of the control groups (PBS and *B. longum* not expressing WT1) at all dosages (1, 5, and 10 mg/body of B440). These mouse doses were considered to correspond with practical oral administration doses of 0.2, 1, and 2 g/body for humans. Taken together, these results suggest that the B440 WT1 oral cancer vaccine can be developed as a novel oral immuno-oncology drug to treat CRPC as a monotherapy or as an adjunct to immune checkpoint inhibitors.

Introduction

In 2012, the World Health Organization (WHO) reported that prostate cancer was the second most common cancer and the fifth leading cause of cancer-related death in men worldwide (1) (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx). Although androgen-deprivation therapy (ADT) initially shows a high response rate in metastatic and/or recurrent prostate cancer after androgen-deprivation therapy (ADT) initially shows a high response rate in metastatic and/or recurrent prostate cancer after surgery or radiotherapy, most prostate cancer will progress to castration-resistant status (castration-resistant prostate cancer, CRPC), defined as biochemical or radiological progression despite the suppression of serum testosterone levels by ADT (2). Recently, metastatic CRPC (mCRPC) has been sequentially treated with novel antiandrogen agents, such as abiraterone; a CYP17 inhibitor; enzalutamide; an androgen receptor antagonist; and antiimmune agents, such as docetaxel and cabazitaxel (3). Despite the clinical benefits of these therapeutic agents, the effective period of these new drugs remains limited.

Because the development of immune checkpoint inhibitors (ICI), such as programmed cell death 1 (PD-1), programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) inhibitors, cancer immunotherapy has become part of standard cancer care (4). Recently, the PD-1 inhibitors pembrolizumab and nivolumab have shown efficacy in the treatment of urological cancers, including urothelial cancer and renal cancer (5), but no ICIs have shown proven efficacy in CRPC (6). ICIs can achieve long-lasting antitumor effects with few side effects by inhibiting the immunosuppressive signals protecting cancer cells. The response rate to ICI alone is currently limited to approximately 30% (7). The major principle of cancer immunotherapy is the induction of tumor-specific cytotoxic T lymphocyte (TIL) activity. The development of novel active immunotherapy, such as a therapeutic cancer vaccine that can forcibly induce tumor antigen-specific TIL, is strongly desired. In 2010, a dendritic cell (DC)-based vaccine, Sipuleucel-T, was approved by the US FDA for the treatment of mCRPC without visceral metastasis (8) but achieved only modest clinical efficacy. Furthermore, production of this vaccine by culturing autologous DCs is labor intensive and extremely costly.

Previously, we constructed a recombinant *Bifidobacterium longum* displaying a partial mouse Wilms’ tumor 1 (WT1) protein (*B. longum* 420) as an oral cancer vaccine using a bacterial vector.
and demonstrated that oral administrations of *B. longum* 420 could induce WT1-specific cellular immunity with significant antitumor effects in a syngeneic mouse tumor model using C1498-WT1 cells and C57BL/6-origin recombinant murine leukemia cells stably expressing murine WT1 protein (9). On the basis of the reports that over 50% of prostate cancer expresses WT1 protein, especially in high-grade and invasive prostate cancer (10), we explored the feasibility of using this WT1 oral cancer vaccine to treat CRPC. In our previous study (11), we compared the antitumor effects of *B. longum* 420 versus a WT1 peptide vaccine, Db126 (11), in a syngeneic mouse tumor model using TRAMP-C2, C57BL/6-origin murine CRPC cells naturally expressing WT1 protein (12). Although the *B. longum* 420 vaccine carries most of the WT1 protein (a.a.117–419), the Db126 peptide vaccine consists of nine amino acids (a.a.126–134, RMFPNAPYL), identified as the major histocompatibility complex (MHC)-class I binding peptide in both humans (HLA-A0201) and C57BL/6 mice (H-2Db; ref. 13). Although both *B. longum* 420 and Db126 vaccines induce RMFPNAPYL-specific CTLs, only the *B. longum* 420 oral vaccine, but not the Db126 peptide vaccine, could inhibit TRAMP-C2 tumor growth (11).

In the present study, we performed further evaluations of cellular immunity induced by the *B. longum* 420 and Db126 vaccines and examined the synergistic effect of combining *B. longum* 420 with anti-PD-1 antibody in the TRAMP-C2 syngeneic mouse tumor model. We further constructed *B. longum* 440, a recombinant *B. longum* displaying a partial human WT1 protein (a.a. 117–419, with a single amino acid substitution, M—Y at a.a. 236), for clinical development. We produced B440, the lyophilized powder of inactivated *B. longum* 440, as the pharmaceutical formulation. For the clinical application of the B440 vaccine, *B. longum* 440 was inactivated by heating to comply with the biosafety regulations for living modified organisms (LMO) in accordance with the Cartagena Act (14). In addition, we performed a preclinical dosage study of B440 using the TRAMP-C2 syngeneic mouse tumor model to determine the clinical dose and schedule. These preclinical studies warrant further clinical development of B440 for the treatment of CRPC.

### Materials and Methods

#### Bacterial strains and media

A recombinant *B. longum* 420 strain that expresses a partial murine-WT1 protein (a.a. 117–419) fused to galacto-N-biose-lacto-N-biose I binding protein (GL-BP) was constructed by shuttle-vector electroporation into *B. longum* 105-A (the Japan Collection of Microorganisms, RKien Bioresource Center, Tsukuba, Japan) in our previous study (9). GL-BP is a membrane protein in the ATP-binding cassette transporter on the wild-type *B. longum* cell wall, which we used as an anchor to display antigen on the bacterial cell surface. A recombinant *B. longum* 2012 strain with a plasmid carrying GL-BP only was also constructed in our previous study and used as a control (15). A recombinant *B. longum* 440 strain that expresses a partial human protein (a.a. 117–419, GenBank: 7490; UniProtKB: P19544.2) was constructed using the same methods as the other recombinant strains. The amino acid at residue 236 of human WT1 protein in *B. longum* 440 was changed from M to Y to increase immunogenicity (16). All recombinant strains were anaerobically cultured overnight in Gifu anaerobic medium (Nissui, Tokyo, Japan) with 50 µg/mL spectinomycin at 37°C. We confirmed that the recombinant strains could be completely inactivated by heating for 5 minutes at 65°C.

#### B440 production

B440, lyophilized *B. longum* 440, was produced as follows. The recombinant *B. longum* 440 was inoculated in the optimized medium and incubated at 37°C for 24 hours under anaerobic conditions in a 2 L jar fermenter. *B. longum* 440 cells were harvested and washed in distilled water. *B. longum* 440 was heated for inactivation and then lyophilized. The amount of recombinant WT-1 protein contained in the lyophilized B440 powder was measured with ELISA using an anti-WT1 protein antibody (Abcam Inc.).

#### TRAMP-C2 cell line and media

TRAMP-C2, an androgen-independent murine prostate cancer cell line endogenously expressing murine-WT1 protein derived from C57BL/6 (17), was purchased from the ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4 mM/l L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 5% heat-inactivated FBS, 5% Nu-Serum IV, 0.005 mg/mL bovine insulin and 10 mM/L dehydroisoandrosterone. The cells were regularly verified to be free of mycoplasma contamination by PCR assay using TaKaRa PCR Mycoplasma Detection Set (Takara Bio, Inc.). TRAMP-C2 cells endogenously express PD-L1, and the expression was increased by stimulation with recombinant interferon-γ (data not shown).

#### Immunization with *B. longum* 420, *B. longum* 440, or WT1 peptide vaccine

Male C57BL/6 mice were orally administered 100µL of PBS with or without 1.0 × 10⁶ colony-forming units (c.f.u) of *B. longum* 420, *B. longum* 2012, or *B. longum* 440, 5 times a week for 6 weeks (days 0–39), using a feeding needle. One hundred micrograms/dose of WT1-peptide vaccine (RMFPNAPYL; Eurofins Genomics, Tokyo, Japan) with incomplete Freund’s adjuvant (Wako, Osaka, Japan) was injected intraperitoneally into the mice once a week for 6 weeks to compare WT1-specific immune responses (18). After vaccination, mice were euthanized, and spleen cells were isolated for evaluation of immune responses by the following in vitro assays.

#### Proliferation assay for immunized splenocytes

Isolated splenocytes were maintained in RPMI-1640 medium supplemented with 10% FBS, 10 mM/l L-HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM/l nonessential amino acids, 50 µM/l L-2-mercaptoethanol, and 1 mM/l L-sodium pyruvate. For evaluation of splenocyte proliferation, a sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt (XTT) assay was performed. Briefly, 1 × 10⁶ splenocyte cells were cultured with 1 × 10⁵ mitomycin-C-treated TRAMP-C2 cells for in vitro restimulation. After 9 days of culture, cell proliferation was determined using a Cell Proliferation Kit II (XTT; Roche, Basel, Switzerland), and the absorbance was read at 450 nm. The stimulation index (SI), the ratio between stimulated and nonstimulated cells, was calculated as described elsewhere (19).
Intracellular cytokine staining for immunized splenocytes

A total of 2.0 × 10⁶ splenocytes were cultured with 2.0 × 10⁶ mitomycin-C-treated TRAMP-C2 cells in vitro. After 48 or 72 hours of culture, GolgiStop (BD Biosciences) was added to the culture, and the cells were then cocultured for an additional 12 hours. For intracellular cytokine staining (ICCS), cells were collected and processed using a BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences) according to our previous study (9). Briefly, collected cells were blocked and stained with PerCP-anti-mouse CD3, FITC-anti-mouse CD4, and APC-anti-CD8 antibodies. Then, the washed cells were permeabilized with BD Fixation/Permeabilization solution. Cells were then washed and stained with PE-anti-IFN-γ, PE-anti-mouse TNFα, or PE-anti-mouse IL-2 (BD) for intracellular staining. Stained cells were analyzed by flow cytometry.

Measurement of cytotoxic T-cell activity

A total of 3.0 × 10⁶ splenocytes were cultured with 3.0 × 10⁶ mitomycin-C-treated TRAMP-C2 cells in the presence of IL-2 in vitro for 6 days to generate effector cells. After culture, effector cells were cocultured with TRAMP-C2 or EL4, a WT1-negative murine leukemia cell line, at a ratio of 20:1, 10:1, and 5:1 for 8 hours. Then, the culture supernatant was collected, and specific CTL activity was measured using an LDH Cytotoxicity Assay Kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega) according to the manufacturer’s instructions. The percentage of specific killing was calculated by the following formula: percentage-specific killing = (experimental release–effector spontaneous release–target spontaneous release)/target maximum release–target spontaneous release) x 100.

Combination treatment of *B. longum* 420 and anti–PD-1 antibody

To investigate the antitumor effect against prostate cancer in vivo, we performed a tumor challenge with TRAMP-C2 according to previously described methods (11). Anti–mouse PD-1 antibody (InvivoPlus anti-mouse PD-1, clone RMP1-14) and Rat IgG2a Isotype Control (InvivoPlus Rat IgG2a Isotype Control, clone 2A3) were purchased from BioXcell, Inc. On day 0, 1 × 10⁶ TRAMP-C2 cells were injected subcutaneously into male C57Bl/6 mice. Following tumor injection, mice were randomly assigned to 7 treatment groups (n = 8): *B. longum* 420 + anti–PD-1, *B. longum* 420 + IgG isotype control, *B. longum* 420, *B. longum* 2012 + anti–PD-1, *B. longum* 2012 + IgG isotype control, *B. longum* 2012, and PBS. Oral administration of 100 μL of PBS with or without 1 × 10⁹ cfu of the recombinant *B. longum* strains was carried out 5 times a week using a feeding needle. The treatment regimen was determined in our previous study (9). After 2 weeks of vaccination, 250 μg of anti–PD-1 or IgG isotype control was intraperitoneally injected into mice 2 times a week for 5 weeks (days 28, 31, 35, 38, 42, 45, 49, 52, 56, and 59). During treatment, tumor volume was monitored and expressed by the following formula: (longest diameter)² x (shortest diameter)² x 0.5. Kaplan–Meier survival curves were generated. Mice were euthanized when the tumor diameter was >20 mm. Tumors were resected and fixed with 4% paraformaldehyde-PBS and embedded with paraffin for immunohistochemical study.

**Immunohistochemical study**

Paraffin embedded tumor tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed in Bond epitope retrieval buffer (pH6.0 for CD3 and CD8a, pH9.0 for CD4; Leica Microsystems, Wetzlar, Germany) at 98°C for 20 minutes. Immunohistochemical staining was performed in automatic tissue processor (Leica Microsystems Bond) according to the manufacturer’s standard protocol. Briefly, tissue sections were incubated at RT for 15 minutes with rabbit anti-mouse CD3 antibody (1:100, Abcam), rabbit anti-mouse CD4 antibody (1:1,000, Abcam) or rabbit anti-mouse CD8a antibody (1:400, Cell Signaling Technology Japan, Tokyo, Japan). After washing, sections were incubated with horseradish peroxidase–conjugated secondary antibodies. After washing, sections were incubated with 3,3′-diaminobenzidine and were counterstained with hematoxylin. Resulting tissue slides were observed under microscope BZ-X710 (Keyence, Osaka, Japan).

**Table 1. Human WT1 amino acid sequence and peptides used in this study**

<table>
<thead>
<tr>
<th>Peptides used in this study</th>
<th>Amino acid sequence</th>
<th>MHC Restriction</th>
<th>CD4/CD8</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Db126</td>
<td>RMFPNAPYL</td>
<td>HLA-A*0201</td>
<td>CD8</td>
<td>Oka et al. (13)</td>
</tr>
<tr>
<td>WH187</td>
<td>SLGEOQYSV</td>
<td>HLA-A*0201</td>
<td>CD8</td>
<td>Oka et al. (13)</td>
</tr>
<tr>
<td>mp235</td>
<td>CYTWNQMNL</td>
<td>HLA-A*0202</td>
<td>CD8</td>
<td>Tsuobi et al. (9)</td>
</tr>
<tr>
<td>Db1332</td>
<td>KRYFKLSHLQMSRKH</td>
<td>HLA-DRB1*0405</td>
<td>CD4</td>
<td>Fujiki et al. (45)</td>
</tr>
<tr>
<td>A24</td>
<td>RWPSQCKRF</td>
<td>HLA-A*0202</td>
<td>CD8</td>
<td>Ohnimani et al. (40)</td>
</tr>
</tbody>
</table>

*NOTE: The amino acid sequence of the WT1 protein displayed on *B. longum* 440 is underlined. Amino acids heterologous to mouse WT1 are written in red letters.*

*Peptides Db126 (green), WH187 (light blue), mp235 (blue), Db1332 (yellow), and A24 (purple) were used for the stimulation of the splenocytes in the ICCS study.*

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efficacy studies used a dose of $1 \times 10^9$ cfu/body (25 g; estimated mouse body weight) of B. longum 420. B440 is estimated to contain $1 \times 10^{11}$ cfu/g of B. longum 440 (data not shown); thus, 10 mg/body of B440 is considered the previous mouse dose of B. longum 420 and 440. The mouse dose of B440 can be converted to a human dose of approximately 2 g/body (60 kg; estimated human body weight) by correcting for the body surface area and working weight range (divisor 12.3; ref. 20). On the basis of the above estimation, in this study, body surface area and working weight range (divisor 12.3; can be converted to a human dose of approximately 2 g/body mouse dose of

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**Results**

**Comparison of the TRAMP-C2–specific cellular immunity induced by B. longum 420 oral vaccine versus Db126 peptide vaccine**

Our previous study (11) demonstrated that both B. longum 420 and Db126 could induce Db126-RMFPNAPYL-specific CTLs by the tetramer assay using the MHC tetramer of the H-2D^d-restricted WT1 peptide (RMFPNAPYL). However, only B. longum 420 could induce a significant tumor growth inhibitory effect in the TRAMP-C2 tumors. Db126 could not induce these effects compared with those of the B. longum 2012 control group. To investigate the superior antitumor effect of B. longum 420 over Db126, we compared the TRAMP-C2–specific cellular immunities induced by B. longum 420 versus Db126 by cell proliferation, ICCS, and *in vitro* cytotoxicity assays.

Splenocytes isolated from *B. longum* 420–vaccinated mice showed increased cell proliferation after stimulation with TRAMP-C2

Splenocytes isolated from mice vaccinated with B. longum 420, Db126, or *B. longum* 2012 were restimulated with mitomycin-C–treated TRAMP-C2 cells, and an *in vitro* cell proliferation assay was performed. As shown in Fig. 1A, splenocytes isolated from the B. longum 420 group showed significantly higher cell proliferation than those isolated from PBS or *B. longum* 2012 after 9 days of culture (*P* < 0.05), whereas the Db126 peptide vaccine did not result in a significant increase in cell proliferation compared with that of the other groups (Fig. 1A). This result suggested that splenocytes isolated from *B. longum* 420 mice responded better to TRAMP-C2 cells than those isolated from Db126 mice.

*B. longum* 420 and Db126 induced cytokine-producing T cells

To investigate the induction of activated T cells by *B. longum* 420 or Db126, we performed ICCS using splenocytes restimulated

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Figure 1. WTI-specific proliferation and cytokine production in splenocytes induced by oral vaccination of *B. longum* 420. **A**, After the last vaccination, splenocytes were isolated and restimulated with mitomycin C–treated TRAMP-C2 in *vitro*. Then, cell proliferation was determined, and the proliferation activity is shown as a stimulation index. Each data point represents the average of each group (bars, ±SE). *B. longum* 420 significantly induced splenocyte proliferation compared with PBS and Db126 group. **B**, splenocytes were restimulated with TRAMP-C2 (*P* < 0.05). **B**, For intracellular cytokine staining, splenocytes were isolated and restimulated with mitomycin C–treated TRAMP-C2 for 48 hours or 72 hours in *vitro* and intracellular cytokine staining was performed for detection of cytokine-producing CD4+ T and CD8+ T cells. Each data point represents the average of each group (bars, ±SE). *B. longum* 420 significantly induced IFN-γ, IL-2, and TNF-α–producing CD4+ T and CD8+ T cells compared with other treatments when splenocytes were restimulated with TRAMP-C2 (*P* < 0.05).
B. longum augmented antitumor effects and improved survival in the B. longum neal injections of Db126 with incomplete Freund’s adjuvant. These results suggested that B. longum 420 but not Db126 could significantly increase the number of CD4⁺ and CD8⁺ T cells producing IFN-γ compared with PBS and B. longum 2012.

B. longum 420 induced significantly higher cytotoxic CTL activity against TRAMP-C2 cells than Db126

To investigate CTL activity against TRAMP-C2 cells, we performed an in vitro cytotoxicity assay using effector cells, which were splenocytes isolated from mice vaccinated with B. longum 420 or Db126. In vitro cytotoxicity of the effector cells against target TRAMP-C2 cells or EL4 cells, which did not express WT1 protein, was measured. B. longum 420 and Db126 induced significantly higher CTL activities against TRAMP-C2 at effector/target ratios of 20:1, 10:1, and 5:1 than PBS and B. longum 2012 (Fig. 2A, P < 0.05; Fig. 1B). In the Db126 peptide vaccine group, only IL-2- and TNF-α-producing T cells showed a significantly higher frequency than those in the PBS and B. longum 2012 groups. Representative dot plots of each treatment group and gating for staining were shown in Supplementary Fig. S1. These results suggested that B. longum 420 but not Db126 could significantly increase the number of CD4⁺ and CD8⁺ T cells producing IFN-γ compared with PBS and B. longum 2012.

B. longum 420 combined with anti–PD-1 antibody showed augmented antitumor effects and improved survival in the TRAMP-C2 mouse CRPC model

To investigate the synergistic antitumor effect of combining B. longum 420 and anti–PD-1 antibody, we performed an animal study using the TRAMP-C2 mouse CRPC model with the treatment regimen illustrated in Fig. 3A. We treated the mice with oral administrations of B. longum 420 or B. longum 2012 with or without intraperitoneal injections of anti–PD-1 antibody, starting two weeks after the TRAMP-C2 cell inoculation (Fig. 3A). All the B. longum 440 treatment groups showed significantly inhibited tumor growth compared with the PBS and B. longum 2012 groups, and the combination of B. longum 420 with anti–PD-1 antibody markedly inhibited tumor growth compared with that of the other treatment groups through the treatment period. At day 74, the combination of B. longum 420 and anti–PD-1 antibody significantly prolonged the survival period compared with that of the other groups (Fig. 3C, P < 0.01).

Combination of B. longum 420 and anti–PD-1 antibody increased the numbers of tumor infiltrating CD3⁺, CD4⁺, and CD8⁺-positive cells

As the result of immunohistochemical study, we observed the highest numbers of tumor infiltrating CD3⁺, CD4⁺ and CD8⁺ positive cells in the combination of B. longum 420 and anti–PD-1 antibody group compared with the other groups (Fig. 4). In addition, we detected more CD4⁺ and CD8⁺-positive cells in B. longum 440 alone group compared with PBS and B. longum 2012 with anti–PD-1 antibody groups (Fig. 4). These results suggested that B. longum 420 could induce the tumor infiltration of CD4⁺ and CD8⁺-positive cells and these immunological responses were enhanced by anti–PD-1 antibody.

B. longum 440 successfully induced cellular immunity specific to multiple WT1 epitopes

For the clinical development of this WT1 oral cancer vaccine, we constructed B. longum 440, which displays a partial human WT1 protein (a.a. 117–419, Table 1). The homology between human and mouse WT1 amino acid sequences is more than 97%. We also modified the human amino acid sequence in B. longum 440 with
one amino acid substitution from M to Y at residue 236 to increase immunogenicity (12). To determine whether B. longum 440 could induce WT1 epitope-specific cellular immunity, we performed an ICCS assay using the 5 peptides previously known as human CD8 or CD4 WT1 epitopes (Table 1). The results showed that splenocytes isolated from Db126-treated mice had significantly higher numbers of CD4+ and CD8+ T cells producing IL-2 than splenocytes isolated from PBS or B. longum 2012 mice when the splenocytes were stimulated with Db126 peptide only (Fig. 5A and B). Representative dot plots of each treatment group with significant data and gating for staining were shown in Supplementary Fig. S2. However, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing IFN-γ or IL-2 than splenocytes isolated from the PBS, B. longum 2012 or Db126 groups when the splenocytes were stimulated with Db126 peptide. In addition, splenocytes isolated from B. longum 440-treated mice showed significantly higher numbers of CD4+ and CD8+ T cells producing
or mp235 (restimulated with 5 types of peptides (Db126, WH187, mp235, Db332, or A24) for 48 hours or 72 hours in vitro, and intracellular cytokine staining was performed for the detection of cytokine-producing CD4+ T and CD8+ T cells. Each data point represents the average of each group (bars, ±SE). B. longum 440 significantly induced IFN-γ (A) and IL-2 (B)-producing CD4+ T and CD8+ T cells compared with other treatments when splenocytes were restimulated with Db126 or mp235 (*, P < 0.05).

IL-2 than those isolated from the PBS, B. longum 2012 or Db126 mice when the splenocytes were stimulated with mp235 (Fig. 5A and B). These results suggested that although the Db126 peptide vaccine could induce only Db126 epitope-specific cellular immunity, the B. longum 440 oral vaccine could induce cellular immunity specific to multiple WT1 epitopes (Db126 and mp235).

**B440 inhibited tumor growth and prolonged survival in the TRAMP-C2 mouse CRPC model in a dose-independent manner**

For the clinical development of this WT1 oral cancer vaccine, we produced B440, which is the lyophilized powder of inactivated B. longum 440, as the pharmaceutical formulation. To investigate the efficacy of B440 and to determine its dosage, we performed a preclinical dosage study of B440 using the TRAMP-C2 mouse CRPC tumor model. B440 at all dosages, which were high, middle and low doses at once, three times, and five times a week frequencies for one and three month periods, showed a significant tumor growth inhibitory effect compared with those of all the PBS and B. longum 2012 control groups (P < 0.05; Fig. 6A). At 71 days after the tumor inoculation, all B440, B. longum 440, and B. longum 420 treatment groups showed a significant tumor growth inhibitory effect compared with all PBS and B. longum 2012 control groups (P < 0.01; Fig. 6B). However, there was no significant difference among the B440, B. longum 420, and B. longum 440 groups. In addition, no significant difference was detected among any B440 administration doses, frequencies, and periods. Table 2 shows the survival ratio at 123 days after the tumor inoculation. There was no significant difference between 1 and 3 months of B440 administration or among the total doses of B440 administered. Figure 7 shows the survival curve of the B440 administration groups. All B440, B. longum 440, and B. longum 420 treatment groups showed significantly prolonged survival compared with all PBS and B. longum 2012 control groups (P < 0.01; Fig. 7). However, there was no significant difference among the administration doses and frequencies. These results suggested that B440 could induce significant tumor growth inhibitory effects and significantly prolong survival in the TRAMP-C2 mouse CRPC model in a dose-independent manner.
Discussion

In recent years, novel findings of an association between gut microbiota, including *Bifidobacterium*, and cancer immunotherapy have accumulated (21, 22). In addition, when *Bifidobacteria* were orally administered to mice, the bacterial cells were detected in Peyer’s patches after 1 hour and in mesenteric lymph nodes (MLN) within 20 hours (23). Using this natural tropism of *Bifidobacterium*, we developed an oral vaccine platform with *Bifidobacterium* as the antigen delivery vehicle to the gut immune system (24) and generated a WT1 oral cancer vaccine expressing the WT1 protein (9), which is highly ranked as a tumor-associated antigen for cancer vaccines (25).

Previously, many short peptide vaccines with the WT1 CD8 epitope (8 to 10 amino acids) were developed, but the results of these vaccines were disappointing (26, 27). For the induction of strong tumor-specific CTLs, the tumor antigen should be taken up inside the DCs and processed, and its epitope needs to be expressed on the DC surface together with MHC (28). Short peptide directly binds to MHC class I expressed on DC, T-cell, and B-cell surfaces, fails to induce strong CTL, and may induce immune tolerance (29, 30). However, the WT1 oral cancer vaccine displaying the greatest length of the WT1 protein can deliver the WT1 protein into DCs and induce vastly improved antitumor effects compared with the conventional peptide vaccine in the mouse CRPC model (11). In the present study, when the splenocytes isolated from the vaccinated mice were stimulated with mitomycin-C–treated TRAMP-C2 cells, only the splenocytes isolated from mice vaccinated with *B. longum* 420, and not those from mice vaccinated with the Db126 peptide vaccine, showed significantly higher cell proliferation and higher numbers of CD4+ and CD8+ T cells producing IFN-γ compared with those from PBS and *B. longum* 2012-treated mice (Fig. 1). In addition, the splenocytes isolated from *B. longum* 420-vaccinated mice showed significantly higher in vitro cytotoxicity than those from the peptide-vaccinated mice at a 5:1 effector/target cell ratio (Fig. 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival (%)</th>
<th>Total dose mg/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-3-1</td>
<td>80%</td>
<td>150</td>
</tr>
<tr>
<td>H-5-1</td>
<td>80%</td>
<td>250</td>
</tr>
<tr>
<td>M-1-1</td>
<td>80%</td>
<td>25</td>
</tr>
<tr>
<td>L-3-3</td>
<td>60%</td>
<td>48</td>
</tr>
<tr>
<td>L-5-3</td>
<td>60%</td>
<td>400</td>
</tr>
<tr>
<td>H-1-3</td>
<td>40%</td>
<td>160</td>
</tr>
<tr>
<td>L-5-1</td>
<td>40%</td>
<td>25</td>
</tr>
<tr>
<td>440 IA</td>
<td>40%</td>
<td>N/A</td>
</tr>
<tr>
<td>440 LV</td>
<td>40%</td>
<td>N/A</td>
</tr>
<tr>
<td>H-3-3</td>
<td>20%</td>
<td>480</td>
</tr>
<tr>
<td>H-5-3</td>
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<td>800</td>
</tr>
<tr>
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<td>16</td>
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<td>L-3-1</td>
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<tr>
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<td>20%</td>
<td>125</td>
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<tr>
<td>M-5-3</td>
<td>20%</td>
<td>400</td>
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<tr>
<td>420 IA</td>
<td>20%</td>
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</tr>
<tr>
<td>H-1-1</td>
<td>0%</td>
<td>50</td>
</tr>
<tr>
<td>M-1-3</td>
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<td>80</td>
</tr>
<tr>
<td>PBS 1mo</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>PBS 3mo</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>2012 IA</td>
<td>0%</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Abbreviation: N/A, not applicable.

**Table 2. Survival ratio at day 123**

**Figure 6.**
*In vivo* antitumor effect of B440 oral administrations against TRAMP-C2. **A**, Tumor growth curve of TRAMP-C2. After tumor formation, mice were divided into 24 treatment groups as follows: B440 (1, 5, or 10 mg per body, once, 3 or 5 times per week, 1 or 3 months of vaccination), *B. longum* 440, *B. longum* 420, *B. longum* 2012, and PBS. Each data point represents the average of each group (n = 5). Oral administration of B440 significantly inhibited the tumor growth of TRAMP-C2 compared with that of all PBS and *B. longum* 2012 control groups (P < 0.01). IA, inactivated vaccine; LV, live vaccine. **B**, At 71 days after the tumor inoculation, all B440, *B. longum* 440, and *B. longum* 420 treatment groups showed a significant tumor growth inhibitory effect compared with all PBS and *B. longum* 2012 control groups (P < 0.01). However, there was no significant difference among the B440, *B. longum* 420, and *B. longum* 440 groups (P > 0.05).
p epinephrine with five known peptides of WT1 epitopes: Db126, WH187, mp235, Db332 and A24 (13, 35–37) (Table 1). Although it is not clear whether these peptides, except for Db126, could bind to C57BL mouse MHC molecules, when the splenocytes isolated from mice vaccinated with B. longum 440 were stimulated by Db 126 and mp235, the numbers of CD4+ and CD8+ T cells producing IL-2 were significantly increased (Fig. 5B). However, the numbers of CD4+ and CD8+ T cells producing IL-2 in splenocytes isolated from Db126 vaccinated mice were increased only when the splenocytes were stimulated with Db126 peptide (Fig. 5B). These results suggested that B. longum 440 could induce cellular immunity specific to multiple WT1 epitopes, whereas the Db126 peptide vaccine could induce only Db126-specific cellular immunity.

For further clinical development, we produced B440, a lyophilized powder of inactivated B. longum 440, as a pharmaceutical formulation. We confirmed that 1 g of B440 contained approximately 0.08 mg of WT1 protein by ELISAs, which was estimated to correlate with 1X1011 cfu of B. longum 440 (data not shown). We also performed a preclinical dosage study of B440 for CRPC using the TRAMP-C2 mouse syngeneic tumor model. All B440 treatment groups of 10, 5, or 1 mg with B440 administered once, twice or three times a week for 1 or 3 months, the inactivated B. longum 420 and B. longum 440 groups, and the live B. longum 440 group significantly suppressed tumor growth compared with the control groups administered PBS and B. longum 2012. We measured the tumor volumes until day 71, when the control groups started to die (Fig. 6). For survival, 80% of mice in the L-3-3 (10 mg 3 times a week for 1 month), L-5-3 and M-1-1 groups were alive on day 123 (Table 2). All three of these groups’ administration periods were a month, and the doses were high (10 mg) or medium (5 mg). However, the second best survival groups with a 60% survival ratio at day 123 were L-3-3 and L-5-3, with administration periods of three months and low doses (1 mg). Furthermore, the survival curve of mice treated for one month indicated that there was no significant difference in survival among administration doses or frequencies (Fig. 7). Because cancer immunotherapies induce the host immune system to attack cancer cells, selecting the optimal dose and schedule is complicated. Previous clinical trials with ICIs, nivolumab and pembrolizumab indicated the dose-independent efficacies of the ICIs. In the phase 1 clinical trial of nivolumab for patients with advanced solid tumors, the objective responses were observed at doses of 1.0, 3.0, and 10.0 mg/kg body weight (38). In the phase II/III study of pembrolizumab, KEYNOTE-010, for non-small-cell lung cancer, there was no difference in efficacy between 2 and 10 mg/kg body weight (39). In our B440 preclinical dosage study, we confirmed the antitumor activity without any toxicity at doses of 1, 5 and 10 mg/body, and these doses were considered to correspond with 0.2, 1 and 2 g/body for humans. These results suggest that it is reasonable to set the doses of B440 in the first in human study with practical oral doses between 0.2 to 2 g/body after the additional preclinical dosage study to determine detailed clinical doses.

In conclusion, we developed a WT1 oral cancer vaccine, recombinant B. longum displaying a partial WT1 protein, for the treatment of CRPC. We confirmed that this oral vaccine induced higher cellular immune responses against TRAMP-C2 mouse CRPC cells and tumors than the Db126 peptide vaccine, and its antitumor activity could be augmented by anti–PD-1 antibody. Furthermore, we produced a B440 pharmaceutical formula, the
lyophilized powder of inactivated *B. longum* 440 displaying the partially modified human WT1 protein. We also confirmed that *B. longum* 440 could induce cellular immunity specific to multiple WT1 epitopes. The dosage study demonstrated that B440 could induce a significant antitumor effect at the clinically practical oral administration doses without any toxicity. These results suggest that the WT1 oral cancer vaccine, B440, can be developed as a novel immuno-oncology drug for the treatment of CRPC as a monotherapy or an adjunct to ICIs.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K. Kitagawa, R. Gono, M. Tanumini, M. Kadowaki, T. Shirakawa

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K. Kitagawa, Y. Hashii, T. Shirakawa

**Writing, review, and/or revision of the manuscript:** K. Kitagawa, Y. Hashii, T. Shirakawa

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** K. Kitagawa, T. Shirakawa

**Study supervision:** M. Fujisawa, T. Shirakawa

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Koichi Kitagawa, Reina Gonoi, Maho Tatsumi, et al.

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