ORAL ADMINISTRATION OF GENETICALLY MODIFIED BIFIDOBACTERIUM
DISPLAYING HCV-NS3 MULTI-EPITOPE FUSION PROTEIN COULD INDUCE AN HCV-NS3
-SPECIFIC SYSTEMIC IMMUNE RESPONSE IN MICE

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2 ABSTRACT

3 More than 170 million people worldwide are chronic HCV (Hepatitis C virus) carriers, and about
4 30% of them will develop progressive liver disease, such as cirrhosis and hepatocellular carcinoma.
5 A combination of pegylated interferon- α with ribavirin, the standard treatment for HCV infection,
6 has been effective in fewer than 50% of patients infected with HCV genotype 1. A strong T cell
7 response against the nonstructural protein 3 (NS3) is important for recovery from acute HCV
8 infection, and an early multi-specific CD4⁺ helper and CD8⁺ cytotoxic T cell response is critical for
9 HCV clearance. In the present study, we successfully constructed a genetically modified
10 *Bifidobacterium longum* (*B. longum*) displaying recombinant HCV-NS3 peptides containing some
11 CD4 and CD8 epitopes located in the HCV-NS3 region as an oral vaccine against chronic HCV
12 infection. The oral administration of this vaccine could induce NS3-specific **immune responses** in
13 mice through intestinal mucosal immunity. Our findings suggest that this novel oral vaccine has
14 great potential as a novel oral vaccine against chronic HCV infection.

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16 Key words: Bifidobacterium, mucosal vaccine, HCV, NS3

17 Abbreviated title: HCV-NS3 oral vaccine
18

1. Introduction

It is estimated that more than 170 million people are infected with the hepatitis C virus worldwide, and these chronic carriers are at risk of developing liver cirrhosis and cancer [1]. HCV is an enveloped, plus-strand RNA virus of the family *Flaviviridae* and is classified into six major genotypes [2]. Genotype 1b is the most prevalent in Japan and is widespread in the United States and Europe. This genotype also has a high rate of resistance to interferon-based treatments. The standard treatment for HCV infection is based on a combination of pegylated (PEG)-interferon (IFN) and an antiviral agent, such as ribavirin (RBV), although the virus eradication rates do not exceed approximately 50% with HCV genotype 1b infection, while that for genotypes 2 and 3 approaches 80% [3-5]. There are new anti-HCV drugs that directly target NS3, including the drugs BOC and TVR, which block the NS3/4A protease of HCV. A triple therapy with BOC or TVR, in combination with the standard treatment, PEG-IFN α and RBV, has increased the rate of sustained virological response (SVR) from under 50% to about 70% in patients with HCV genotype 1 [3, 6-8]. But approximately 30% of patients do not respond to this therapy and some suffer serious side effects such as fatigue, flu-like symptoms, anemia, and severe depression. Since there is no effective vaccine against HCV at present, it is still important to develop novel vaccine strategies to prevent HCV infection, or to increase the cure rate of the current interferon-based combinational therapy or to otherwise establish an interferon-free treatment regimen [9].

HCV genome RNA is constructed by four structural protein regions (C-E1-E2-P7) and six non-structural protein regions (NS2-NS3-NS4A-NS4B-NS5A-NS5B). The NS3 protein, which has a serine protease domain in its N-terminal first third and a helicase domain in the C-terminal second third, is essential for viral replication [10]. NS3-specific immune responses are higher and more frequently observed in patients with resolving infections than in chronically infected patients. These facts suggest that the NS3 protein might play a key role in HCV clearance [11]. In addition,

numerous CTL epitopes have been identified in NS3 region, it could be an ideal target for the development of a novel vaccine [12, 13].

In the present study, we focused on a vaccine development strategy using genetically modified probiotic bacteria as an oral vaccine for the treatment of HCV chronic infection. *Bifidobacterium* is one of the principal probiotic bacteria and possesses many advantages as a mucosal vaccine platform. Candela et al. demonstrated that *B. longum* and *B. lactis* were strongly adhesive to caco-2 cells and showed characteristics of human intestinal cells [14]. In addition, *B. longum* strains induced large amounts of IFN- γ and TNF- α *in vitro*, suggesting that *B. longum* induced high activation of Th1 cell-mediated immune responses [15]. These findings suggest that genetically modified *B. longum* could be used as an antigen delivery vehicle for a vaccine against many pathogens, including HCV. For antigen expression on the cell surface, we used the galacto-*N*-biose/lacto-*N*-biose I-binding protein (GLBP), the ABC transporter of *B. longum*, as an anchor [16]. By inserting the antigen at the C-terminal end of GLBP, the recombinant *B. longum* expressed the GLBP-antigen fusion protein on its cell surface. Previously, we constructed a recombinant *B. longum* displaying *Salmonella*-flagellin as an oral typhoid vaccine and confirmed in a mouse experimental model that this oral vaccine could induce a flagellin-specific systemic immune-response and could protect mice challenged with a lethal dose of *S. Typhimurium* [17].

In the present study, we constructed a genetically modified *B. longum* displaying recombinant HCV-NS3 protein, containing CD4 and CD8 epitopes, and examined whether this novel oral vaccine could induce an NS3-specific immune response using a mouse experimental model.

2. Materials and methods

2.1. Strains and media

Escherichia coli DH5 α was obtained from Toyobo Co., Ltd. (Tokyo, Japan) and used as a host for routine genetic engineering. *Bifidobacterium longum* (*B. longum*) JCM1217 was obtained from the Japan Collection of Microorganisms, RIKEN Bioresource Center. *E. coli* cells were grown in Luria-Bertani (LB) medium at 37°C. *B. longum* strains JCM1217 and 105-A [18] were grown anaerobically in GAM broth (Nissui, Tokyo, Japan) at 37°C.

2.2. Construction of a recombinant *B. longum* cell expressing the GLBP-NS3 fusion protein

Proper folding of NS3 requires the presence of NS4A peptide *in trans* [19] and, accordingly, the expression of an intact NS3 protein in bacteria was considered to be difficult. In the present study, we exploited one NS3 sub-domain (β - α - β -domain, 1216–1350 amino acid residues) and the preceding linker region (1196–1215 aa) [20] as a “core structure” to express the specified polypeptide at the cell surface of bifidobacteria (**Fig. S1a**). The core region, i.e. 1196–1350 aa, contains two CD4 epitopes (1201–1220 and 1321–1340) [21] and one CD8 epitope (1291–1299) of NS3 [22], to which the other epitopes can be attached at either end. Two variants were constructed: one consisting of 1073–1081 aa (containing CD8 epitope) [23], the core region, and 1373–1380 aa (CD8 epitope) [24] in that order (**Fig. S1b**), and the other consisting of 1073–1081 aa (containing CD8 epitope), the core region, 1373–1380 aa (CD8 epitope), 1406–1415 aa (CD8 epitope) [25], and 1436–1454 aa (CD8 epitope) [26, 27] (**Fig. S1c**). The former was designated NS3 short peptides containing two CD4 epitopes and three CD8 epitopes, while the latter was designated NS3 long peptides containing two CD4 epitopes and five CD8 epitopes. The genes for these polypeptides were synthesized by GenScript (NJ, USA), by reference to the Codon Usage Database (<http://www.kazusa.or.jp/codon/>). These synthetic variants were fused to GLBP, and the corresponding genes were ligated with the *E. coli*-*B. longum* shuttle vector, pJW241, as described previously [17]. The resulting plasmids carrying GLBP-NS3(short) and GLBP-NS3(long) were

introduced into *B. longum* 105-A by electroporation [18] to generate strains 2164 and 2165, respectively. Strain 2012 with a plasmid carrying GLBP only was also constructed and used as a control. **Figure S1 b and c** show a schematic drawing of the antigen surface displaying system of *B. longum*.

2.3. Western blotting

Western blotting was employed to determine whether the recombinant *B. longum* successfully expressed the GLBP-NS3 protein. *B. longum* grown in GAM broth overnight was harvested by centrifugation, washed in PBS three times, and resuspended in PBS. An equal volume of 2×sample buffer and 10% 2-mercaptoethanol (Wako, Tokyo, Japan) were added to the sample. The samples were heated for 5 min at 100°C just before the run. The gels were composed of 12% acrylamide and 0.4% bisacrylamide containing 0.1% (wt/vol) SDS. SDS-PAGE was carried out at 80 V, 20 mA for 90 min and the proteins were transferred onto a PVDF membrane. After blocking with 3% BSA and 0.1% Tween 20 in PBS overnight at 4°C and washing in PBS with Tween 20 (0.1%), the membrane was incubated for 1 h at room temperature with the primary rabbit anti-HCV-NS3 antibody (Operon, Zaragoza, Spain) against the both short and long NS3-peptides, 1:1000. After washing in PBS Tween, the membrane was incubated with the secondary antibody, goat anti-rabbit IgG HRP conjugated (Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 for 1 h at room temperature. Antibody binding to proteins was detected by enhanced chemiluminescence LAS 3000 mini, using the ECL Western Blotting Analysis System (GE Healthcare Japan, Tokyo, Japan).

2.4. Immunocytochemical study

Immunocytochemical staining was performed to examine the surface expression of the GLBP-NS3 protein on the recombinant *B. longum*. Bacterial cells were grown in GAM broth

overnight, collected, washed in PBS three times, and blocked with 1% BSA in PBS for 30 min at 37°C. After blocking, the cells were washed in PBS twice and resuspended with the primary antibody (Operon, Zaragoza, Spain) in 1% BSA PBS, 1:50. After 30 min of incubation at 37°C, they were washed in PBS twice and resuspended with the secondary antibody (Alexa Fluor™ 594 goat anti-rabbit IgG antibody, Molecular Probes Inc., Eugene, OR) in 1% BSA PBS, 1:100. After being washed in PBS twice, they were viewed under a fluorescent microscope (KEYENCE, Osaka, Japan).

2.5. Animal experiment

2.5.1. Immunization and sampling for evaluation of immune responses

Female BALB/C mice, 8-12 weeks of age, were purchased from Clea Japan, Inc. (Tokyo, Japan). Forty mice were randomly assigned to five experimental groups as follows: group 1, recombinant *B. longum* 2164 displaying NS3 short peptides immunization (eight mice); group 2, recombinant *B. longum* 2165 displaying NS3 long peptides immunization (eight mice); group 3, recombinant *B. longum* 2012 expressing GLBP protein control (eight mice); group 4, parental *B. longum* 245 control (eight mice); group 5, PBS control (eight mice). The recombinant *B. longum* or parental *B. longum* (5×10^7 colony-forming units /100 µl of PBS), or 100 µl of PBS, was orally administered directly into the stomach using a feeding needle three times a week for 4 weeks (at day 1, 3, 6, 8, 10, 13, 15, 17, 20, 22, 24, and 27), the dose and period of immunization were determined by our previous study [17]. Fecal samples were collected on days 0, 14, and 28 and were freeze dried. The feces were vortexed in PBS containing 5% non-fat milk, 0.1 mg of soybean trypsin inhibitor/ml, 2 mM phenylmethylsulphonyl fluoride, and 5 µl/mg dry feces, and were then centrifuged at 13,000 rpm for 20 min at 4°C. We also collected blood samples from the tail vein on days 0, 14, and 28, incubated them overnight at 4°C, and centrifuged them to obtain the serum samples. The supernatants were then frozen until analysis. In addition, spleens were removed on day 29 for *in*

vitro splenocyte stimulation with GST-NS3 peptide. All aspects of the experimental design and procedure were reviewed and approved by the institutional ethics and animal welfare committees of the Kobe University School of Medicine.

2.5.2. Enzyme-linked immunosorbent assay (ELISA) for antibodies induction

Nunc-Immunoplate MaxiSorp F96 plates (Nalgae Nunc, Inc., Rochester, NY, USA) were coated with GST-NS3 peptide (4 µg/mL), which was synthesized by the previously described method [28], in PBS and incubated overnight at 4°C. The plates were blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature and washed three times with PBS Tween. The anti-sera or extracts of feces were diluted to appropriate concentrations in PBS and added to the plates. The plates were then incubated for 3 h and washed. The secondary antibodies: Anti IgG Mouse Goat Poly-HRP (1/1000) and Anti IgA Mouse Goat Poly-HRP (1/1000), were added and incubated for 3 h at room temperature. The absorbance was measured at 450 nm in an Ultrospec Visible Plate Reader II96 (GE Healthcare Japan, Tokyo, Japan).

2.5.3. In vitro splenocyte stimulation with GST-NS3 peptide

The eight mice were sacrificed in each of five groups on day 29. Their spleens were punctured with a cell scraper and then dispersed through a 70 µm cell strainer to obtain single-cell suspensions. After centrifugation (2,000 rpm, 5 min), 5 ml of red blood cell lysis buffer (0.83% [w/v] NH₄Cl) was added to the pellet for 5 min. After two washes in sterile PBS, the cells were resuspended in culture medium (RPMI 1640 including 10% fetal bovine serum, penicillin [100 U/ml], streptomycin [100 µg/ml], and 5% MEM NEAA) and counted. The cells were plated (4×10⁵/well) with a total volume of 200 µl/well into a sterile 96-well microplate and stimulated with the GST-NS3 peptide (2 µg/well) or the negative control (sterile PBS). Cultures were incubated for 66 h at 37°C in 5% CO₂. Then the

cells were harvested for real-time PCR assay by centrifugation at $2,000 \times g$ for 5 min, and the supernatants were collected for ELISA and frozen at -20°C until use.

2.5.4. Expression level of cytokines mRNAs in splenocyte by real-time PCR assay

Expression levels of cytokines, *IFN- γ* , *IL-12*, and *IL-4* mRNAs in splenocytes were measured by real time RT-PCR. Total RNA was isolated from the stimulated and non-stimulated splenocytes using Trizol (Life Technologies Corporation, Carlsbad, CA). Real-time RT-PCR was performed by using TaKaRa RNA PCR Kit (AMV) Ver.3.0 (Takara Bio, Ohtsu, Japan) and SYBR Green (Life Technologies Corporation). The sets of primer sequences for *IFN- γ* , *IL-12*, *IL-4* and β -actin were shown in **Table S1**. Results were normalized according to *β -actin* mRNA expressions.

2.5.5. Expression level of *IFN- γ* in splenocyte by ELISA assay

The productions of *IFN- γ* in the supernatants of the cultivations of splenocytes stimulated and non-stimulated with GST-NS3 peptide were determined by using an ELISA Quantikine Mouse Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Optical densities were measured at a wave length of 450 nm [17,29-31].

2.5.6. The frequency of CD4^{+} and CD8^{+} T cells producing *IFN- γ*

In addition, the frequency of CD4^{+} and CD8^{+} T cells producing *IFN- γ* in splenocytes cultured with GST-NS3 peptide was examined by the intracellular cytokine staining (ICCS). Twelve mice were assigned to three experimental groups as follows: group 1, recombinant *B. longum* 2164 displaying NS3 short peptides immunization (four mice); group 2, recombinant *B. longum* 2165 displaying NS3 long peptides immunization (four mice); group 3, recombinant *B. longum* 2012 expressing GLBP protein control (four mice), and they were vaccinated, and their

splenocytes were isolated and cultured as described above. ICCS was performed by using the BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. In brief, 61 h after addition of stimulants, Golgistop (0.8 µl) was added to each well and the plates were incubated at 37°C in 5% CO₂ for an additional 5 h. Cells were washed twice with sterile PBS and aliquots of 1×10⁶ cells were transferred to FACS tubes. Cells were incubated with 10 µg/ml Purified anti-mouse CD16/32 Antibody (BioLegend) for 20 min on ice and washed. Cells were then stained with either FITC-conjugated anti-mouse CD4 antibody (clone RM4-5; PharMingen) or PE-conjugated anti-mouse CD8a antibody (clone 53-6.7; PharMingen), at a concentration of 2 µg/ml in staining buffer (0.1% (w/v) sodium azide, 1% heat-inactivated FCS in PBS) for 30 min on ice in the dark. Cells were washed twice with staining buffer and incubated with Fixation/Permeabilization solution (250 µl) for 20 min on ice in the dark. Cells were washed twice and stained with 5 µg/ml APC-conjugated anti-mouse IFN-γ antibody (clone XMG1.2; PharMingen) for 30 min on ice in the dark. Cells were washed twice and resuspended in staining buffer. Staining was assessed by FACSCalibur (BD Biosciences, San Jose, CA) and analyzed using CellQuest software.

2.6. Data analysis

Statistical comparisons between groups were performed using a one-way ANOVA followed by the Tukey's multiple comparison test. Differences among means were considered significant when $p < 0.05$.

3. Results

3.1 The expression of the GLBP-NS3 fusion protein

We performed western blotting to determine whether the recombinant *B. longum* successfully expressed the GLBP-NS3 proteins. As seen in **Fig. S2**, the whole cell lysates of both the recombinant *B. longum*, 2164 and 2165, demonstrated production of GLBP-NS3 fusion proteins with molecular masses similar to the theoretical molecular masses of 66 kDa for strain 2164 and 69 kDa for strain 2165. We also performed immunocytochemical staining to confirm the cell surface display of NS3 proteins, as seen in **Fig. 1**, and the fluorescences were observed in the recombinant *B. longum*, 2164 (**Fig. 1e**) and 2165 (**Fig. 1f**), and not in the parental *B. longum* (**Fig. 1d**).

3.2 NS3-specific IgA in feces and IgG in sera from mice immunized with the recombinant *B. longum*

We examined the production of NS3-specific secretory IgA in murine feces to determine whether the recombinant *B. longum*, 2164 and 2165, could induce a local humoral immune responses in mice. We orally administered the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* displaying NS3 long peptides (group 2), the recombinant *B. longum* 2012 expressing only GLBP protein (group 3), parental *B. longum* 245 (group 4), and PBS control (group 5) directly into the stomach every other day for 4 weeks and measured NS3-specific IgA in feces on days 0, 14, and 28. Group 2 showed significantly higher levels of NS3-specific secretory IgA on day 28 as compared with the other groups (**Fig. 2**). We also examined the production of NS3-specific IgG in sera to determine the induction of a systemic immune response with the recombinant *B. longum* 2164 and 2165. Significantly higher levels of NS3-specific IgG were observed in groups 1 and 2 on days 14 and 28 as compared with the other groups (**Fig. 3**).

3.3. The expressions of IFN- γ , IL-12, and IL-4 mRNAs in splenocytes isolated from the vaccinated mice

To determine if mice vaccinated with the recombinant *B. longum* 2164 and 2165, induced Th1

and Th2 cell-mediated response *in vivo*, we isolated the splenocytes from the mice and measured the mRNA expression levels of Th1 cytokine, *IFN-γ* and *IL-12*, and Th2 cytokine, *IL-4*, in the splenocytes with or without the pulsation of GST-NS3 peptide by real-time RT PCR. As in Th1 cytokines of *IFN-γ*, and *IL-12*, mRNA levels of *IFN-γ*, and *IL-12* were significantly increased in the splenocytes pulsed with the GST-NS3 *in vitro* isolated from the group 2 mice vaccinated with the recombinant *B. longum* 2165 (long NS3 peptides) compared with the splenocytes pulsed with the GST-NS3 isolated from the PBS control mice, group 5 (**Fig. 4 a and b**). Also in group 2 mice, the expression level of *IL-12* mRNA in the splenocytes pulsed with the GST-NS3 was significantly increased compared with that in the splenocytes without pulsation (**Fig. 4 b**). As in Th2 cytokine of *IL-4*, mRNA level of *IL-4* in the splenocytes pulsed with the GST-NS3 was significantly increased in group 2 compared with that in group 5 (**Fig. 4 c**).

3.4. The production of *IFN-γ* in splenocytes by NS3 stimulation

To determine if mice vaccinated with the recombinant *B. longum*, 2164 and 2165, induced a robust Th1 cell-mediated response *in vivo*, we harvested splenocytes from mice and measured their *IFN-γ* secretion in response to the GST-NS3 peptide using an ELISA *in vitro*. As seen in **Fig. 5**, the splenocytes from group 2 mice secreted significantly higher *IFN-γ* following pulsation with the GST-NS3 peptide as compared with the other groups. Additionally, the splenocytes from group 1 mice secreted higher levels of *IFN-γ*, but the difference was not significant.

3.5. The frequency of CD4⁺ and CD8⁺ T cells producing *IFN-γ*

We performed ICCS to investigate the frequencies of CD4⁺ and CD8⁺ T cells producing *IFN-γ* in splenocytes isolated from the mice vaccinated with the recombinant *B. longum*, 2164 or 2165. The representative flow cytometric dot plots of each group of the recombinant *B.*

1 *longum* 2164 displaying NS3 short peptides, the recombinant *B. longum* 2165 displaying NS3
2 long peptides, and the recombinant *B. longum* 2012 expressing only GLBP protein, were shown
3 in figure 6 a. The frequency of CD4+ and CD8+ T cells producing *IFN-γ* in *B. longum* 2165
4 group were significantly higher than that in *B. longum* 2164 and *B. longum* 2012 groups
5 ($p < 0.001$) (figure 6b). Also the frequency of CD4+ and CD8+ T cells producing *IFN-γ* in *B.*
6 *longum* 2164 group were significantly higher than that in *B. longum* 2012 groups (** $p < 0.01$,
7 *** $p < 0.001$) (figure 6b).

4. Discussion

The majority of vaccines in use today are delivered by injection to the intramuscular, subcutaneous, or intradermal spaces, although this practice is painful, requires a needle and syringe, and poses a risk of transmission of infections among patients, health workers, and the community, especially in developing countries [32]. The initial infection with most human pathogens occurs at the gastrointestinal, respiratory, or genital mucosa. Theoretically, oral vaccines could induce effective mucosal and/or systemic immunity, and could be easily and safely administered without a needle and syringe to a large number of people in a short period of time. Despite these advantages, oral vaccines have been used relatively infrequently against infectious diseases such as poliomyelitis, typhoid, rotavirus, and adenovirus infections. In most cases, oral vaccines are employed against enteric infections using attenuated live pathogens. These facts suggest the difficulty of inducing systemic immunity using oral vaccines.

We previously developed a genetically modified *B. longum* displaying *Salmonella*-flagellin for use as an oral typhoid vaccine, and demonstrated that this vaccine could induce both humoral and cellular immunity, and protected immunized mice against a lethal challenge of *Salmonella* Typhimurium [17]. In the intestinal immune system, the vaccine using bifidobacteria platform administered orally is recognized by microfold cells (M cells) that are responsible for antigen up-take into mucosa-associated lymphoid tissue (MALT), such as Peyer's patches. Dendritic cells present the antigen to lymphocytes, such as T cells and B cells, and also activate them. Moreover, an oral vaccine can induce not only local mucosal immunity, but also effective systemic cell-mediated immunity [33]. Based on these benefits, we developed a genetically modified *B. longum* displaying HCV-NS3 peptides for use as an oral therapeutic vaccine for the treatment of chronic HCV hepatitis. Although the NS3 protein could be an ideal antigen for a therapeutic vaccine to treat chronic HCV

infection, it has been considered difficult for bacteria such as bifidobacteria to express the whole intact NS3 protein. Therefore, we constructed two variants of recombinant peptides from the NS3 protein, which can be expressed by bifidobacteria and contain two CD4 epitopes and three CD8 epitopes, or two CD4 epitopes and five CD8 epitopes, as described in the “Materials and Methods” section. In the present study, we confirmed that the two recombinant bifidobacteria, 2164 [short peptides] and 2165 [long peptides], could produce the NS3 peptides and display them on the cell surface, using western blotting ([Fig. S2](#)) and immunocytochemical staining ([Fig. 1](#)).

As in the present experiments to evaluate the induction of NS3-specific antibodies for mucosal and systemic humoral immunity, the recombinant *B. longum* displaying NS3 long peptides induced the highest levels of NS3-specific IgA antibody in stool samples and IgG antibody in blood samples ([Fig. 2, 3](#)). In addition, we found that the mRNA level of *IL-4*, considered as Th2 cytokine, was significantly increased in the splenocytes pulsed with the GST-NS3 peptide isolated from the mice vaccinated with the recombinant *B. longum* displaying NS3 long peptides compared with that isolated from PBS control mice ([Fig. 4 c](#)). Nevertheless, the most important object of treatment of chronic HCV infection is the elimination of the HCV virus from the hepatocytes of patients in order to inhibit the advance of liver cirrhosis and hepatocarcinoma. During viral clearance, adaptive cellular immunity plays a central role in eliminating the HCV virus by recognizing and injuring antigen-expressing hepatocytes. Any therapeutic vaccine for chronic HCV infection must induce cell-mediated immunity, especially an NS3-specific CTL response [9, 34]. In the present study, we confirmed the induction of an NS3-specific Th1 response by immunization with recombinant *B. longum* 2165 displaying long NS3 peptides. We observed that the mRNA levels of *IFN-γ* and *IL-12*, considered as Th1 cytokines, were significantly increased in the splenocytes pulsed with the GST-NS3 peptide isolated from the mice vaccinated with the recombinant *B. longum* displaying NS3 long peptides compared with that isolated from PBS control mice ([Fig. 4 a, b](#)). Furthermore we

observed that the secretion level of IFN- γ in splenocytes isolated from *B. longum* 2165-immunized mice was significantly increased by pulsation with the NS3 protein, **and also the intracellular cytokine staining (ICCS) revealed that after the pulsation of the splenocytes with the NS3 protein, the frequencies of CD4⁺ and CD8⁺ T cells producing IFN- γ from *B. longum* 2165-immunized mice were significantly higher than those from *B. longum* 2164-immunized mice (p<0.001, figure 6a and 6b).** Interestingly, the recombinant *B. longum* 2165 displaying long NS3 peptides, which is added two more CD8 epitopes to short NS3 peptides, showed significant Th1 and Th2 responses in the present study. These additional two CD8 epitopes may play important role in the induction of NS3-specific adaptive immunity. Bifidobacteria contain DNA with a high GC content and hence a high fraction of unmethylated CpG motifs that affect the immune system by interacting with Toll-like-receptor (TLR) 9, triggering the production of proinflammatory cytokines and promoting the Th-1 response [35, 36]. These features of bifidobacteria could be exploited to advantages for antigen delivery vehicle system superior to other types of vehicles such as adenovirus [37], vaccine virus [38], and DNA vaccine [39].

In summary, we developed recombinant bifidobacteria displaying NS3 peptides containing several CD4 and CD8 epitopes for use as an oral vaccine against chronic HCV infection, and confirmed this novel vaccine could induce both humoral and cellular immunity in a mouse experimental model. In the future, this needle/syringe-free and cold-chain-free oral vaccine could be applied to the large number of patients with chronic HCV infection to markedly improve the cure rate of the current interferon-based combinational therapy and, further, may help establish interferon-free treatment regimens.

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Fig. 1. Immunocytochemical staining with anti-NS3 primary antibody of the recombinant *B. longum*, 2164 and 2165, and the parental *B. longum*. Bright-field images of the parental *B. longum* (a) and the recombinant *B. longum*, 2164 and 2165 (b and c), and immunofluorescence images of the parental *B. longum* (d) and the recombinant *B. longum*, 2164 and 2165 (e and f).

Fig. 2. Anti-NS3 IgA antibody levels in feces following oral administration (n=8 in each group) of the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 2), the recombinant *B. longum* 2012 expressing only GLBP protein (group 3), parental *B. longum* 245 (group 4), and PBS control (group 5). Fecal samples were collected on days 0, 14, and 28. Each data point represents the average of each group; bars, $\pm$ SE. A significantly higher level of anti-NS3 IgA antibody was observed in vaccine group 2 at day 28 (***p < 0.05**).

Fig. 3. Anti-NS3 IgG antibody levels in blood sera following oral administration (n=8 in each group) of the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 2), the recombinant *B. longum* 2012 expressing only GLBP protein (group 3), parental *B. longum* 245 (group 4), and PBS control (group 5). Blood samples were collected on days 0, 14, and 28. Each data point represents the average of each group; bars, $\pm$ SE. A significantly higher level of anti-NS3 IgG antibody was observed in vaccine groups 1 and 2 at days 14 and 28 (****p < 0.01**).

Fig. 4. Relative mRNA levels of *IFN- γ* (a), *IL-12* (b) and *IL-4* (c) in splenocytes of mice at day 29 after oral administration (n=6 in each group) of the recombinant *B. longum* 2164 displaying NS3

short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 2), the recombinant *B. longum* 2012 expressing only GLBP protein (group 3), parental *B. longum* 245 (group 4), and PBS control (group 5). The splenocytes were cultured with GST-NS3 (GST-NS3 peptide pulsing) or without GST-NS3 (no pulsing). Each data point represents the relative values of each group's average. A significant increase of *IL-12* expression by NS3-pulsing compared with each level of no pulsing was detected only in group 2 (**p < 0.01) (b), which was vaccinated with the recombinant *B. longum* 2165 with NS3 long peptides. The significantly higher expression of *IFN-γ*, *IL-12* and *IL-4* was observed in splenocytes pulsing with GST-NS3 peptide isolated from group 2 compared with that from group 5 (*p < 0.05) (a, b, c).

Fig. 5. IFN-γ production in splenocytes of mice at day 29 after oral administration (n=8 in each group) of the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 2), the recombinant *B. longum* 2012 expressing only GLBP protein (group 3), parental *B. longum* 245 (group 4), and PBS control (group 5). The splenocytes were cultured with GST-NS3 (GST-NS3 peptide pulsing) or without GST-NS3 (no pulsing). Each data point represents the average of each group; bars, ±SE. A significant increase in IFNγ by NS3-pulsing compared with each level of no pulsing was detected only in group 2 (**p < 0.01), which was vaccinated with the recombinant *B. longum* 2165 with NS3 long peptides.

Fig. 6. Intracellular cytokine staining of splenocytes isolated from the mice at day 29 after oral administration (n=4 in each group) of the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 2), and the recombinant *B. longum* 2012 expressing only GLBP protein (group 3). Cells were stimulated with GST-NS3 peptide for 66 h, and the frequency of IFN-γ producing CD4⁺ or

CD8⁺ T cells was flow detected by flow cytometry. The representative flow cytometric dot plots
in each group were shown in (a). Each data point represents the average of each group; bars,
±SE (b). The frequency of CD4⁺ and CD8⁺ T cells producing IFN- γ in *B. longum* 2165 group
were significantly higher than that in *B. longum* 2164 and *B. longum* 2012 groups (**
p<0.001) (b). Also the frequency of CD4⁺ and CD8⁺ T cells producing IFN- γ in *B. longum* 2164
group were significantly higher than that in *B. longum* 2012 groups (**p<0.01, *** p<0.001)
(b).

Fig 1

Fig 2

Fig 3

Fig 4

Fig 4

Fig 5

Fig 6

(a)

IFN- γ $\uparrow$

CD4 $\rightarrow$

CD8 $\rightarrow$

Fig 6