NS3 monoclonal antibody. (B) Serine protease analysis of NS3wt, various NS3 mutants and NS3/4A. Huh-7.5 cells were transiently transfected with each of the NS3 expression plasmids together with pNS5A/5BΔC (as a substrate). Cell lysates were subjected to immunoblot analysis using anti-NS3 and anti-NS5A monoclonal antibodies to detect NS3 (top panel) and NS5A/5BΔC and NS5A (middle panel), respectively. The amounts of GAPDH (bottom panel) were measured as an internal control to verify equal amounts of sample loading. (C) Effects of NS3wt or NS3 mutants on RIG-I-mediated IFN-β promoter activity. Huh-7 cells were transfected with a plasmid expressing NS3wt or each NS3 mutant together with pSG5-NS4A, pEF1A/N-RIG-I-FLAG, pIFN-β-luc and pRL-TK. Firefly luciferase activity was measured 48 h post transfection and normalized to Renilla luciferase activity. Data represent mean ± SEM of the data from three independent experiments. *, $p<0.01$; †, $p<0.05$, compared with NS3wt. (D) RNA helicase analysis of NS3wt and its mutant. NS3 helicase assay was performed using GST-NS3wt, GST-NS3(K210N) and GST as a negative control, as described in the Materials and methods section. The mean activity obtained with the GST control was subtracted from those obtained with test samples. The mean activity of GST-NS3wt was arbitrarily expressed as 100%. *, $p<0.05$, compared with NS3wt.
doi:10.1371/journal.pone.0098877.g002

were carried out for 60 min at 37°C. To stop the reactions, the wells were washed with 150 mM NaCl and dried at room temperature for 15 min. The wells were then washed with a detection washing buffer (100 mM maleic acid, 150 mM NaCl and 0.3% Tween 20, pH 7.5), incubated with a 10% BSA-containing blocking solution (100 mM maleic acid and 150 mM NaCl, pH 7.5) for 30 min followed by incubation with 20 μl of alkaline phosphatase-labeled anti-digoxygenin antibody solution (Roche Applied Science, Germany; 1:10,000 dilution in the blocking solution) for 30 min. After being washed with a detection buffer (100 mM Tris-HCl, pH 9.5, and 100 mM NaCl), 20 μl of a working solution containing CSPD chemiluminescence substrate (Roche) was added to each well and the plates were incubated for 5 min at 17°C. The wells were then drained and dried, and the luminescence in each well was counted in a luminescence multi-well plate reader. Helicase activities were determined by the reduction of the luminescence, which reflects the release of the digoxygenin-labeled oligonucleotides from the otherwise DNA duplex substrate.

Luciferase Reporter Assay

Huh-7 cells cultured in a 24-well tissue culture plate were transiently transfected with pSG5-NS3wt or each NS3 mutant (0.25 μg), together with pSG5-NS4A (0.25 μg), pIFN-β-Luc (0.2 μg), pEF1A/N-RIG-I-FLAG (0.05 μg) and pRL-TK (0.01 μg). After 48 h, cells were harvested and a luciferase assay was performed by using Dual-Luciferase Reporter Assay system (Promega). Firefly and Renilla luciferase activities were measured by using a GloMax 96 Microplate Luminometer (Promega).

Mice and Immunizations

BALB/c mice (H-2^d) were purchased from CLEA Japan, Inc. Mice were maintained in specific pathogen-free conditions according to institutional guidelines. All of the animal experiments were carried out according to the protocol approved by the Ethics Committee for Animal Experiments at Kobe University (Permit Number: P121002). All surgery was performed under isoflurane anesthesia, and efforts were made to minimize suffering. Eight-week-old female BALB/c mice were immunized with 200 μg of a plasmid, 100 μg each into both quadriceps, by intramuscular

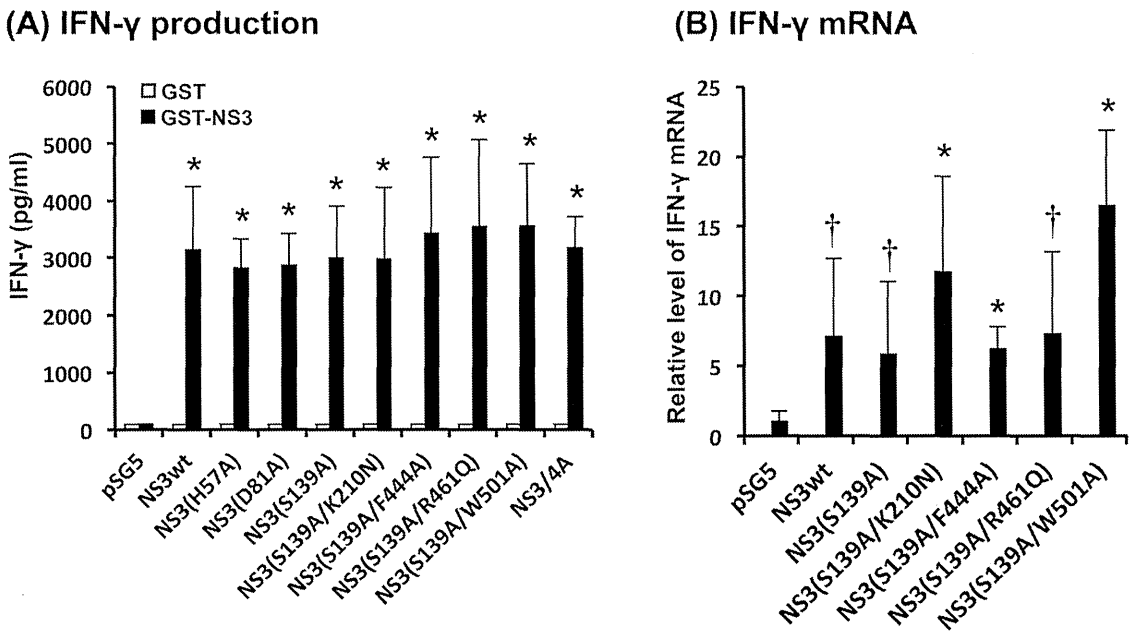

Figure 3. IFN-γ production induced by NS3 DNA vaccination. (A) IFN-γ production by splenocytes obtained from immunized mice. BALB/c mice (2 mice/group) were immunized with each of the DNA vaccines expressing NS3wt, various NS3 mutants or NS3/4A. Splenocytes obtained from the immunized mice were cultured in the presence of GST-NS3 (5 μg/ml) for 72 h. The amounts of IFN-γ in culture supernatants were measured with ELISA. Data represent mean ± SEM of the data from three independent experiments. *, $p<0.01$ compared with the mock-immunized control. (B) IFN-γ mRNA expression. Splenocytes obtained from immunized mice were cultured in the presence of GST-NS3 (5 μg/ml) for 24 h. The amounts of IFN-γ mRNA were determined by real-time quantitative RT-PCR analysis and normalized to GAPDH mRNA expression levels. Data represent mean ± SEM of the data from three independent experiments. The value for splenocytes from the mock-immunized control was arbitrarily expressed as 1.0. *, $p<0.01$; †, $p<0.05$, compared with the control.
doi:10.1371/journal.pone.0098877.g003

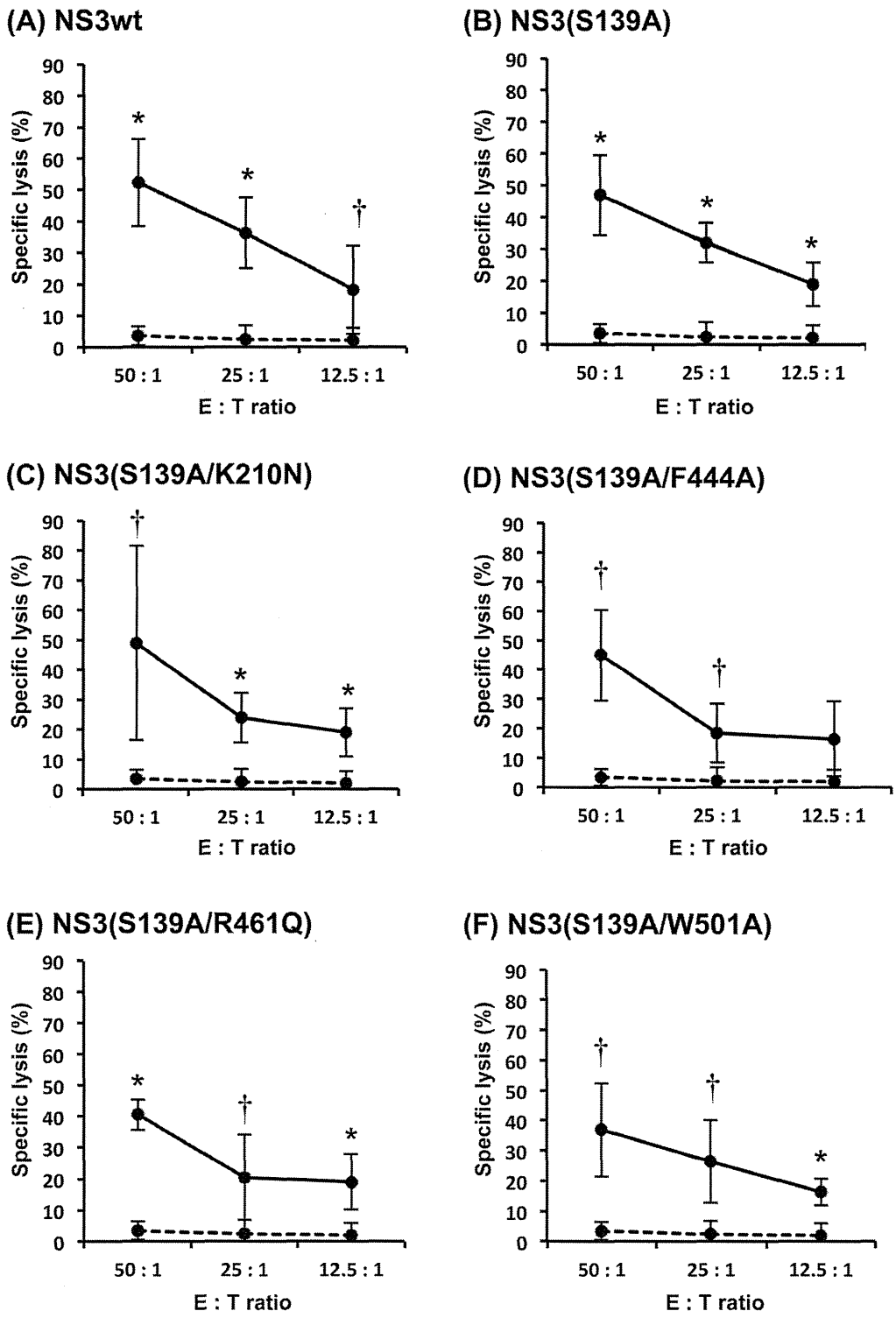

Figure 4. NS3-specific CTL activity induced by DNA vaccination. BALB/c mice (2 mice/group) were immunized with each of the DNA vaccines expressing NS3wt, various NS3 mutants or NS3/4A. Splenocytes obtained from the immunized mice were stimulated in vitro for 5 days with P815-NS3 cells and GST-NS3wt (5 μg/ml). Effectors and targets (P815-NS3) were cocultured for 4 h with the ratios of 50:1, 25:1, and 12.5:1. Released LDH was measured and the percentage of specific killing was calculated. Specific CTL activity of splenocytes obtained from NS3-immunized mice and the mock-immunized control are shown with solid and dashed lines, respectively. Data represent mean ± SEM of the data from three independent experiments. *, $p<0.01$; †, $p<0.05$, compared with the mock-immunized control.
doi:10.1371/journal.pone.0098877.g004

injection using a needle-free injector (Twin-Jector EZ II, JCR Pharmaceuticals Co., Ltd., Japan). We adopted the injection dosage according to previous studies [41,42]. The needle-free jet injection has been reported to enhance the immunological

responses induced by DNA vaccines [43]. Mice were boosted with the same plasmid (100 μg) at 4 and 6 weeks after the first injection. Control mice were injected with the empty pSG5 vector.

Splenocytes Culture

Eight weeks after the first immunization, spleens were resected and crushed with the use of a 22G needle. Splenocytes were strained with a cell strainer (40 μM, BD Falcon, USA) and treated for 5 min with 0.75% ammonium chloride buffer (pH 7.65) to lyse red blood cells. The splenocytes were suspended in RPMI1640 medium supplemented with 2 mM L-glutamine, 10% heat inactivated FCS, 50 U/ml penicillin, 50 U/ml streptomycin and 55 mM 2-mercaptoethanol.

IFN-γ Secretion Assay

Splenocytes seeded in 96-well (flat-bottom) plates at a concentration of 4×10^5 cells per well in 200 μl complete medium were stimulated with GST-NS3, or GST as a control, at a concentration of 5 μg/ml for 72 h. The amounts of IFN-γ in the culture supernatants were measured using an ELISA kit (Quantikine Mouse IFN-γ, R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

Real-time Quantitative RT-PCR

Total RNA was extracted from GST-NS3-stimulated mouse splenocytes using a ReliaPrep RNA cell miniprep system (Promega) according to the manufacturer's instructions. One μg of total RNA was reverse transcribed using a GoScript Reverse Transcription system (Promega) with random primers and was subjected to quantitative real-time PCR analysis using SYBR Premix Ex Taq (TaKaRa Bio Inc., Kyoto, Japan) in a MicroAmp 96-well reaction plate and an Applied Biosystems 7500 fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primers used to amplify IFN-γ mRNA were 5-CCTGCGGCCTAGCTCTGA-3′ (sense) and 5′-CAGCCA-GAAACAGCCATGAG-3′ (antisense). As an internal control, murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were measured using primers 5′-CATCGCCTTCCGTGTTCCTA-3′ (sense) and 5′-GCGGCACGTCAGATCCA-3′ (antisense).

CTL Assay

Splenocytes obtained from NS3-immunized mice were cultured for 5 days with P815-NS3 cells and 5 μg/ml of GST-NS3 to generate effector cells. The effector splenocytes and target P815-NS3 cells (1×10^4 cells) were cocultured in 96-well plates (round-bottom) for 4 h at 37°C in 5% CO_2 with ratios of 50:1, 25:1, and 12.5:1. Specific CTL activity was measured using a Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega). Released LDH was measured according to the manufacturer's protocol. The percentage of specific killing was calculated by the following formula: % specific killing = (experimental release − effector spontaneous release − target spontaneous release)/(target maximum release − target spontaneous release) × 100.

Statistical Analysis

Student's t-test was used to compare the data between two different groups. For multiple comparisons, a one-way analysis of variance (ANOVA) was used. A p-value of <0.05 was considered to be statistically significant.

Results

Characterization of Wild Type NS3 (NS3wt) and NS3 Mutants Expressed by DNA Vaccines

We constructed plasmids expressing NS3 mutants lacking the serine protease and the NTPase/RNA helicase activities to avoid potential risks posed by those enzymes (Fig. 1). The NS3 mutants were expressed efficiently in Huh-7.5 cells, as demonstrated by immunofluorescence (Fig. 2A) and immunoblotting assays (Fig. 2B, top panel). Importantly, all the NS3 mutants, either protease-deficient single-mutants or protease/helicase-deficient double-mutants, lacked the serine protease activity, as shown by the absence of the cleaved-off product of NS5A (Fig. 2B, middle panel). Equal loading of the samples was verified by GAPDH staining (Fig. 2B, bottom panel). The serine protease activity of NS3 is also known to cleave the RIG-I-associated adaptor protein MAVS (also known as Cardif, IPS-1 and VISA) and, therefore, blockade the RIG-I-mediated induction of IFN-β gene expression [44,45]. We confirmed that all the NS3 mutants lost their abilities to blockade the RIG-I-mediated IFN-β gene expression (Fig. 2C).

As for the NTPase/RNA helicase activities of NS3, it has been well documented that introduction of either one of the K210N, F444A, R461Q and W501A mutations severely affects the NS3 helicase activity [34,35,36]. Indeed, we confirmed that NS3 helicase activity was markedly impaired by the introduction of the K210N mutation (Fig. 2D).

Induction of IFN-γ Production by NS3-specific T cells after Immunization with NS3 DNA Vaccines

In order to evaluate the possible efficacy of the NS3 plasmids as DNA vaccines, BALB/c mice were injected intramuscularly with each of the plasmids, followed by booster injections at 4 and 6 weeks after the first injection. Two weeks after the last immunization, splenocytes were obtained from the mice, stimulated with GST-NS3 *in vitro* and the levels of IFN-γ production in the culture supernatants were measured. The results obtained revealed that protease-deficient single-mutants, i.e., NS3(H57A), NS3(D81A) and NS3(S139A), induced high levels of IFN-γ production, which were comparable to that induced by NS3wt and NS3/4A (Fig. 3A). Moreover, protease/helicase-deficient double-mutants with the backbone of NS3(S139A), i.e., NS3(S139A/K210N), NS3(S139A/F444A), NS3(S139A/R461Q) and NS3(S139A/W501A), induced IFN-γ production to the same extent as observed with the single-mutants. Consistently, real-time quantitative RT-PCR analysis revealed that the levels of IFN-γ mRNA expression were significantly higher in splenocytes obtained from NS3-immunized mice than those from mock-immunized control (Fig. 3B).

Induction of NS3-specific CTL Activities by Immunization with NS3 DNA Vaccines

We measured CTL activities induced by the NS3 DNA vaccines. Splenocytes obtained from the vaccinated mice two weeks after the last immunization were stimulated with GST-NS3 and P815-NS3 cells for 5 days and the effector splenocytes were mixed with the target P815-NS3 cells to determine the levels of CTL activities. Protease/helicase-deficient double-mutants, NS3(S139A/K210N), NS3(S139A/F444A), NS3(S139A/R461Q) and NS3(S139A/W501A), induced strong CTL activities against the target P815-NS3 cells to the level equivalent to that induced by NS3wt and a protease-deficient single-mutant NS3(S139A) (Fig. 4).

Discussion

Effective therapeutic vaccines against virus infection must induce sufficient levels of cell-mediated immune responses against the target viral epitope(s) and also must avoid concomitant risk factors, including potential carcinogenic properties. The HCV NS3 is considered to be an important target for development of HCV therapeutic vaccines because NS3-specific $CD4^+$ and $CD8^+$ T cell responses correlate well with resolution of the infection [46,47,48] and have been described as an indicator for viral clearance both in humans and chimpanzees [48,49,50]. On the other hand, NS3 possesses serine protease and NTPase/RNA helicase activities, which are necessary for the viral polyprotein processing and viral RNA replication, respectively [1,2]. In addition to the essential role in the virus life cycle, the NS3 serine protease interferes with normal cellular functions, such as blockade of IFN-β production [3,18,19,20] and deregulation of EGF signaling [22]. Also, the NTPase/RNA helicase of NS3 may interferes with cellular RNA helicases, which are involved in RNA folding/remodeling [51], enhancement of polymerase processivity [52], and/or genome encapsidation [53]. Importantly, perturbations of cellular RNA helicases are implicated in cancer development [23]. In the present study, therefore, we aimed to develop DNA vaccines that express NS3 mutants lacking both serine protease and NTPase/RNA helicase activities (Fig. 1) in order to avoid concomitant potential risks caused by the viral enzymes.

We first introduced single-point mutations into each of the catalytic triad of the NS3 serine protease (H57A, D81A and S139A) and found that all of the NS3 mutants efficiently induced IFN-γ production by splenocytes obtained from the vaccinated mice (Fig. 3A). Since His at position 57 is located within a well-characterized $CD4^+/CD8^+$ epitope [14,54], we decided not to choose pNS3(H57A) as a vaccine candidate. We then introduced a point mutation (K210N, F444A, R461Q and W501A) [34,35,36] to pNS3(S139A) to impair NTPase/RNA helicase activities. All the resultant DNA vaccine candidates, pNS3(S139A/K210N), pNS3(S139A/F444A), pNS3(S139A/R461Q) and pNS3(S139A/W501A), which express double-mutants lacking both serine protease and NTPase/RNA helicase activities, efficiently induced IFN-γ production by splenocytes of the vaccinated mice. We also observed that the protease-deficient single-mutant pNS3(S139A) and all of the four protease/helicase-deficient double-mutants induced NS3-specific CTL activities to the same extent compared to the non-mutated pNS3wt (Fig. 4). All but H57A mutation of NS3 examined in this study are located outside the human CD4 and CD8 epitopes reported so far, with the H57A mutation being located at the C-terminal edge of an epitope [7,8,9,11]. Therefore, these findings suggest that a single mutation in the protease and NTPase/RNA helicase domains would not interfere with immunogenicity of NS3 as a whole in mice and human.

In general, DNA vaccines mediate antigen expression only transiently in the vaccinees and, therefore, the possible side effects caused by the NS3 enzymatic activities through DNA vaccination would be rather marginal. However, when NS3 is expressed by means of a long-lasting live vaccine, such as a recombinant attenuated varicella zoster virus vaccine, it might potentially exert certain harmful effects after a long period of time. Currently, we aim to generate a recombinant attenuated varicella zoster virus expressing HCV NS3. For this purpose, an NS3 mutant lacking both protease and helicase activities and yet maintaining a full range of antigenic epitopes would be more appropriate than NS3wt.

In summary, we propose that plasmids expressing NS3 protease/helicase-deficient double-mutants, pNS3(S139A/K210N), pNS3(S139A/F444A), pNS3(S139A/R461Q) and pNS3(S139A/W501A), would be good candidates for safe and efficient therapeutic DNA vaccines against HCV infection.

Acknowledgments

The authors are grateful to Dr. T. Fujita, Kyoto University, for providing pIFNβ-Luc.

Author Contributions

Conceived and designed the experiments: SLR DPJ CA PS LD HH. Performed the experiments: SLR DPJ IS LD. Analyzed the data: SLR DPJ. Contributed reagents/materials/analysis tools: HH. Wrote the paper: SLR DPJ HH.

References

1. Lindenbach BD, Rice CM (2005) Unravelling hepatitis C virus replication from genome to function. Nature 436: 933–938.
2. Scheel TK, Rice CM (2013) Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. Nat Med 19: 837–849.
3. Morikawa K, Lange CM, Gouttenoire J, Meylan E, Brass V, et al. (2011) Nonstructural protein 3–4A: the Swiss army knife of hepatitis C virus. J Viral Hepat 18: 305–315.
4. Micallef JM, Kaldor JM, Dore GJ (2006) Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. J Viral Hepat 13: 34–41.
5. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST (2013) Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. Hepatology 57: 1333–1342.
6. Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, et al. (2011) Telaprevir for retreatment of HCV infection. N Engl J Med 364: 2417–2428.
7. Castelli FA, Leleu M, Pouvelle-Moratille S, Farci S, Zarour HM, et al. (2007) Differential capacity of T cell priming in naive donors of promiscuous CD4+ T cell epitopes of HCV NS3 and Core proteins. Eur J Immunol 37: 1513–1523.
8. Mashiba T, Udaka K, Hirachi Y, Hiasa Y, Miyakawa T, et al. (2007) Identification of CTL epitopes in hepatitis C virus by a genome-wide computational scanning and a rational design of peptide vaccine. Immunogenetics 59: 197–209.
9. Day CL, Lauer GM, Robbins GK, McGovern B, Wurcel AG, et al. (2002) Broad specificity of virus-specific CD4+ T-helper-cell responses in resolved hepatitis C virus infection. J Virol 76: 12584–12595.
10. Bowen DG, Walker CM (2005) Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature 436: 946–952.
11. Takaki A, Wiese M, Maertens G, Depla E, Seifert U, et al. (2000) Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med 6: 578–582.
12. Penna A, Missale G, Lamonaca V, Pilli M, Mori C, et al. (2002) Intrahepatic and circulating HLA class II-restricted, hepatitis C virus-specific T cells: functional characterization in patients with chronic hepatitis C. Hepatology 35: 1225–1236.
13. MacDonald AJ, Duffy M, Brady MT, McKiernan S, Hall W, et al. (2002) CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. J Infect Dis 185: 720–727.
14. Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, et al. (2002) Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J Immunol 169: 3447–3458.
15. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, et al. (1998) The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 4: 1065–1067.
16. Levrero M (2006) Viral hepatitis and liver cancer: the case of hepatitis C. Oncogene 25: 3834–3847.
17. Banerjee A, Ray RB, Ray R (2010) Oncogenic potential of hepatitis C virus proteins. Viruses 2: 2108–2133.
18. Gale M Jr, Foy EM (2005) Evasion of intracellular host defence by hepatitis C virus. Nature 436: 939–945.
19. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, et al. (2005) Complete replication of hepatitis C virus in cell culture. Science 309: 623–626.
20. Foy E, Li K, Sumpter R Jr, Loo YM, Johnson CL, et al. (2005) Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proc Natl Acad Sci U S A 102: 2986–2991.

21. Kaukinen P, Sillanpaa M, Kotenko S, Lin R, Hiscott J, et al. (2006) Hepatitis C virus NS2 and NS3/4A proteins are potent inhibitors of host cell cytokine/chemokine gene expression. Virol J 3: 66.
22. Brenndorfer ED, Karthe J, Frelin L, Cebula P, Erhardt A, et al. (2009) Nonstructural 3/4A protease of hepatitis C virus activates epithelial growth factor-induced signal transduction by cleavage of the T-cell protein tyrosine phosphatase. Hepatology 49: 1810–1820.
23. Robert F, Pelletier J (2013) Perturbations of RNA helicases in cancer. Wiley Interdiscip Rev RNA 4: 333–349.
24. Botlagunta M, Vesuna F, Mironchik Y, Raman A, Lisok A, et al. (2008) Oncogenic role of DDX3 in breast cancer biogenesis. Oncogene 27: 3912–3922.
25. Abdelhaleem M (2004) Do human RNA helicases have a role in cancer? Biochim Biophys Acta 1704: 37–46.
26. Hidajat R, Nagano-Fujii M, Deng L, Tanaka M, Takigawa Y, et al. (2005) Hepatitis C virus NS3 protein interacts with ELKS-{delta} and ELKS-{alpha}, members of a novel protein family involved in intracellular transport and secretory pathways. J Gen Virol 86: 2197–2208.
27. Deng L, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y, Ikeda M, et al. (2006) NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. J Gen Virol 87: 1703–1713.
28. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, et al. (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5: 730–737.
29. Love RA, Parge HE, Wickersham JA, Hostomsky Z, Habuka N, et al. (1996) The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. Cell 87: 331–342.
30. Lin C, Pragai BM, Grakoui A, Xu J, Rice CM (1994) Hepatitis C virus NS3 serine proteinase: trans-cleavage requirements and processing kinetics. J Virol 68: 8147–8157.
31. Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM (1993) Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J Virol 67: 2832–2843.
32. Tomei L, Failla C, Santolini E, De Francesco R, La Monica N (1993) NS3 is a serine protease required for processing of hepatitis C virus polyprotein. J Virol 67: 4017–4026.
33. Martinez MA, Clotet B (2003) Genetic screen for monitoring hepatitis C virus NS3 serine protease activity. Antimicrob Agents Chemother 47: 1760–1765.
34. Frick DN (2007) The hepatitis C virus NS3 protein: a model RNA helicase and potential drug target. Curr Issues Mol Biol 9: 1–20.
35. Kuang WF, Lin YC, Jean F, Huang YW, Tai CL, et al. (2004) Hepatitis C virus NS3 RNA helicase activity is modulated by the two domains of NS3 and NS4A. Biochem Biophys Res Commun 317: 211–217.
36. Tai CL, Pan WC, Liaw SH, Yang UC, Hwang LH, et al. (2001) Structure-based mutational analysis of the hepatitis C virus NS3 helicase. J Virol 75: 8289–8297.
37. Blight KJ, McKeating JA, Rice CM (2002) Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol 76: 13001–13014.
38. Deng L, Shoji I, Ogawa W, Kaneda S, Soga T, et al. (2011) Hepatitis C virus infection promotes hepatic gluconeogenesis through an NS5A-mediated, FoxO1-dependent pathway. J Virol 85: 8556–8568.
39. Hicham Alaoui-Ismaili M, Gervais C, Brunette S, Gouin G, Hamel M, et al. (2000) A novel high throughput screening assay for HCV NS3 helicase activity. Antiviral Res 46: 181–193.
40. Vlachakis D, Brancale A, Berry C, Kossida S (2011) A rapid assay for the biological evaluation of helicase activity. *Protocol Exchange doi:10.1038/protex.2011.275.* http://www.nature.com/protocolexchange/protocols/2282#/.
41. Hegde R, Liu Z, Mackay G, Smith M, Chebloune Y, et al. (2005) Antigen expression kinetics and immune responses of mice immunized with noninfectious simian-human immunodeficiency virus DNA. J Virol 79: 14688–14697.
42. Zhou F, Wang G, Buchy P, Cai Z, Chen H, et al. (2012) A triclade DNA vaccine designed on the basis of a comprehensive serologic study elicits neutralizing antibody responses against all clades and subclades of highly pathogenic avian influenza H5N1 viruses. J Virol 86: 6970–6978.
43. Raviprakash K, Porter KR (2006) Needle-free injection of DNA vaccines: a brief overview and methodology. Methods Mol Med 127: 83–89.
44. Sumpter R Jr, Loo YM, Foy E, Li K, Yoneyama M, et al. (2005) Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol 79: 2689–2699.
45. Moradpour D, Penin F, Rice CM (2007) Replication of hepatitis C virus. Nat Rev Microbiol 5: 453–463.
46. Diepolder HM, Gerlach JT, Zachoval R, Hoffmann RM, Jung MC, et al. (1997) Immunodominant CD4+ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. J Virol 71: 6011–6019.
47. Diepolder HM, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, et al. (1995) Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 346: 1006–1007.
48. Missale G, Bertoni R, Lamonaca V, Valli A, Massari M, et al. (1996) Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J Clin Invest 98: 706–714.
49. Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, et al. (2001) Determinants of viral clearance and persistence during acute hepatitis C virus infection. J Exp Med 194: 1395–1406.
50. Nascimbeni M, Mizukoshi E, Bosmann M, Major ME, Mihalik K, et al. (2003) Kinetics of CD4+ and CD8+ memory T-cell responses during hepatitis C virus rechallenge of previously recovered chimpanzees. J Virol 77: 4781–4793.
51. Rajkowitsch L, Chen D, Stampfl S, Semrad K, Waldsich C, et al. (2007) RNA chaperones, RNA annealers and RNA helicases. RNA Biol 4: 118–130.
52. Jarvis TC, Kirkegaard K (1991) The polymerase in its labyrinth: mechanisms and implications of RNA recombination. Trends Genet 7: 186–191.
53. Sun S, Rao VB, Rossmann MG (2010) Genome packaging in viruses. Curr Opin Struct Biol 20: 114–120.
54. Wertheimer AM, Miner C, Lewinsohn DM, Sasaki AW, Kaufman E, et al. (2003) Novel CD4+ and CD8+ T-cell determinants within the NS3 protein in subjects with spontaneously resolved HCV infection. Hepatology 37: 577–589.

Vaccine 32 (2014) 3066–3074

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Oral administration of genetically modified *Bifidobacterium* displaying HCV-NS3 multi-epitope fusion protein could induce an HCV-NS3-specific systemic immune response in mice

Saki Takei[a], Chika Omoto[b], Koichi Kitagawa[b], Naoya Morishita[a], Takane Katayama[c], Katsumi Shigemura[f], Masato Fujisawa[f], Masato Kawabata[a,d], Hak Hotta[d,e], Toshiro Shirakawa[a,b,d,f,*]

[a] Division of Infectious Disease Control, Department of Microbiology and Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan
[b] Division of Infectious Disease Control, Department of International Health, Kobe University Graduate School of Health Sciences, Kobe, Japan
[c] Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Ishikawa, Japan
[d] Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan
[e] Division of Microbiology, Department of Microbiology and Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan
[f] Department of Surgery Related Urology, Kobe University Graduate School of Medicine, Kobe, Japan

ARTICLE INFO

Article history:
Received 28 August 2013
Received in revised form 24 February 2014
Accepted 6 March 2014
Available online 21 March 2014

Keywords:
Bifidobacterium
Mucosal vaccine
HCV
NS3

ABSTRACT

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

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 approach 80% [3–5]. There are new anti-HCV drugs that directly target NS3, including the drugs boceprevir (BOC) and telaprevir (TVR), which block the NS3/4A protease of HCV. A triple therapy with BOC or TVR, in combination with the standard treatment, PEG-IFNα 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

* Corresponding author at: Center for Infectious Diseases, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-Cho, Chuo-Ku, Kobe 650-0017, Japan. Tel.: +81 78 382 5686; fax: +81 78 382 5715.
E-mail address: toshiro@med.kobe-u.ac.jp (T. Shirakawa).

http://dx.doi.org/10.1016/j.vaccine.2014.03.022
0264-410X/

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 *Bifidobacterium longum* and *Bifidobacterium 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 *Salmonella* 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 DH5α 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. Fig. S1b and c shows 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 (2000 rpm, 5 min), 5 ml of red blood cell lysis buffer (0.83% [w/v] NH_4Cl) 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 (4×10^5/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% CO_2. Then the cells were harvested for real-time PCR assay by centrifugation at 2000 × *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% CO_2 for an additional 5 h. Cells were washed twice with sterile PBS and aliquots of 1×10^6 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*,

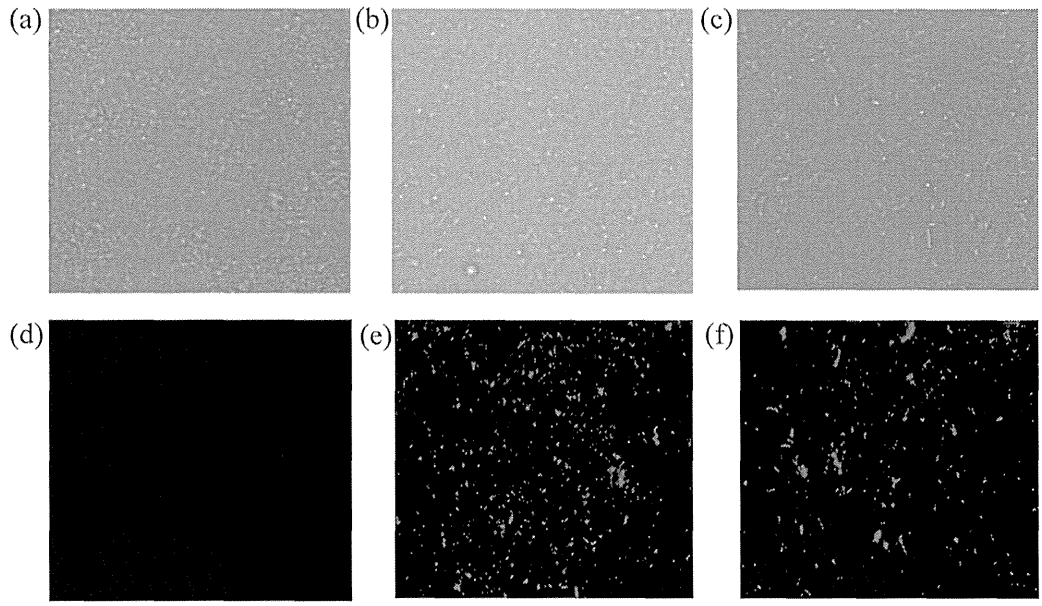

Fig. 1. Immunocytochemical staining with anti-NS3 primary antibody of the recombinant *B. longum*, 2164 and 2165, and the parental *B. longum*. Bright-field images of the parental *B. longum* (a) and the recombinant *B. longum*, 2164 and 2165 (b and c), and immunofluorescence images of the parental *B.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

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 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 systemic immune responses 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

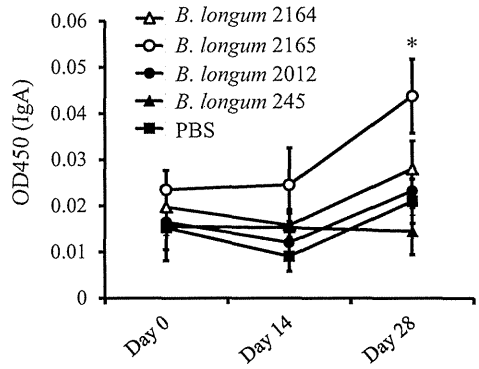

Fig. 2. Anti-NS3 IgA antibody levels in feces following oral administration ($n = 8$ in each group) of the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 2), the recombinant *B. longum* 2012 expressing only GLBP 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, ±SE. A significantly higher level of anti-NS3 IgA antibody was observed in vaccine group 2 at day 28 (*$p \leq 0.05$).

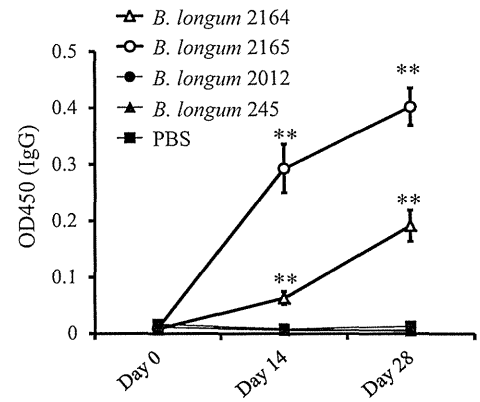

Fig. 3. Anti-NS3 IgG antibody levels in blood sera following oral administration ($n = 8$ in each group) of the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 2), the recombinant *B. longum* 2012 expressing only 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, ±SE. A significantly higher level of anti-NS3 IgG antibody was observed in vaccine groups 1 and 2 at days 14 and 28 (**$p \leq 0.01$).

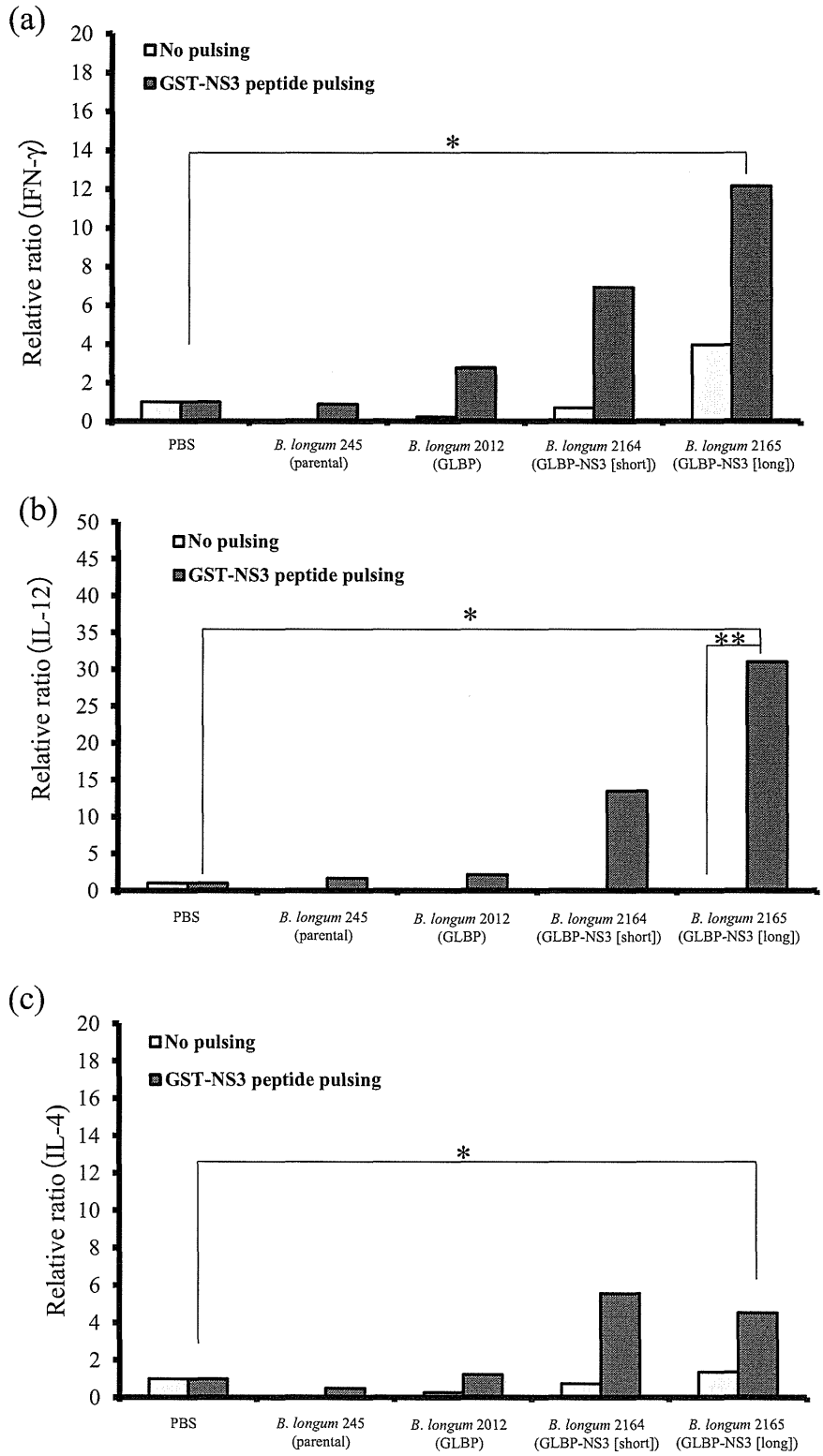

Fig. 4. Relative mRNA levels of *IFN-γ* (a), *IL-12* (b) and *IL-4* (c) in splenocytes of mice at day 29 after oral administration ($n = 6$ in each group) of the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 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–c).

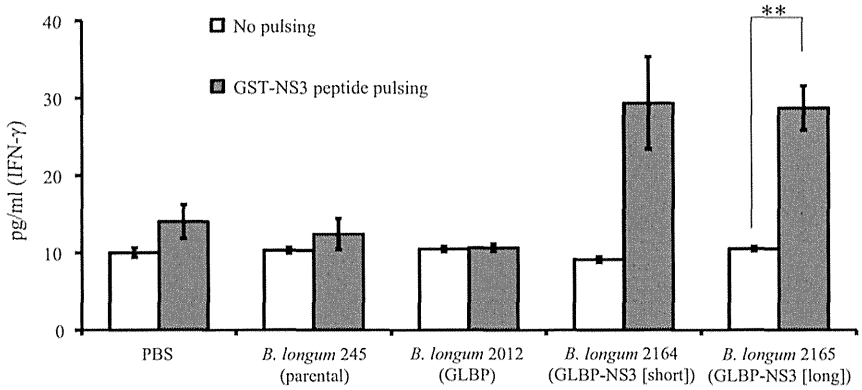

Fig. 5. *IFN-γ* production in splenocytes of mice at day 29 after oral administration ($n = 8$ in each group) of the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 2), the recombinant *B. longum* 2012 expressing 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.

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. 4a 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. 4b). 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. 4c).

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. longum* 2164 displaying NS3 short peptides, the recombinant *B. longum* 2165 displaying NS3 long peptides, and the recombinant *B. longum* 2012 expressing only GLBP protein, were shown in Fig. 6a. 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 ($p \leq 0.001$) (Fig. 6b). 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 (**$p \leq 0.01$, ***$p \leq 0.001$) (Fig. 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 Section 2. 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

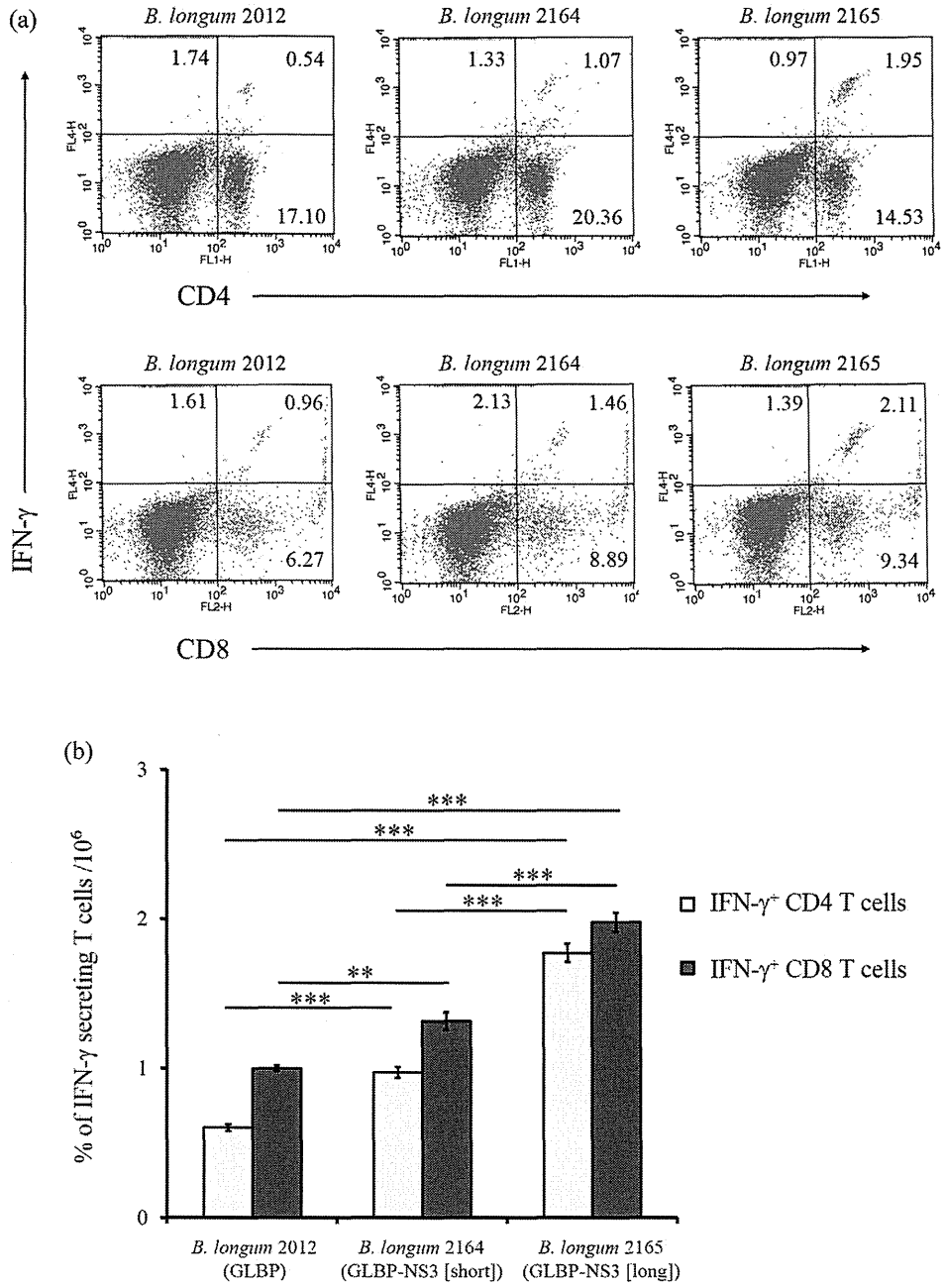

Fig. 6. Intracellular cytokine staining of splenocytes isolated from the mice at day 29 after oral administration ($n=4$ in each group) of the recombinant *B. longum* 2164 displaying NS3 short peptides (group 1), the recombinant *B. longum* 2165 displaying NS3 long peptides (group 2), and the recombinant *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 (***$p \leq 0.001$) (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 (**$p \leq 0.01$, ***$p \leq 0.001$) (b).

the highest levels of NS3-specific IgA antibody in stool samples and IgG antibody in blood samples (Figs. 2 and 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. 4c). 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. 4a and 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 ($p \leq 0.001$, Fig. 6a and b). 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.

Acknowledgments

This study was supported in part by a grant-in-aid for Scientific Research (No. 23591475) from the Japan Society for the Promotion of Science (JSPS), a grant-in-aid through the Program of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and a Science and Technology Research Partnerships for Sustainable Development (SATREPS) from the Japan Science and Technology Agency (JST) and the Japan International Cooperation Agency (JICA).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.03.022.

References

[1] World Health Organization. Hepatitis C: global prevalence. Wkly Epidemiol Rec 1997;72(46):341–8.
[2] Bukh J, Purcell RH, Miller RH. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc Natl Acad Sci U S A 1994;91(17):8239–43.
[3] Brody H. Hepatitis C. Nature 2011;474(7350):S1–7.
[4] Li K, Lemon SM. Innate immune responses in hepatitis C virus infection. Semin Immunopathol 2013;35(1):53–72.
[5] Di Bisceglie AM, Hoofnagle JH. Optimal therapy of hepatitis C. Hepatology 2002;36(5 Suppl 1):S121–7.
[6] Flamm SL, Lawitz E, Jacobson I, Bourlière M, Hezode C, Vierling JM, et al. Boceprevir with peginterferon alfa-2a-ribavirin is effective for previously treated chronic hepatitis C genotype 1 infection. Clin Gastroenterol Hepatol 2013;11(1):81–7.
[7] Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. J Hepatol 2012;56(1):78–84.
[8] Esteban R, Buti M. Triple therapy with boceprevir or telaprevir for treatment naïve HCV patients. Best Pract Res Clin Gastroenterol 2012;26(4):445–53.
[9] Torresi J, Johnson D, Wedemeyer H. Progress in the development of preventive and therapeutic vaccines for hepatitis C virus. J Hepatol 2011;54(6):1273–85.
[10] Kolykhalov AA, Mihalik K, Feinstone SM, Rice CM. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication in vivo. J Virol 2000;74(4):2046–51.
[11] Yu H, Babiuk LA, van Drunen Littel-van den Hurk S. Priming with CpG-enriched plasmid and boosting with protein formulated with CpG oligodeoxynucleotides and Quil A induces strong cellular and humoral immune responses to hepatitis C virus NS3. J Gen Virol 2004;85(Pt 6):1533–43.
[12] El-Gogo S, Staib C, Lasarte JJ, Sutter G, Adler H. Protective vaccination with hepatitis C virus NS3 but not core antigen in a novel mouse challenge model. J Gene Med 2008;10(2):177–86.
[13] Cerny A, McHutchison JG, Pasquinelli C, Brown ME, Brothers MA, Grabscheid B, et al. Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. J Clin Invest 1995;95(2):521–30.
[14] Candela M, Perna F, Carnevali P, Vitali B, Ciati R, Gionchetti P, et al. Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production. Int J Food Microbiol 2008;125(3):286–92.
[15] López P, Gueimonde M, Margolles A, Suárez A. Distinct *Bifidobacterium* strains drive different immune responses in vitro. Int J Food Microbiol 2010;138(1–2):157–65.
[16] Suzuki R, Wada J, Katayama T, Fushinobu S, Wakagi T, Shoun H, et al. Structural and thermodynamic analyses of solute-binding protein from *Bifidobacterium longum* specific for core 1 disaccharide and lacto-*N*-biose I. J Biol Chem 2008;283(19):13165–73.
[17] Yamamoto S, Wada J, Katayama T, Jikimoto T, Nakamura M, Kinoshita S, et al. Genetically modified *Bifidobacterium* displaying *Salmonella*-antigen protects mice from lethal challenge of *Salmonella* Typhimurium in a murine typhoid fever model. Vaccine 2010;28(41):6684–91.
[18] Matsumura H, Takeuchi A, Kano Y. Construction of *Escherichia coli–Bifidobacterium longum* shuttle vector transforming *B. longum* 105-A and 108-A. Biosci Biotechnol Biochem 1997;61(7):1211–2.
[19] Lin C, Thomson JA, Rice CM. A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex in vivo and in vitro. J Virol 1995;69(7):4373–80.
[20] Yao N, Reichert P, Taremi SS, Prosise WW, Weber PC. Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease-helicase. Structure 1999;7(11):1353–63.
[21] Schulze zur Wiesch J, Lauer GM, Day CL, Kim AY, Ouchi K, Duncan JE, et al. Broad repertoire of the CD4+ Th cell response in spontaneously controlled hepatitis C virus infection includes dominant and highly promiscuous epitopes. J Immunol 2005;175(6):3603–13.
[22] Mashiba T, Udaka K, Hirachi Y, Hiasa Y, Miyakawa T, Satta Y, et al. Identification of CTL epitopes in hepatitis C virus by a genome-wide computational scanning and a rational design of peptide vaccine. Immunogenetics 2007;59(3):197–209.
[23] Wertheimer AM, Miner C, Lewinsohn DM, Sasaki AW, Kaufman E, Rosen HR. Novel CD4+ and CD8+ T-cell determinants within the NS3 protein in subjects with spontaneously resolved HCV infection. Hepatology 2003;37(3):577–89.
[24] Ito K, Shiraki K, Funatsuki K, Ishiko H, Sugimoto K, Murata K, et al. Identification of novel hepatitis C virus-specific cytotoxic T lymphocyte epiotpe in NS3 region. Hepatol Res 2006;36(4):294–300.
[25] López-Labrador FX, He XS, Berenguer M, Cheung RC, Wright TL, Greenberg HB. The use of class-I HLA tetramers for the detection of hepatitis C virus NS3-specific CD8(+) T cells in patients with chronic infection. J Immunol Methods 2004;287(1–2):91–9.
[26] Neumann-Haefelin C, Frick DN, Wang JJ, Pybus OG, Salloum S, Narula GS, et al. Analysis of the evolutionary forces in an immunodominant CD8 epitope in hepatitis C virus at a population level. J Virol 2008;82(7):3438–51.
[27] Erickson AL, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, et al. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunology 2001;15(6):883–95.
[28] Hidajat R, Nagano-Fujii M, Deng L, Tanaka M, Takigawa Y, Kitazawa S, et al. Hepatitis C virus NS3 protein interacts with ELKS-δ and ELKS-α, members of a novel protein family involved in intracellular transport and secretory pathways. J Gen Virol 2005;86(Pt 8):2197–208.
[29] Chen YS, Hsiao YS, Lin HH, Yen CM, Chen SC, Chen YL. Immunogenicity and anti-*Burkholderia pseudomallei* activity in Balb/c mice immunized with plasmid DNA encoding flagellin. Vaccine 2006;24(6):750–8.
[30] Strindelius L, Degling Wikingsson L, Sjöholm I. Extracellular antigens from *Salmonella enteritidis* induce effective immune response in mice after oral vaccination. Infect Immun 2002;70(3):1434–42.
[31] Fu S, Xu J, Li X, Xie Y, Qiu Y, Du X, et al. Immunization of mice with recombinant protein CobB or AsnC confers protection against *Brucella abortus* infection. PLoS One 2012;7(2):e29552.
[32] Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev 2006;58(1):68–89.
[33] Kim SH, Lee KY, Jang YS. Mucosal Immune System and M Cell-targeting Strategies for Oral Mucosal Vaccination. Immune Netw 2012;12(5):165–75.
[34] Krishnadas DK, Li W, Kumar R, Tyrrell DL, Agrawal B. HCV-core and NS3 antigens play disparate role in inducing regulatory or effector T cells in vivo: implications for viral persistence or clearance. Vaccine 2010;28(9):2104–14.
[35] Jiang W, Pisetsky D. Enhancing immunogenicity by CpG DNA. Curr Opin Mol Ther 2003;5(2):180–5.

[36] Raghavan S, Nystrom J, Fredriksson M, Holmgren J, Harandi AM. Orally administered CpG oligodeoxynucleotide induces production of CXC and CC Chemokines in the gastric mucosa and suppresses bacterial colonization in a mouse model of *Helicobacter pylori* infection. Infect Immun 2003;71(12):7014–22.

[37] Hosseini SY, Sabahi F, Moazzeni SM, Modarressi MH, Saberi Firoozi M, Ravanshad M. Construction and preparation of three recombinant adenoviruses expressing truncated NS3 and core genes of hepatitis C virus for vaccine purposes. Hepat Mon 2012;12(8):e6130.

[38] Sekiguchi S, Kimura K, Chiyo T, Ohtsuki T, Tobita Y, Tokunaga Y, et al. Immunization with a recombinant vaccinia virus that encodes nonstructural proteins of the hepatitis C virus suppresses viral protein levels in mouse liver. PLoS One 2012;7(12):e51656.

[39] Lang KA, Yan J, Draghia-Akli R, Khan A, Weiner DB. Strong HCV NS3- and NS4A-specific cellular immune responses induced in mice and *Rhesus macaques* by a novel HCV genotype 1a/1b consensus DNA vaccine. Vaccine 2008;26(49):6225–31.

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i24.7555

World J Gastroenterol 2014 June 28; 20(24): 7555-7569
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Impact of hepatitis C virus heterogeneity on interferon sensitivity: An overview

Ahmed El-Shamy, Hak Hotta

Ahmed El-Shamy, Hak Hotta, Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
Ahmed El-Shamy, Department of Virology, Suez Canal University Faculty of Veterinary Medicine, Ismalia 41522, Egypt
Author contributions: El-Shamy A and Hotta H contributed equally to this work.
Supported by A Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan, a SATREPS Grant from Japan Science and Technology Agency and Japan International Cooperation Agency, and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) program from the Ministry of Education, Culture, Sports, Science and Technology, Japan
Correspondence to: Hak Hotta, MD, PhD, Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. hotta@kobe-u.ac.jp
Telephone: +81-78-3825500 Fax: +81-78-3825519
Received: November 15, 2013 Revised: February 18, 2014
Accepted: April 21, 2014
Published online: June 28, 2014

Abstract

Hepatitis C virus (HCV) is a major cause of liver disease worldwide. HCV is able to evade host defense mechanisms, including both innate and acquired immune responses, to establish persistent infection, which results in a broad spectrum of pathogenicity, such as lipid and glucose metabolism disorders and hepatocellular carcinoma development. The HCV genome is characterized by a high degree of genetic diversity, which can be associated with viral sensitivity or resistance (reflected by different virological responses) to interferon (IFN)-based therapy. In this regard, it is of importance to note that polymorphisms in certain HCV genomic regions have shown a close correlation with treatment outcome. In particular, among the HCV proteins, the core and nonstructural proteins (NS) 5A have been extensively studied for their correlation with responses to IFN-based treatment. This review aims to cover updated information on the impact of major HCV genetic factors, including HCV genotype, mutations in amino acids 70 and 91 of the core protein and sequence heterogeneity in the IFN sensitivity-determining region and IFN/ribavirin resistance-determining region of NS5A, on virological responses to IFN-based therapy.

Key words: Hepatitis C virus; Interferon; Genotype; Core protein; Nonstructural proteins 5A; Interferon sensitivity-determining region; Interferon/ribavirin resistance-determining region

Core tip: This review aims to cover recent updates on the impact of major hepatitis C virus (HCV) genetic factors, including HCV genotype, mutations in amino acids 70 and 91 of the core protein and sequence heterogeneity in interferon (IFN) sensitivity-determining region and IFN/ribavirin resistance-determining region of Nonstructural proteins 5A, on virological responses to IFN-based therapy.

El-Shamy A, Hotta H. Impact of hepatitis C virus heterogeneity on interferon sensitivity: An overview. *World J Gastroenterol* 2014; 20(24): 7555-7569 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i24/7555.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i24.7555

INTRODUCTION

Since its discovery in 1989[1,2], hepatitis C virus (HCV) has been the subject of intense research and clinical investigations as its major role in human disease has emerged. Globally, HCV is estimated to infect 180 million people, who represent about 3% of the world's population. HCV is a major cause of chronic liver disease, such as chronic

hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC)[3-6]. HCC is the third most common cause of cancer-related mortality worldwide[7]. In particular, HCV infection accounts for 30%-90% of HCC cases in Western Europe, United States and Asia[8]. Although the treatment of HCV infection is available, it is costly and requires long-term medical support and follow-up. Moreover, current therapies are still impractical for a substantial proportion of HCV-infected patients. The development of a protective vaccine remains a distant prospect.

HCV is an enveloped virus with a positive-strand RNA molecule of approximately 9600 nucleotides. HCV lacks a DNA intermediate; thus, it is incapable of integrating into host chromosomal DNA. Despite this, unlike most RNA viruses, HCV is capable of establishing persistent infection. This ability is central to HCV pathogenesis because it allows chronic infection to occur in 60% to 90% of infected individuals, and virtually all clinically significant HCV-related liver damage takes place during the chronic phase of infection[9,10].

The HCV genome encodes a single open reading frame that encodes a large polyprotein of approximately 3000 amino acids (aa). The polyprotein is processed by host cell peptidases and viral proteases to generate three structural (core, E1, E2) and seven non-structural (p7, NS2 to NS5B) proteins[11]. Both ends of the HCV genome contain highly conserved untranslated regions (5'- and 3'-UTRs) that are critical for genome replication and viral protein translation[12,13]. The 5'-UTR contains the HCV internal ribosome entry site (IRES), an RNA structural element that mediates ribosome binding for translation in a cap-independent manner, while the 3'-UTR is required for HCV RNA replication[14-17].

HCV displays a high nucleotide mutation rate that is estimated to be 1.44×10^{-3} nucleotide changes per site per year over the whole genome[18,19]. This mainly arises from the error-prone nature of its RNA-dependent RNA polymerase, which lacks 3'-to-5' exonuclease proofreading activity. This has resulted in diversification of HCV into distinct genotypes and subtypes. HCV exists in the host as quasispecies, which are a dynamic distribution of non-identical but closely related genomes[20,21]. This genetic diversity plays a vital role in HCV's ability to establish persistent infection and to evade the various selective pressures exerted by immune responses and antiviral therapy. Also, different HCV genotypes exhibit different treatment responses and different pathogenicity. Consequently, the impact of sequence heterogeneity within particular regions of the HCV genome, such as the core, E2, NS3 and NS5A, on treatment responses has been a subject of interest for many researchers. In this review, we will discuss the updated information about major HCV genetic factors, including viral genotype and sequence heterogeneity within certain regions of the HCV genome, in particular the core and NS5A regions, that influence the outcome of interferon (IFN)-based therapy.

HCV GENOTYPE AND SUSCEPTIBILITY TO IFN-BASED THERAPY

HCV exhibits genetic variability at several different levels. Most obvious is the genetic divergence of the main genotypes of HCV. Phylogenetic analysis of nucleotide sequences recovered from infected individuals in different geographical regions has classified HCV into seven major genotypes and series of subtypes[22]. HCV genotypes differ in 30%-35% of nucleotide sites over the whole genome, while the subtypes within a given genotype differ in 20%-25% of their nucleotide sites, with more sequence variability concentrated in such regions as the E1 and E2 glycoproteins, while more sequence conservation is found in the 5'- and 3'-UTR, the core gene and some of the nonstructural protein genes, such as NS3[23].

HCV genotypes differ in three major properties that highlight the importance of genetic diversity among the different HCV genotypes: (1) the prevalence of certain HCV genotype is frequently associated with certain geographical ranges; for example, HCV genotype 1 is prevalent in North America and Japan, genotype 3 is most common on the Indian subcontinent, genotype 4 is the most common genotype in Africa and the Middle East, genotype 5 can be found in South Africa and genotype 6 in Southeast Asia[24]; (2) the pathogenicity of HCV infection varies among the different genotypes; for example, HCV genotype 3 infection is associated with a higher degree of liver steatosis[25-27] and genotype 1 infection associated with a higher risk of HCC development[28,29]; and (3) the response rates to IFN-based therapy vary significantly between the different HCV genotypes[30-34].

To date, IFN represents the backbone of HCV therapeutic options. Pegylated-IFN and ribavirin (PEG-IFN/RBV) combination therapy is the standard of care for the treatment of chronic hepatitis C infection of different HCV genotypes. Recently, new direct-acting antivirals (DAAs) have been introduced to the treatment of HCV genotype 1 infection[35,36]. HCV genotype is an important determinant of both treatment strategy and outcome. HCV genotype 1 and genotype 4 infections need longer (48 wk) treatment period than genotypes 2 and 3 (24 wk); HCV genotypes 1 and 4 are less responsive to PEG-IFN/RBV treatment, with a sustained virological response (SVR) rate hovering around 50%[30,37-41], while the SVR rate in HCV genotype 2 and genotype 3 infections approaches 80%[30,37]. These differences in the SVR rates observed among different HCV genotypes (Figure 1) have suggested that viral genome variability could play a role in determining treatment outcome. However, it is still unclear which genetic element(s) within the HCV genome accounts for the difference in treatment response rates among different HCV genotypes. Interestingly, a series of detailed phylogenetic analyses have shown that there is a significant correlation between the relative evolutionary age of HCV genotypes and the response

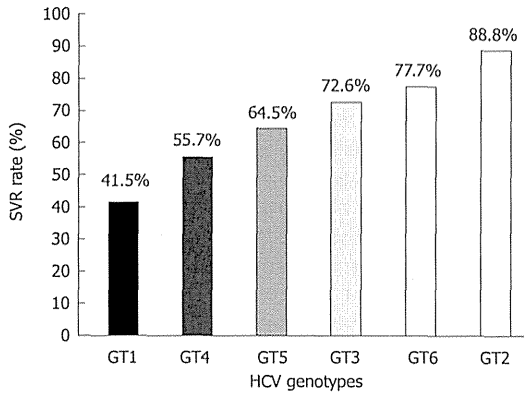

Figure 1 Average values of sustained virological response to pegylated-interferon and ribavirin combination therapy in patients infected with different hepatitis C virus genotypes. Average of sustained virological response (SVR) rates were calculated based on SVR rates achieved in clinical studies cited in references for genotype 1 (GT1)[30,31,37,152-154], GT2[32,155-160], GT3[32,155-158,160], GT4[30,38-40,161-163], GT5[164,165] and GT6[166-172]. In those studies, standard 48-wk treatment regimens were used for GT1 and GT4, and 24-wk treatment regimens were used for GT2 and GT3. Since the treatment regimens for GT5 and GT6 have not been established yet, the treatment duration varied among different studies where it ranged from 24 to 52 wk. HCV: Hepatitis C virus.

rates to IFN-based therapy[42]. In these analyses, HCV genotype 2 branched first, genotypes 1 and 4 branched last, and genotypes 3, 5 and 6 branched in between. Thus, it has been hypothesized that genotypes that emerged earlier exhibit better treatment outcomes, while newly-emerged genotypes have higher rates of resistance to IFN-based therapy than their ancestors. This correlation might be attributable to selective pressures generated by the host immune system, which the IFN-based therapy relies on to a large extent.

MUTATIONS IN GENOMIC REGIONS OF HCV AND RESISTANCE TO IFN-BASED THERAPY

As stated above, the response rates to IFN-based therapy vary between different HCV genotypes. More importantly, the sensitivity to IFN also varies between different HCV isolates of a given genotype and subtype. This has further highlighted a possible role for certain viral genetic factors in determining IFN responsiveness. In this context, genetic variations within two genomic regions, the core and NS5A, have been widely discussed for their correlation with treatment outcome. We will cover updated information regarding the impact of sequence heterogeneity within the core and NS5A regions of different HCV genotypes on treatment outcome, as described below.

CORE PROTEIN

At both the RNA and protein levels, HCV core plays a critical role in the virus life cycle. At the RNA level, a limited region of the first 45 to 60 nucleotides of the core gene, together with the 5'-UTR, is required for IRES function that initiates translation of the HCV polyprotein[14-17,43]. At the protein level, the HCV core is an RNA-binding protein that forms the capsid shell to protect the HCV genome while the virus passes from one cell to another or from one person to another. Furthermore, the core protein plays an important role(s) in the pathogenicity of HCV by modulating host cellular signaling pathways through interaction with a variety of cellular proteins. The core protein has been implicated in IFN resistance, liver steatosis, insulin resistance and hepatocellular carcinoma[44-49].

The HCV core protein shares high homology among different HCV genotypes. There are, however, certain polymorphisms, which are closely associated with the clinical outcome of IFN-based therapy. Akuta *et al*[50] first reported that aa mutations at positions 70 and 91 of the HCV genotype 1b (HCV-1b) core protein were associated with virological responses to PEG-IFN/RBV treatment. They found a significant correlation between core mutations at aa 70 (Arg^{70} to Gln^{70}/His^{70}) and/or aa 91 (Leu^{91} to Met^{91}) and poor treatment response. Gln^{70}/His^{70} and/or Met^{91} were found in 100% of ultimate resistance cases, who tested positive for HCV RNA at the end of 48-wk PEG-IFN/RBV treatment, but in only 42% of responders. Subsequently, several clinical studies were conducted by the same investigators and others, including our group, on Japanese patients infected with HCV-1b to follow up this observation. Most of these studies corroborate the initial observation that identified the aa mutation at position 70 of the core protein as a negative predictive marker for resistance to PEG-IFN/RBV treatment[51-60]. The polymorphism at aa 70 is also a useful determinant for the virologic response to extended 72-wk PEG-IFN/RBV treatment[61]. However, it is worth noting that although the significance of the point mutation at aa 70 (Gln^{70}) in prediction of poor treatment response is consistent among most clinical studies, the possible significance of the mutation at aa 91 is contradictory.

Since the discovery of their importance in IFN resistance, mutations at aa 70 and 91 have been the subject of intensive investigation for different aspects of chronic HCV infection including disease progression and pathogenesis. In HCV-1b infection, Gln^{70}/His^{70} and/or Met^{91} were significantly associated with severe insulin resistance[62], the severity of liver disease, elevated gamma-glutamyl transpeptidase (γ-GTP) levels, low platelet count and low albumin levels[63]. More importantly, Gln^{70}/His^{70} and/or Met^{91} (non-wild core) are significantly associated with an increased risk of HCC development[64-69]. Notably, Gln^{70} is the only HCV point mutation associated with both an increased risk of HCC and IFN treatment failure in multiple studies. Because increased HCC risk and IFN treatment failure are associated with the same viral point mutation, it is possible that both adverse outcomes result from disruption of the same cellular pathway, specifically, the IFN signaling pathway that is involved in both anti-

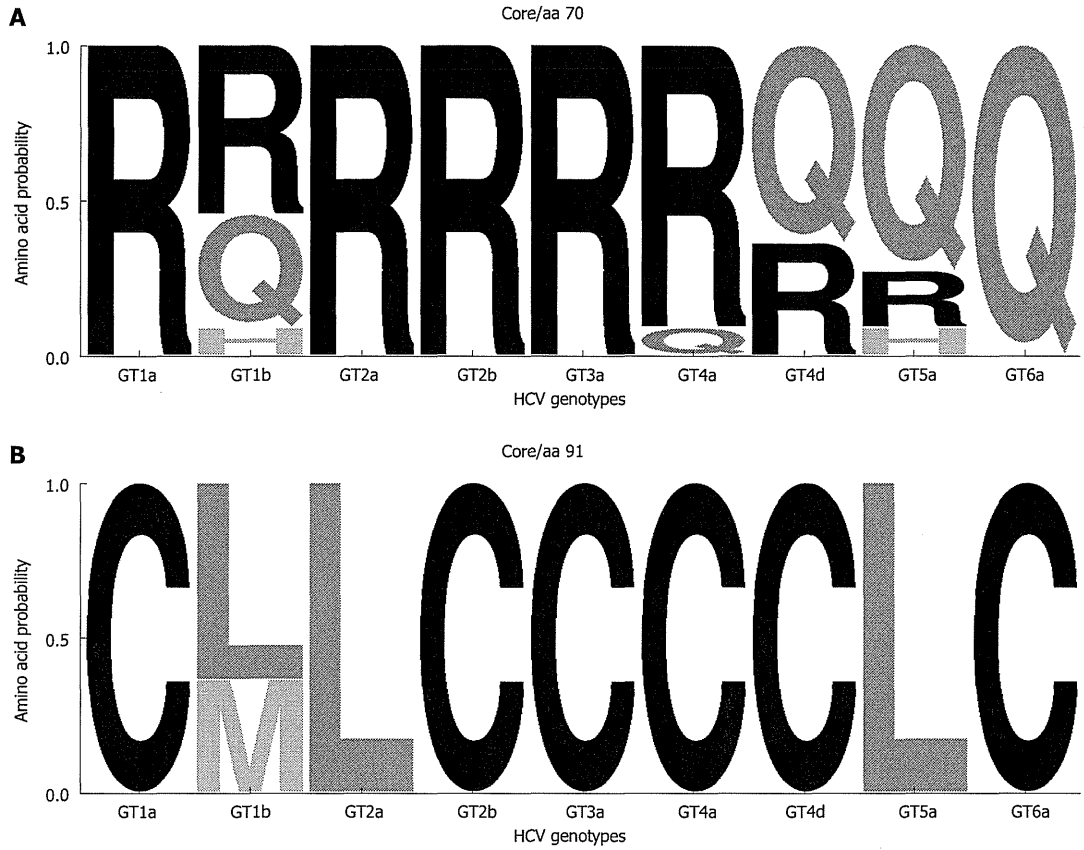

Figure 2 Probability of different aa residues at positions 70 (A) and 91 (B) of the core protein of different hepatitis C virus subtypes. The probability was estimated based on the core protein sequences available in hepatitis C virus (HCV) database (http://hcv.lanl.gov/content/index). The length of an aa symbol-letter represents its probability for a given HCV subtype. R: Arginine; Q: Glutamine; H: Histidine; L: Leucine; C: Cysteine; M: Methionine.

proliferative and antiviral functions.

It is important to note that the majority of the studies that demonstrated the importance of aa 70 and 91 mutations of the core protein were carried out on Japanese patients infected with HCV-1b. This raised two important questions: (1) is the significance of these viral mutations commonly observed among other HCV genotypes/subtypes; and (2) is the significance of these viral mutations commonly observed among other host ethnicities? To answer the first question, the correlation between the core protein mutations and IFN treatment outcome was investigated in non-HCV-1b infections, such as HCV-1a, -2a, -2b and -4a[70-76]. There was no significant correlation between these mutations and IFN treatment outcome in non-HCV-1b infections, where sequence patterns were quite conserved at these positions. The probability with different aa residues appear at positions 70 and 91 of the core protein of different HCV genotypes is shown in Figure 2. In contrast, point mutations at positions 4 and 110 of the core protein of HCV-2a were significantly associated with responses to PEG-IFN/RBV treatment[70,71]. As for the second question regarding host ethnicity, to the best of our knowledge, there are only three independent studies that were carried out on American[77], Swedish[76] and Saudi patients[75] infected with HCV-1b to investigate the possible correlation between mutations at aa 70 and 91 and IFN treatment outcome. In those studies, the point mutation at aa 70, but not at aa 91, was significantly associated with PEG-IFN/RBV treatment outcome. Overall, the results thus far obtained suggest that the mutation at codon 70 of the HCV core protein can be used as a predictive marker of IFN treatment outcome in only HCV-1b (and possibly HCV-5a) infection, regardless of host ethnicity.

As observed with the standard PEG-IFN/RBV combination therapy, Gln^{70} of the core protein is also significantly correlated with poor response to the recently approved triple therapy of PEG-IFN/RBV/protease inhibitor (telaprevir) for HCV-1b infection[78-81]. Interestingly, Gln^{70} showed significant linkage disequilibrium with minor genotypes (T/G and G/G) of the rs8099917 single nucleotide polymorphism (SNP) near the IL28B gene, which has recently been proposed as the strongest host genetic factor that is associated with poor response to PEG-IFN/RBV combination therapy in HCV genotype 1 infection[78,80,82].

Despite accumulated clinical evidence that strongly supports the correlation between HCV-1b core protein

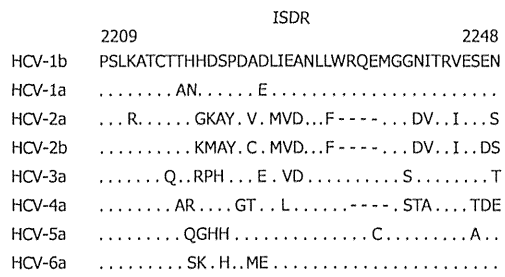

Figure 3 Sequence alignment of interferon sensitivity-determining region of major hepatitis C virus subtypes. References of aligned sequences are: hepatitis C virus (HCV)-1b, Enomoto *et al*[90]; HCV-1a, AF009606; HCV-2a and -2b, Murakami *et al*[104]; HCV-3a, GU814263; HCV-4a, El-Shamy *et al*[73]; HCV-5a, AF064490; HCV-6a, DQ480512. ISDR: Interferon sensitivity-determining region.

mutations and responses to IFN-based treatment, the molecular mechanism underlying this correlation is still obscure. Three experimental attempts aimed primarily to investigate this issue; however these studies produced conflicting results. In two of the studies, there was no significant difference in IFN resistance between the wild-type core protein and mutants at aa 70 and 91[83,84], while in the third study, there was a strong association between the core protein polymorphisms and IFN resistance *via* IL-6-induced and SOCS3-mediated suppression of IFN signaling[85]. These contradictions may, in part, be attributable to the different experimental models used by each study. Furthermore, Eng *et al*[86] identified a novel family of HCV core isotypes, referred to as minicores, which contain the C-terminal portion of the classical core protein, but lack the N-terminal portion. Interestingly, the N-termini of two major minicore proteins are at or near aa 70 and 91, and importantly, mutations in aa 70 and 91 regulate the expression levels of 70 and 91 minicores. Accordingly, it was hypothesized that these clinically important mutations at aa 70 and 91 of the core protein alter HCV function through altered expression levels of minicores, which might lead to IFN resistance. Further investigations using biologically relevant experimental models are needed to elucidate the molecular mechanism(s) underlying the role for the core protein mutations in HCV pathogenesis.

NS5A

The NS5A protein has generated a wide range of interest in HCV research because of its ability to modulate host cell functions, including responses to IFN. NS5A is multifunctional phosphoprotein that is found in a basally phosphorylated form of 56 kDa and a hyperphosphorylated form of 58 kDa[87,88]. NS5A modulates HCV replication through interaction with other viral proteins and certain host proteins to form the HCV replication complex. Moreover, NS5A has been implicated in various forms of viral pathogenesis through interactions with a wide variety of cellular proteins. Thus, NS5A clearly plays multiple roles in mediating viral replication, host-cell interactions, and viral pathogenesis[89]. NS5A is most extensively studied among all the HCV proteins for its relationship with IFN responsiveness. We review recent information regarding the clinical implication of NS5A sequence heterogeneity in predicting treatment outcome of IFN-based therapy.

IFN SENSITIVITY-DETERMINING REGION OF NS5A

Initially, in the era of IFN monotherapy, Enomoto *et al*[90,91] gained a key insight into the clinical influence of NS5A sequence heterogeneity on responses to IFN treatment. In their study, they amplified an NS5A fragment by RT-PCR from sera of HCV-infected patients, determined their sequences at both the nucleotide and amino acid levels, and compared them with a standard (reference) sequence of a given HCV subtype (Figure 3) to determine the number of amino acid substitutions in a sample of a given patient. By comparing the data obtained from patients who successfully responded to IFN therapy and those who did not, the authors identified a region called the IFN sensitivity-determining region (ISDR) spanning from aa 2209 to 2248 of NS5A of HCV-1b, whose sequence heterogeneity was closely associated with IFN treatment outcome. They found that Japanese patients infected with HCV-1b isolates having four or more mutations in the ISDR (ISDR $\geqslant$ 4) compared to that of the HCV-1b prototype (HCV-J) successfully responded to IFN therapy whereas patients infected with viral isolates having ISDR $\leqslant$ 3 were non-responders. Since this discovery, the ISDR has been the subject of intense clinical and experimental investigations. Although subsequent studies conducted in Japan were consistent with the initial ISDR report[92-95], results obtained from studies conducted in Europe and North America were quite controversial[96-99]. This might be explained in part by the fact that HCV isolates in western countries have lower overall degree of sequence heterogeneity, particularly in the ISDR, than HCV isolates circulating in Japan; the prevalence of HCV isolates with ISDR $\geqslant$ 4 is lower in western countries than in Japan. Host genetic differences between western and Japanese populations and the difference in treatment regimens may also contribute, at least partially, to the apparent discrepancy. Despite this, a meta-analysis conducted by Pascu *et al*[100] on 1230 ISDR sequences obtained from European and Japanese patients infected with HCV-1b clearly confirmed the importance of the ISDR in determining IFN treatment outcome. In this connection, it should be noted that, in the majority of studies dealing with the ISDR heterogeneity, nested RT-PCR followed by direct sequencing of the amplified fragments, without subcloning each sequence of quasispecies, was adopted to obtain the ISDR sequences, which, in general, identifies the most dominant population of quasispecies but may miss minor populations of quasispecies present in the patients.

The predictive value of ISDR initially described in the era of IFN monotherapy continues to be significant also

Figure 4 Sequence alignment of interferon/ribavirin resistance-determining region and its vicinity of major hepatitis C virus subtypes. The residues in interferon/ribavirin resistance-determining region (IRRDR) are written in bold face letters. References of aligned sequences are: hepatitis C virus (HCV)-1b, El-Shamy *et al*[122]; HCV-1a, AF009606; HCV-2a and -2b, Murakami *et al*[104]; HCV-3a, GU814263; HCV-4a, El-Shamy *et al*[73]; HCV-5a, AF064490; HCV-6a, DQ480512.

in the era of PEG-IFN/RBV therapy[58,59,101,102]. However, the original criterion of ISDR $\geq$ 4 to predict SVR was substituted by ISDR $\geq$ 2. This might result from the selective impact of IFN monotherapy, whereby the prevalence of sensitive isolates with ISDR $\geq$ 4 was decreased while that of HCV isolates of ISDR $\leq$ 3 was increased. Subsequently, those IFN monotherapy-resistant isolates with ISDR $\geq$ 2 were selected by PEG-IFN/RBV as sensitive isolates and those with ISDR $\leq$ 1 as resistant ones[52,58,102,103]. As for the different genotypes, the degree of sequence heterogeneity in ISDR was significantly correlated with SVR rate in Japanese patients infected with HCV-2a, the second most prevalent genotype in Japan[71,104,105]. In contrast, ISDR sequence heterogeneity did not associate with treatment outcome in patients infected with HCV-2b, -3a or -4a[73,104,106]. Whether the value of ISDR varies with different HCV genotypes and host ethnicity needs further investigation.

The molecular mechanism of ISDR-mediated IFN resistance is still unclear. Some studies have revealed that NS5A binds to and suppresses the function of the IFN-induced double-stranded RNA-activated protein kinase (PKR)[107-109]. PKR is known to inhibit viral replication by inhibiting viral protein synthesis through phosphorylation of eukaryotic initiation factor (eIF)-2. The PKR-binding domain (PKR-BD) of NS5A, spanning from aa 2209 to 2274, consists of the ISDR and its downstream region of 26 aa. The NS5A-PKR interaction was shown to be weakened by the ISDR mutations observed with IFN-sensitive HCV isolates, which would result in weaker suppression of PKR activity. In this context, a significant correlation between sequence variation in PKR-BD and IFN responsiveness was also reported[110-112]. On the other hand, we and other investigators have proposed that NS5A may also play a role(s) in IFN-resistance in an ISDR-independent manner. We have reported that an N-terminal portion of NS5A (aa 1-148) that lacks the ISDR and PKR-BD physically interacts with and inhibits the antiviral activity of 2',5'-oligoadenylate synthetase in cultured cells[113]. It has also been demonstrated *in vitro* that NS5A induces the expression of IL-8 at both the mRNA and protein levels. IL-8 is known to inhibit IFN-α signaling pathway. In agreement with this experimental observation, clinical data were reported that IL-8 levels in pretreatment sera were significantly higher in non-responders than in SVR patients[114,115].

IFN/RBV RESISTANCE-DETERMINING REGION OF NS5A

Despite the controversy about the ISDR concept in the era of IFN monotherapy, correlation between NS5A sequence heterogeneity and IFN responsiveness continued to be the subject of interest for many researchers. In this context, by using the same methodology as that for the ISDR analysis, a correlation between sequence heterogeneity of variable region 3 (V3) in a C-terminal portion of NS5A (aa 2356-2379) and responses to IFN-based therapy was also reported[110,112,116-120]. Our group further expanded this observation and gained a key insight when we identified a new region near the C-terminus of NS5A of HCV-1b spanning from aa 2334 to 2379, which we referred to as the IFN/RBV resistance-determining region (IRRDR)[121,122]. The IRRDR consists of the V3 region and its flanking upstream region, pre-V3 (aa 2334-2354). The reference sequences of different HCV subtypes are shown in Figure 4. In our initial study carried out on 47 Japanese patients infected with HCV-1b, we found that a higher degree of IRRDR sequence heterogeneity was closely associated with an early virological response (EVR) at week 16 of the 48-wk PEG-IFN/RBV treatment course[116]. Most importantly, in a follow-up study, the degree of sequence heterogeneity of the IRRDR was significantly associated with SVR. In particular, 16 (76%) of 21 SVR, but only 2 (8%) of 24 Non-SVR, had HCV with 6 or more mutations in the IRRDR (IRRDR $\geq$ 6). Accordingly, IRRDR $\geq$ 6 could predict SVR with a positive predictive value of 89% (16/18), while IRRDR $\leq$ 5 could predict non-SVR with a negative predictive value of 81% (22/27). Thus, we proposed that the degree of sequence heterogeneity of the IRRDR would be a useful positive and negative predictive marker for PEG-IFN/RBV treatment outcomes in HCV-1b infection[121,122]. Following this report, several reports were published by