Journal of Medical Virology 85:241–249 (2013)

# Synthetic Lipophilic Antioxidant BO-653 Suppresses HCV Replication

**Fumihiko Yasui,[1] Masayuki Sudoh,[2] Masaaki Arai,[3] and Michinori Kohara[1]***

[1]*Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Setagaya-ku, Tokyo, Japan*
[2]*Kamakura Research Laboratories, Chugai Pharmaceutical Co., Ltd., Kanagawa, Japan*
[3]*Biologics Research Department, Advanced Medical Research Laboratories, Mitsubishi Tanabe Pharma Corporation, Osaka, Japan*

The influence of the intracellular redox state on the hepatitis C virus (HCV) life cycle is poorly understood. This study demonstrated the anti-HCV activity of 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653), a synthetic lipophilic antioxidant, and examined whether BO-653's antioxidant activity is integral to its anti-HCV activity. The anti-HCV activity of BO-653 was investigated in HuH-7 cells bearing an HCV subgenomic replicon (FLR3-1 cells) and in HuH-7 cells infected persistently with HCV (RMT-tri cells). BO-653 inhibition of HCV replication was also compared with that of several hydrophilic and lipophilic antioxidants. BO-653 suppressed HCV replication in FLR3-1 and RMT-tri cells in a concentration-dependent manner. The lipophilic antioxidants had stronger anti-HCV activities than the hydrophilic antioxidants, and BO-653 displayed the strongest anti-HCV activity of all the antioxidants examined. Therefore, the anti-HCV activity of BO-653 was examined in chimeric mice harboring human hepatocytes infected with HCV. The combination treatment of BO-653 and polyethylene glycol-conjugated interferon-α (PEG-IFN) decreased serum HCV RNA titer more than that seen with PEG-IFN alone. These findings suggest that both the lipophilic property and the antioxidant activity of BO-653 play an important role in the inhibition of HCV replication. ***J. Med. Virol. 85:241–249, 2013.*** © 2012 Wiley Periodicals, Inc.

**KEY WORDS:** BO-653; antioxidant activity; chemical structure; HCV replication; chimeric mice

## INTRODUCTION

Hepatitis C virus (HCV) causes persistent infection, leading to chronic liver diseases including chronic hepatitis, cirrhosis, and hepatocellular carcinoma. In 2009, the number of patients with HCV infection worldwide was estimated to be 130–170 million [Lavanchy, 2009]. Recent years have seen the development of several promising treatments for patients infected with HCV. The addition of a protease inhibitor (boceprevir or telaprevir) to polyethylene glycol-conjugated interferon-α (PEG-IFN) and ribavirin improved dramatically the sustained virological response rates in treatment-naive patients with genotype 1 infections. However, the sustained virological response rate of triple therapy with a telaprevir-based regimen in null responders treated with PEG-IFN/ribavirin is only 30% [Fontaine and Pol, 2011; Kumada et al., 2012]. There is concern that high-risk groups such as patients with the *IL28B* minor allele (re8099917 SNP; GT/GG), the elderly, or those with fibrosis will be resistant to the triple therapy [Suppiah et al., 2009; Tanaka et al., 2009]. Therefore, new therapeutic strategies are required to treat HCV infection.

Chronic HCV infection is closely associated with oxidative stress. Oxidative stress reflects an imbalance between the production of reactive oxygen species (ROS) and the activity of intracellular antioxidant systems. The cumulative evidence from experimental

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant sponsor: Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation of Japan; Grant sponsor: Ministry of Health, Labor and Welfare of Japan.

Conflict of interest: Dr. Sudoh is an employee of Chugai Pharmaceutical Co., Ltd. The other authors declare no potential conflicts of interest.

*Correspondence to: Michinori Kohara, Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 2-1-6, Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan. E-mail: kohara-mc@igakuken.or.jp

Accepted 9 October 2012

DOI 10.1002/jmv.23466
Published online 28 November 2012 in Wiley Online Library (wileyonlinelibrary.com).

© 2012 WILEY PERIODICALS, INC.

and clinical studies demonstrates that HCV infection causes excessive ROS production and decreased activity of antioxidant enzymes [Kato et al., 2001; Levent et al., 2006]. In addition, previous studies showed that aggravation of oxidative stress in hepatocytes infected with HCV is correlated with the iron overload, while phlebotomy improves oxidative stress markers and liver pathology [Seronello et al., 2007]. Therefore, oxidative stress is a deleterious factor involved in the development of various hepatic diseases ranging from chronic hepatitis to hepatocellular carcinoma. In contrast, the influence of the intracellular redox state on HCV replication is controversial. Exogenous addition of either hydrogen peroxide or unsaturated fatty acid has been shown to induce oxidative stress and inhibit HCV replication in cell culture models [Choi et al., 2004; Huang et al., 2007]. Yano et al. [2007] reported previously that any of several nutrients (including vitamin E, a hydrophobic antioxidant) enhance HCV RNA replication. In contrast, overproduction of the antioxidant enzyme heme oxygenase-1 decreases HCV RNA replication in both full-length and subgenomic replicons [Zhu et al., 2008]. Despite these in vitro results, there have been no reports on the effect of antioxidant or pro-oxidant reagents on the life cycle of HCV in any animal models, such as chimeric mice harboring human hepatocytes infected with HCV.

BO-653 (2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran), a lipophilic (hydrophobic) antioxidant, was previously a clinical candidate for potential treatment of atherosclerosis and the prevention of post-angioplasty restenosis [Cynshi et al., 1998; Meng, 2003]. This compound is an effective inhibitor of lipid peroxidation and inhibits potently oxidation of lipids such as low-density lipoprotein [Noguchi et al., 1997; Tamura et al., 2003]. The present study examined the anti-HCV activity of BO-653 both in vitro and in vivo, and sought to clarify whether the antioxidant activity of the molecule was integral to the observed anti-HCV activity.

## MATERIALS AND METHODS

### Chemicals

BO-653 (molecular weight [MW], 388.6) was a gift of the Chugai Pharmaceutical company (Tokyo, Japan). Probucol [4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol)] was purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*-acetyl cysteine and ascorbic acid (vitamin C) were obtained from Sigma–Aldrich (St. Louis, MO). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and D-α-tocopherol (vitamin E) were obtained from Calbiochem (San Diego, CA) and MP Biomedical LLC (Solon, OH), respectively.

### Viruses and Cells

Patients provided written informed consent prior to blood sample collection.

HuH-7 cells harboring a HCV subgenomic replicon (FLR3-1 cells; genotype 1b, Con-1 strain) were maintained at 37°C in 5% $CO_2$ in Dulbecco's modified Eagle's medium GlutaMAX-I (DMEM-GlutaMax I; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) and 0.5 mg/ml G418 [Inoue et al., 2007].

HuH-7 cells infected persistently with HCV (RMT-tri cells; genotype 1a) were generated in the laboratory as described below and were maintained in DMEM containing 10% FCS, nonessential amino acids, 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 0.4% glucose. Complementary DNA (cDNA) of the full-genome HCV (nucleotides 1–9,598; GenBank accession number AB520610) was prepared from the serum of a patient with acute hepatitis infected with HCV genotype 1a [Inoue et al., 2007]. The resulting serum harbored HCV at a titer of $10^{8.6}$ copies/ml, as detected by a quantitative real-time polymerase chain reaction (qRT-PCR) as described previously [Takeuchi et al., 1999]. The sequence of the final cDNA construct was determined from a consensus of more than 10 clones, and was subcloned under the control of a T7 promoter (pHCV-RMT). The construct of HCV subgenomic replicon was generated from pHCV-RMT. HCV subgenomic RNA was transcribed using T7 RNA polymerase and the MEGAscript in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer's instructions. The resulting synthetic RNA of the HCV subgenomic replicon was transfected into HuH-7 cells by electroporation. Following transfection, the HCV subgenomic replicon-bearing HuH-7 cells was established. Total RNA was extracted by the acid guanidinium–phenol–chloroform method from a sample of the HCV subgenomic replicon-bearing HuH-7 cells and reverse transcribed. Sequence of the resulting cDNA has three nonsynonymous substitutions compared to that of the original HCV subgenomic replicon. Next, three nonsynonymous substitutions were inserted into the original full-length HCV sequence to enhance the replication rate in HuH-7 cells. Full-length RNA was also transcribed as described above. The resulting synthetic RNA of full-length HCV was transfected into HuH-7 cells by electroporation. Following transfection, the HCV RNA level in the transfected cells was measured on a weekly basis, revealing persistent infection at a level of $10^{6.5}$–$10^{6.8}$ copies/μg total cellular RNA over the course of 50 days. Finally, the transfected cell line was designated as RMT-tri.

### Analysis of Anti-HCV Effect of BO-653 in FLR3-1 Cells

The anti-HCV activity of BO-653 in FLR3-1 cells was measured by inhibiting luciferase activity [Inoue et al., 2007]. In brief, FLR3-1 cells were seeded at $4 \times 10^3$ cells/well in 96-well white plates. After 24 hr, the culture medium was replaced with fresh medium containing various concentrations of BO-653

(12–1,000 μM). The culture medium containing 1% MeOH was used as the negative control. After 72 hr incubation, the luciferase activity of the cells was measured using the Bright-Glo luciferase assay (Promega, Madison, WI) according to the manufacturer's instructions.

### Analysis of Anti-HCV Effect of BO-653 in RMT-Tri Cells

RMT-tri cells were seeded at $2.5 \times 10^4$ cells/well in 24-well plates. After 24 hr, the culture medium was replaced with fresh medium containing various concentrations of BO-653 (12–1,000 μM). The culture medium containing 1% MeOH was used as the negative control. After 72 hr incubation, the cell monolayer was harvested by adding 400 μl of 5 M guanidine-isocyanate solution containing 5.6 μl of 2-mercaptoethanol. The total RNA was extracted as above; HCV RNA was quantified by qRT-PCR.

### Comparison of Anti-HCV Activity of Lipophilic and Hydrophilic Antioxidants

The anti-HCV activity of various antioxidants, including hydrophilic and lipophilic compounds, was compared in FLR3-1 cells. BO-653, α-tocopherol, and probucol were used as lipophilic antioxidants; *N*-acetyl cysteine, ascorbic acid, and trolox were used as hydrophilic antioxidants. The anti-HCV activities of these compounds were determined by luciferase assays as described above.

### Cytotoxicity Testing

Simultaneously with the luciferase assays, the cell viability was measured by using a WST-8 cell counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

### Western Blot Analysis

FLR3-1 cells were treated with BO-653 as described above. After 96 hr, the cells were lysed with lysis buffer (protease inhibitor cocktail [Complete, Roche Diagnostics, IN] formulated according to the manufacturer's instructions in 10 mM Tris [pH 7.4], 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40). The cell lysates were resolved by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After incubation with a blocking buffer consisting of 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 [TBS-T], the membranes were incubated with rabbit polyclonal anti-NS3 antibody (R212 clone) and goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as the primary and secondary antibodies, respectively. Labeling was visualized using the Immobilon Western system (Millipore, Billerica, MA). To provide a loading control, β-actin was detected using mouse anti-β-actin monoclonal antibody (Sigma-Aldrich) and sheep anti-mouse IgG HRP-conjugated IgG (GE Healthcare) as the primary and secondary antibodies, respectively.

### Immunofluorescent Staining

FLR3-1 cells treated with 111 μM BO-653 for 96 hr were probed with the primary antibody (anti-NS3) after blocking with TNB blocking buffer (PerkinElmer, Waltham, MA). An anti-rabbit IgG Alexa-Fluor 488 conjugate (Invitrogen, Grand Island, NY) was then applied as the secondary antibody.

### Measurement of Antioxidant Activity of BO-653, α-Tocopherol, and Probucol in Lipid Peroxidation

Oxidation of methyl linoleate (10 mM) was carried out at 37°C under air in acetonitrile solution by adding 0.2 mM AMVN (2,2′-azobis-2,4-dimethylvaleronitrile) as a radical initiator in the presence of various concentrations of BO-653, α-tocopherol, and probucol. These antioxidants were added at concentrations of 0.2–20 μM to the reaction mixture; after 60 min incubation at 37°C, the reactions were stopped by chilling on ice.

The levels of lipid peroxidation were determined by the ferrous oxidation-xylenol orange (FOX) method [Nourooz-Zadeh et al., 1994]. In brief, completed methyl linoleate oxidation reactions were diluted 10-fold with MeOH containing 4.4 mM 2,6-di-*tert*-butyl-4-methylphenol (BHT). Each diluted solution (1.8 ml) was mixed with 0.1 ml of 2 mM xylenol orange solution (in 250 mM $H_2SO_4$) and 0.1 ml of 5 mM ferrous chloride solution (in 250 mM $H_2SO_4$). The mixture was incubated at room temperature for 60 min, at which point the absorbance at 570 nm was measured using a UV/visible light spectrophotometer. Cumene hydroperoxide was used to generate a standard curve for lipid hydroperoxidation.

### Pharmacokinetics of BO-653 in uPA/SCID Mice Harboring Human Hepatocytes

Chimeric uPA/SCID mice harboring human hepatocytes were purchased from PhoenixBio (Hiroshima, Japan). All animal experiments were approved by the Ethics Committee of Tokyo Metropolitan Institute of Medical Science and were performed in accordance with the guidelines of the Animal Experimental Committee of Tokyo Metropolitan Institute of Medical Science. Two chimeric mice were administered BO-653 (at 800 or 2,000 mg/kg in 3% gum arabic solution) by single oral gavage. At 24 hr after administration, blood was collected and the plasma concentration of BO-653 was measured by high-performance liquid chromatography (HPLC). Aliquots of plasma (100 μl) were mixed with 50 μl MeOH containing 10 mM ascorbic acid and 100 μl acetonitrile containing 30 μg/ml MeO-BO-653 as the internal control. The mixtures were centrifuged at 9,100*g* for 5 min, and the resulting supernatants of 30 μl each were separated using

an octadecyl column (Capcell Pak C18 UG120, 3 μm, 4.6 mm × 50 mm; Shiseido, Tokyo, Japan) at 30°C, a detection wavelength of 300 nm, and an eluent (acetonitrile) flow rate of 1.0 ml/min.

### Treatment of HCV-Infected Chimeric Mice With BO-653 and/or PEG-IFN

Chimeric mice also were used as an in vivo model of persistent HCV infection, as described previously [Inoue et al., 2007]. uPA/SCID mice were engrafted with human hepatocytes; 6 weeks later, the chimeric mice were infected by intravenous (IV) injection with patient serum containing $10^6$ copies of HCV genotype 1b (HCR6; GenBank accession no. AY045702). By 4 weeks after infection, the HCV RNA levels reached a plateau of $10^6$–$10^7$ copies/ml of mouse serum. To determine anti-HCV activity of BO-653 in the early phase of the treatment, the chimeric mice (n = 2–5 per group) infected with HCV were given once-daily oral gavage with 2,000 mg/kg BO-653 in 3% gum arabic, and/or twice weekly subcutaneous injection with 30 μg/kg PEG-IFNα-2a (Chugai Pharmaceutical) as shown in Table I. Body weights were monitored daily, and blood for serum was collected prior to the start of treatment (Day-1) and once weekly thereafter (Days 8 and 14). Following the terminal bleed, animals were sacrificed and liver specimens were collected.

### Quantitation of HCV RNA by qRT-PCR

After completion of the treatment, total RNA was purified from the serum and liver specimens by the acid guanidinium-phenol-chloroform method and qRT-PCR was used to quantify HCV RNA from the RNA samples corresponding to 1 μl serum and about 5 $mm^3$ of liver.

### Quantitation of Serum Human Albumin

The human albumin concentration in the blood of chimeric mice was measured in 2-μl serum samples by using an Alb-II kit (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

### Statistical Analysis

Data are presented as mean ± standard deviations (SDs). Statistical analysis was performed by using either Student's *t*-test or ANOVA, followed by Tukey's test or Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

## RESULTS

### Inhibitory Effect of BO-653 on HCV Replication In Vitro

The anti-HCV activity of BO-653 (Fig. 1A) was investigated in cells harboring HCV subgenomic replicons (FLR3-1 cells). BO-653 suppressed the replication of HCV subgenomic replicons in a concentration-dependent manner (Fig. 1B). The half-maximal inhibitory concentration ($IC_{50}$) of BO-653 in FLR3-1 cells was 36.0 μM. In contrast, no cytotoxicity was observed with up to 1,000 μM of BO-653 in FLR3-1 cells (Fig. 1B). Western blotting and immunofluorescent staining of FLR3-1 cells demonstrated that the level of HCV NS3 protein, but not that of β-actin, was reduced as the concentrations of BO-653 increased (Fig. 1C and D). A similar trend was seen in RMT-tri cells for the replication of full-genome HCV genotype 1a (Fig. 1E).

### Comparison of Anti-HCV Activity of Lipophilic Antioxidants

The antioxidant activity of BO-653 has been compared previously with that of probucol and α-tocopherol [Cynshi et al., 1998]. Therefore, the anti-HCV activity of these three lipophilic antioxidants was compared in FLR3-1 cells. At a concentration >37 μM, BO-653 exhibited stronger inhibitory effects against HCV replication than did the two other compounds (Fig. 2A). In addition, the antioxidant activity of these compounds was determined by an in vitro lipid peroxidation system. BO-653 had the strongest antioxidant activity against lipid peroxidation in this in vitro assay (Fig. 2C).

### Comparison of Anti-HCV Activity of Hydrophilic and Lipophilic Antioxidants

The anti-HCV activities of some representative antioxidants were investigated further (Fig. 3A). As noted above, lipophilic antioxidants exhibited anti-HCV activity in cell culture; however, hydrophilic antioxidants (*N*-acetyl cysteine, ascorbic acid, and trolox) did not inhibit the replication of HCV subgenomic replicons at comparable concentrations. None of the

TABLE I. Schedule of Blood Collection and Drug Administration for Chimeric Mice Infected With HCV

| | Day | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | −1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Collection of blood | B | | | | | | | | | B | | | | | | B |
| BO-653 | | BO | BO | BO | BO | BO | BO | BO | BO | BO | BO | BO | BO | BO | BO | |
| PEG-IFN | | I | | | I | | | | I | | | I | | | | |
| BO-653 + PEG-IFN | | BO/I | BO | BO | BO/I | BO | BO | BO | BO/I | BO | BO | BO/I | BO | BO | BO | |

B, sampling of blood; BO, orally administrated BO-653 (2,000 mg/kg); I, subcutaneous injection of PEG-IFN (30 μg/kg).

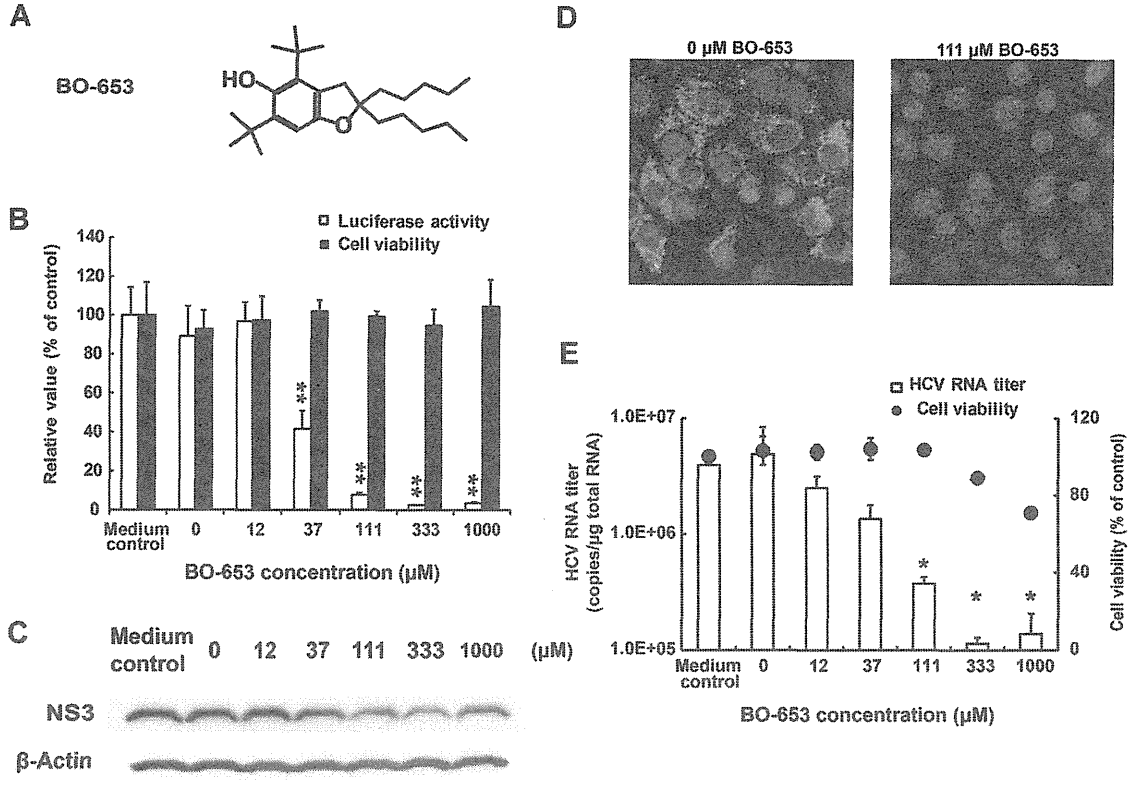



Fig. 1. In vitro anti-HCV activity of BO-653. A: The chemical structure of BO-653. B: The inhibitory effect of BO-653 on the replication of HCV subgenomic replicons in FLR3-1 cells. HCV replication (white bars) and cell viability (black bars) were determined after 72 hr of treatment; $^{**}P < 0.01$, compared with the medium control. C: Effect of BO-653 on the levels of HCV NS3 protein and β-actin (Western blotting). D: Immunofluorescent staining of HCV NS3 protein (green) in FLR3-1 cells in the absence or presence (111 μM) of BO-653; nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). E: Effect of BO-653 on the HCV RNA titer and cell viability of HuH-7 cells infected with HCV genotype 1a (RMT-tri) after 72 hr of treatment; $^{*}P < 0.05$, compared with the medium control. Statistical analyses were performed by using ANOVA with post-hoc Dunnett's (n = 3 replicates).

compounds had any cytotoxicity at the concentrations tested (Fig. 3B).

### Anti-HCV Activity of BO-653 and PEG-IFN in Chimeric Mice Infected With HCV

As demonstrated above using an in vitro assay with FLR3-1 cells, lipophilic antioxidants, including BO-653, exhibited strong anti-HCV activity. The anti-HCV activity of BO-653 was assessed further in vivo by using the compound to treat humanized chimeric mice infected with HCV. First, to measure the pharmacokinetics of BO-653, two chimeric mice were administered orally BO-653 at 800 or 2,000 mg/kg. Twenty-four hours after administration, the mice had mean BO-653 plasma concentrations of 25.0 and 83.1 μM, respectively (Table II). Thus, the BO-653 plasma concentration at the higher dose level exceeded the $IC_{50}$ of BO-653 (36.0 μM) demonstrated previously by the in vitro assay (Fig. 1B), suggesting that oral administration of 2,000 mg/kg BO-653 might be relevant therapeutically for chimeric mice infected with HCV. These mice were infected persistently with HCV genotype 1b by injection with the serum of a HCV-infected patient (see Materials and Methods Section). To determine the anti-HCV activity of BO-653 in early phase of treatment, BO-653 (oral) and/or PEG-IFN (subcutaneous) were then administered over a period of 14 days, according to the schedule shown in Table I, and serum and liver specimens were collected. No adverse effect of the treatment, such as loss of body weight or decreased human albumin secretion, was observed in any of the study groups (Fig. 4A and B). In the mice treated with PEG-IFN, which received a dose 20-fold higher dose than that used in the clinic, the serum HCV RNA titers fell approximately 30-fold and 50-fold in weeks 1 and 2, respectively. Treatment with BO-653 alone at 2,000 mg/kg orally once daily did not reduce the HCV RNA serum titers. However, the combination of BO-653 and PEG-IFN was effective, with the combination demonstrating 200-fold decrease in serum

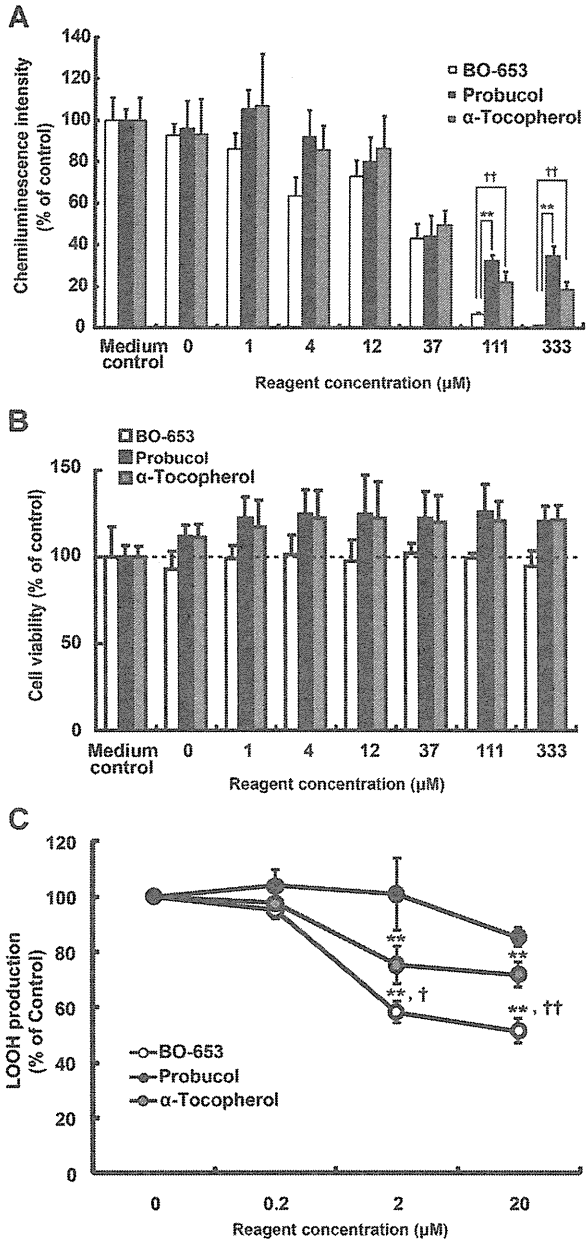



Fig. 2. Comparison of the in vitro anti-HCV activities of lipophilic antioxidants. **A**: The anti-HCV activity and (**B**) cytotoxicity of BO-653, α-tocopherol, and probucol were measured in FLR3-1 cells after 72 hr exposure to the compounds; $^{**}P < 0.01$, compared with probucol at the same concentration, $^{\dagger\dagger}P < 0.01$, compared with α-tocopherol at the same concentration. **C**: Antioxidant activity of BO-653, α-tocopherol, and probucol in the peroxidation of methyl linoleate with AMVN (FOX method); $^{**}P < 0.01$, compared with probucol at the same concentration, and $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, compared with α-tocopherol at the same concentration. Statistical analyses were performed using ANOVA with post-hoc Tukey's.

HCV titer at 2 weeks; the effect was statistically significant compared to treatment with PEG-IFN alone (Fig. 4C). In the liver samples, a decrease in the titer of HCV RNA to 7–34% of the value in untreated mice

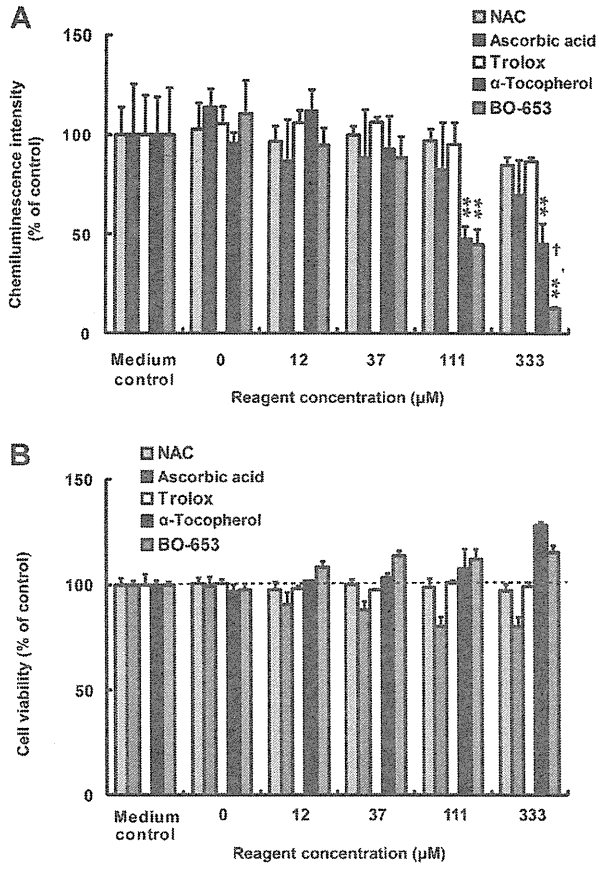



Fig. 3. Comparison of the anti-HCV activity of lipophilic and hydrophilic antioxidants. A: The anti-HCV activity and (**B**) cytotoxicity of BO-653 and α-tocopherol (lipophilic antioxidants) and *N*-acetyl cysteine (NAC), ascorbic acid, and trolox (hydrophilic antioxidants) in FLR3-1 cells after 72 hr of exposure to the compounds. $^{**}P < 0.01$, compared with the hydrophilic antioxidants at the same concentration, and $^{\dagger}P < 0.05$, compared with α-tocopherol at the same concentration. Statistical analyses were performed using ANOVA with post-hoc Tukey's.

was also observed in the mice given the combination therapy (Fig. 4D).

## DISCUSSION

Several lines of evidence indicate that chronic HCV infection is associated with persistently elevated levels of ROS, resulting in oxidative stress and thus contributing to the development of hepatic dam-

TABLE II. Concentration of BO-653 in Plasma

| Dose | Mouse ID | Concentration of BO-653 in plasma (μM) | Mean concentration (μM) |
|---|---|---|---|
| 800 mg/kg | 1 | 30.2 | 25.0 |
| | 2 | 19.8 | |
| 2,000 mg/kg | 1 | 100.9 | 83.1 |
| | 2 | 65.2 | |

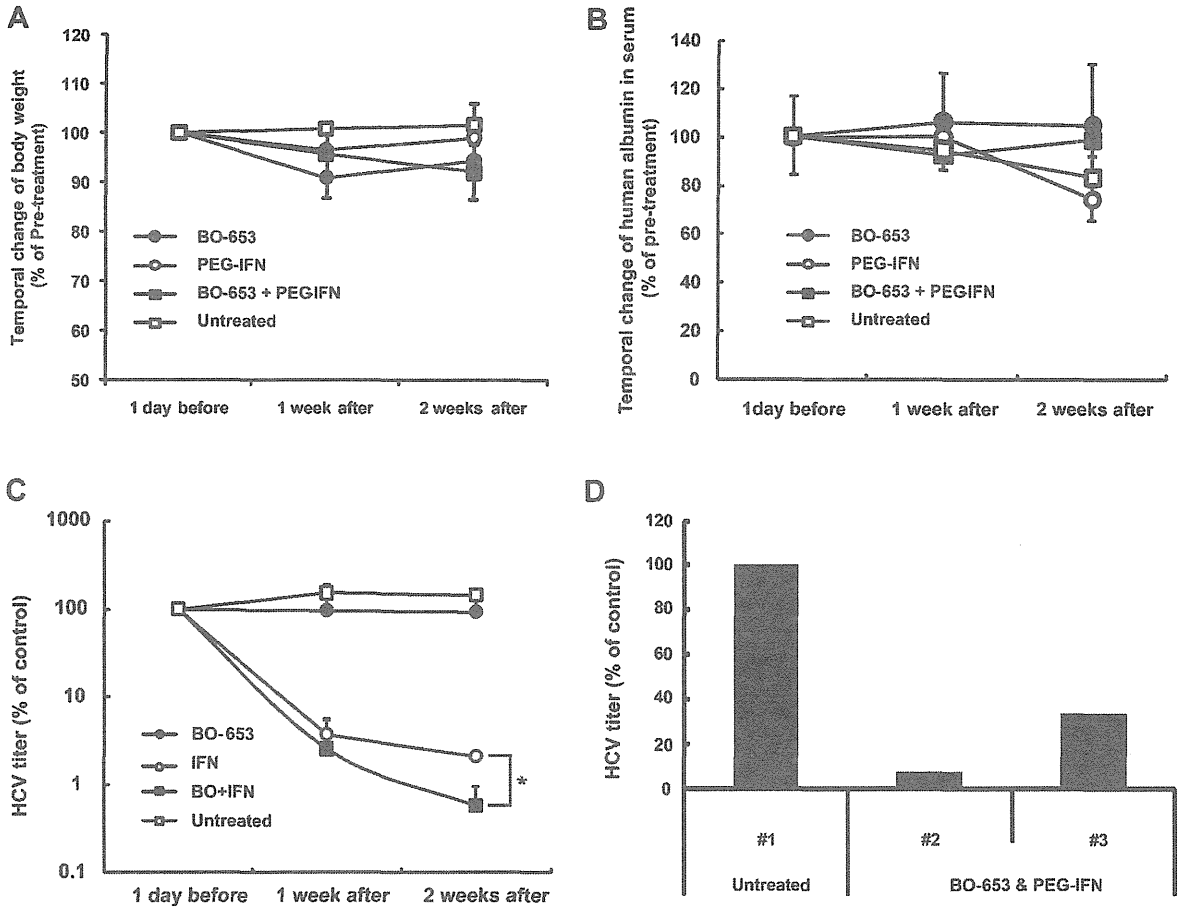



Fig. 4. Anti-HCV effect of BO-653 in chimeric mice infected with HCV. Temporal changes in the (A) body weight, (B) human albumin concentration in the serum, and (C) HCV RNA titer in the serum of chimeric mice infected with HCV after the indicated treatments. All groups included 3–5 mice, except for the untreated group (n = 2). The mice received once-daily oral gavage with 2,000 mg/kg BO-653 and/or twice-weekly subcutaneous injection with 30 μg/kg PEG-IFNα-2a (see Table I). The HCV RNA titers were normalized to the pre-treatment (Day-1) titer in the respective mouse. $^{*}P < 0.05$, PEG-IFN and BO-653 combination treatment (n = 5) was compared with PEG-IFN monotherapy (n = 3) of the same duration. Statistical analyses were performed using Student's *t*-test. D: The HCV RNA titer in the liver of chimeric mice treated with or without PEG-IFN and BO-653 combination treatment (untreated group, n = 1; combination treatment group, n = 2).

age [Shimoda et al., 1994; Bureau et al., 2001; Gong et al., 2001; Waris et al., 2005; Levent et al., 2006]. On the other hand, the influence of the intracellular redox state on HCV replication is controversial. A previous study demonstrated inhibition of HCV subgenomic replicon replication by lipid peroxidation and restoration of the replication by treatment with vitamin E [Huang et al., 2007]. A similar result was reported by Choi et al. [2004], who showed inhibition of HCV replication by exogenous hydrogen peroxide treatment. These results indicate that elevated levels of ROS, higher than those induced by natural HCV infection, can lead to inhibition of HCV replication. Yano et al. [2007] reported that several antioxidants, including vitamin E and β-carotene, enhance the replication of HCV genome-length replicons at relatively low concentrations (<10 μM). In contrast, the antioxidant pyrrolidine dithiocarbamate can suppress HCV replication via the inhibition of STAT-3 activation [Waris et al., 2005]. Notably, these studies have been performed primarily in in vitro systems, using either subgenomic replicon-containing cells, full-genome replicon-containing cells, or cells persistently infected with HCV. Therefore, clarification of the effect of antioxidants on HCV replication will require further work, including the use of in vivo models.

BO-653 (2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran), a lipophilic (hydrophobic) antioxidant, was investigated clinically for potential treatment of atherosclerosis and prevention of post-angioplasty restenosis [Cynshi et al., 1998; Meng, 2003]. Starting in November 2001, a phase II trial tested BO-653

for treatment of atherosclerosis and the prevention of post-angioplasty restenosis, but subsequent trials of the compound were suspended due to lack of efficacy. The present investigation of BO-653 as a potential anti-HCV drug reflects subsequent identification of the compound in random screening for antiviral activity.

In the present study, the anti-HCV activity of BO-653 was assessed in an in vitro system, and analyzed further in an in vivo system that used chimeric mice harboring human hepatocytes infected with HCV. In addition, the anti-HCV activity of BO-653 was compared with that of several other antioxidants, including hydrophilic and lipophilic compounds. Among these antioxidants, BO-653 had the strongest anti-HCV activity against HCV subgenomic replicons, followed by α-tocopherol. A previous study showed that the replication of a HCV replicon was increased in the presence of lower concentrations of vitamin E [Yano et al., 2007]. However, the results of the present study suggest that vitamin E exhibits anti-HCV activity when present in cell culture at concentrations exceeding 37 μM. Also, in chimeric mice infected persistently with HCV, the combination of PEG-IFN and BO-653 was more effective than PEG-IFN alone, although BO-653 alone did not have any anti-HCV activity in this mouse model. Monotherapy with DEBIO-025, a non-immunosuppressive cyclosporine A derivative and novel anti-HCV drug candidate, can decrease the viral load in patients with chronic HCV infection but not in chimeric mice infected with HCV [Inoue et al., 2007; Flisiak et al., 2009]; therefore, BO-653 monotherapy should be evaluated further in patients infected with HCV. Di Bona et al. [2006] reported impaired IFN-α signaling following oxidative stress, suggesting that oxidative stress causes resistance to the antiviral activity of IFN-α in patients infected with HCV. Taken together, these findings imply that lipophilic antioxidants such as BO-653 support the effects of PEG-IFN via antioxidant activity.

Several reports indicate that antioxidants, especially hydrophilic compounds, have antiviral activity [Docherty et al., 1999; Docherty et al., 2006; Ho et al., 2009; Geiler et al., 2010; Tian et al., 2010]. *N*-acetyl cysteine can inhibit the replication of H5N1 influenza A virus in a cell culture system [Geiler et al., 2010], and the antiviral effect of epigallocatechin gallate on enterovirus 71 may be associated with the modulation of the cellular redox state [Ho et al., 2009]. In contrast, the inhibitory effects of the lipophilic antioxidants on HCV RNA replication were stronger than those of the hydrophilic antioxidants. These lipophilic antioxidants inhibited the replication of HCV without cytotoxicity at concentrations higher than 37 μM. The role of the redox state of the lipid membrane on HCV RNA replication is of great interest, given reports of the association of RNA replication with lipid rafts [Shi et al., 2003; Sakamoto et al., 2005]. Further studies will be needed to elucidate the precise mechanism(s) underlying the suppression of HCV replication by BO-653.

In conclusion, the present study demonstrated that lipophilic antioxidants have stronger anti-HCV activity than hydrophilic antioxidants and that BO-653 has the strongest anti-HCV activity of the antioxidants tested. The combination of PEG-IFN and BO-653 was more effective than PEG-IFN alone in chimeric mice infected persistently with HCV. Further development of this compound would require additional considerations such as the route of administration and pharmacokinetics. Taken together, these findings provide insights into the influence of the intracellular redox state on the life-cycle of HCV.

## ACKNOWLEDGMENTS

We thank Isao Maruyama and Hiroshi Yokomichi of PhoenixBio Co., Ltd. for the maintenance of and technical assistance with the chimeric mice, and Haruyoshi Shirai, Isamu Kusanagi and Mayuko Matsuda of the Chugai Pharmaceutical Company for technical assistance with the biochemical assays.

## REFERENCES

Bureau C, Bernad J, Chaouche N, Orfila C, Beraud M, Gonindard C, Alric L, Vinel JP, Pipy B. 2001. Nonstructural 3 protein of hepatitis C virus triggers an oxidative burst in human monocytes via activation of NADPH oxidase. J Biol Chem 276: 23077–23083.

Choi J, Lee KJ, Zheng Y, Yamaga AK, Lai MM, Ou JH. 2004. Reactive oxygen species suppress hepatitis C virus RNA replication in human hepatoma cells. Hepatology 39:81–89.

Cynshi O, Kawabe Y, Suzuki T, Takashima Y, Kaise H, Nakamura M, Ohba Y, Kato Y, Tamura K, Hayasaka A, Higashida A, Sakaguchi H, Takeya M, Takahashi K, Inoue K, Noguchi N, Niki E, Kodama T. 1998. Antiatherogenic effects of the antioxidant BO-653 in three different animal models. Proc Natl Acad Sci USA 95:10123–10128.

Di Bona D, Cippitelli M, Fionda C, Camma C, Licata A, Santoni A, Craxi A. 2006. Oxidative stress inhibits IFN-alpha-induced antiviral gene expression by blocking the JAK-STAT pathway. J Hepatol 45:271–279.

Docherty JJ, Fu MM, Stiffler BS, Limperos RJ, Pokabla CM, DeLucia AL. 1999. Resveratrol inhibition of herpes simplex virus replication. Antiviral Res 43:145–155.

Docherty JJ, Sweet TJ, Bailey E, Faith SA, Booth T. 2006. Resveratrol inhibition of varicella-zoster virus replication in vitro. Antiviral Res 72:171–177.

Flisiak R, Feinman SV, Jablkowski M, Horban A, Kryczka W, Pawlowska M, Heathcote JE, Mazzella G, Vandelli C, Nicolas-Metral V, Grosgurin P, Liz JS, Scalfaro P, Porchet H, Crabbe R. 2009. The cyclophilin inhibitor Debio 025 combined with PEG IFNalpha2a significantly reduces viral load in treatment-naive hepatitis C patients. Hepatology 49:1460–1468.

Fontaine H, Pol S. 2011. Antiviral activity of telaprevir and boceprevir for the treatment of hepatitis C virus infection in treatment-experienced patients. Clin Res Hepatol Gastroenterol 35: S59–S63.

Geiler J, Michaelis M, Naczk P, Leutz A, Langer K, Doerr HW, Cinatl J, Jr. 2010. *N*-acetyl-L-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus. Biochem Pharmacol 79:413–420.

Gong G, Waris G, Tanveer R, Siddiqui A. 2001. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. Proc Natl Acad Sci USA 98:9599–9604.

Ho HY, Cheng ML, Weng SF, Leu YL, Chiu DT. 2009. Antiviral effect of epigallocatechin gallate on enterovirus 71. J Agric Food Chem 57:6140–6147.

Huang H, Chen Y, Ye J. 2007. Inhibition of hepatitis C virus replication by peroxidation of arachidonate and restoration by vitamin E. Proc Natl Acad Sci USA 104:18666–18670.

Inoue K, Umehara T, Ruegg UT, Yasui F, Watanabe T, Yasuda H, Dumont JM, Scalfaro P, Yoshiba M, Kohara M. 2007. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. Hepatology 45:921–928.

Kato J, Kobune M, Nakamura T, Kuroiwa G, Takada K, Takimoto R, Sato Y, Fujikawa K, Takahashi M, Takayama T, Ikeda T, Niitsu Y. 2001. Normalization of elevated hepatic 8-hydroxy-2'-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 61: 8697–8702.

Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. 2012. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. J Hepatol 56:78–84.

Lavanchy D. 2009. The global burden of hepatitis C. Liver Int 29:74–81.

Levent G, Ali A, Ahmet A, Polat EC, Aytac C, Ayse E, Ahmet S. 2006. Oxidative stress and antioxidant defense in patients with chronic hepatitis C patients before and after pegylated interferon alfa-2b plus ribavirin therapy. J Transl Med 4:25.

Meng CQ. 2003. BO-653. Chugai. Curr Opin Invest Drugs 4: 342–346.

Noguchi N, Iwaki Y, Takahashi M, Komuro E, Kato Y, Tamura K, Cynshi O, Kodama T, Niki E. 1997. 2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran: Design and evaluation as a novel radical-scavenging antioxidant against lipid peroxidation. Arch Biochem Biophys 342:236–243.

Nourooz-Zadeh J, Tajaddini-Sarmadi J, Wolff SP. 1994. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjuction with triphenylphosphine. Anal Biochem 220:403–409.

Sakamoto H, Okamoto K, Aoki M, Kato H, Katsume A, Ohta A, Tsukuda T, Shimma N, Aoki Y, Arisawa M, Kohara M, Sudoh M. 2005. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat Chem Biol 1:333–337.

Seronello S, Sheikh MY, Choi J. 2007. Redox regulation of hepatitis C in nonalcoholic and alcoholic liver. Free Radic Biol Med 43:869–882.

Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J Virol 77:4160–4168.

Shimoda R, Nagashima M, Sakamoto M, Yamaguchi N, Hirohashi S, Yokota J, Kasai H. 1994. Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. Cancer Res 54:3171–3172.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100–1104.

Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, Kawaguchi R, Tanaka S, Kohara M. 1999. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 116:636–642.

Tamura K, Kato Y, Ishikawa A, Kato Y, Himori M, Yoshida M, Takashima Y, Suzuki T, Kawabe Y, Cynshi O, Kodama T, Niki E, Shimizu M. 2003. Design and synthesis of 4,6-di-*tert*-butyl-2,3-dihydro-5-benzofuranols as a novel series of antiatherogenic antioxidants. J Med Chem 46:3083–3093.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109.

Tian Y, Jiang W, Gao N, Zhang J, Chen W, Fan D, Zhou D, An J. 2010. Inhibitory effects of glutathione on dengue virus production. Biochem Biophys Res Commun 397:420–424.

Waris G, Turkson J, Hassanein T, Siddiqui A. 2005. Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: Role of STAT-3 in HCV replication. J Virol 79:1569–1580.

Yano M, Ikeda M, Abe K, Dansako H, Ohkoshi S, Aoyagi Y, Kato N. 2007. Comprehensive analysis of the effects of ordinary nutrients on hepatitis C virus RNA replication in cell culture. Antimicrob Agents Chemother 51:2016–2027.

Zhu Z, Wilson AT, Mathahs MM, Wen F, Brown KE, Luxon BA, Schmidt WN. 2008. Heme oxygenase-1 suppresses hepatitis C virus replication and increases resistance of hepatocytes to oxidant injury. Hepatology 48:1430–1439.

Author's personal copy

Vaccine 31 (2013) 5968–5974

Contents lists available at ScienceDirect

Vaccine

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

# DNA vaccine expressing the non-structural proteins of hepatitis C virus diminishes the expression of HCV proteins in a mouse model

Takeshi Wada[a], Michinori Kohara[b], Yasuhiro Yasutomi[a,c,*]

[a] Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, Japan
[b] Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Setagaya-ku, Tokyo, Japan
[c] Division of Immunoregulation, Department of Molecular and Experimental Medicine, Mie University Graduate School of Medicine, Tsu, Mie, Japan

ARTICLE INFO

*Article history:*
Received 20 June 2013
Received in revised form 3 September 2013
Accepted 8 October 2013
Available online 19 October 2013

*Keywords:*
Hepatitis C
DNA vaccine
Mouse model

ABSTRACT

Most of the people infected with hepatitis C virus (HCV) develop chronic hepatitis, which in some cases progresses to cirrhosis and ultimately to hepatocellular carcinoma. Although various immunotherapies against the progressive disease status of HCV infection have been studied, a preventive or therapeutic vaccine against this pathogen is still not available. In this study, we constructed a DNA vaccine expressing an HCV structural protein (CN2), non-structural protein (N25) or the empty plasmid DNA as a control and evaluated their efficacy as a candidate HCV vaccine in C57BL/6 and novel genetically modified HCV infection model (HCV-Tg) mice. Strong cellular immune responses to several HCV structural and non-structural proteins, characterized by cytotoxicity and interferon-gamma (IFN-γ) production, were observed in CN2 or N25 DNA vaccine-immunized C57BL/6 mice but not in empty plasmid DNA-administered mice. The therapeutic effects of these DNA vaccines were also examined in HCV-Tg mice that conditionally express HCV proteins in their liver. Though a reduction in cellular immune responses was observed in HCV-Tg mice, there was a significant decrease in the expression of HCV protein in mice administered the N25 DNA vaccine but not in mice administered the empty plasmid DNA. Moreover, both $CD8^+$ and $CD4^+$ T cells were required for the decrease of HCV protein in the liver. We found that the N25 DNA vaccine improved pathological changes in the liver compared to the empty plasmid DNA. Thus, these DNA vaccines, especially that expressing the non-structural protein gene, may be an alternative approach for treatment of individuals chronically infected with HCV.

© 2013 Elsevier Ltd. All rights reserved.

## 1. Introduction

Infection with hepatitis C virus (HCV) can lead to chronic hepatitis and ultimately death through liver failure or onset of carcinoma [1]. Although various immunotherapies against the progressive disease status of HCV infection have been studied, a preventive or therapeutic vaccine against this pathogen is still not available. Therefore, the development of effective vaccines, especially therapeutic vaccines, is needed to control the progressive disease of HCV.

Acute infections are characterized by high frequencies of HCV-specific CD8 and CD4 T cell responses that can persist for a long time after the clearance of viremia and recovery from the infection [2,3]. On the other hand, individuals who remain chronically infected have weak or undetectable cellular immune responses to HCV antigens [4–6]. It has been reported that HCV evades immune responses by suppression of the activity of effector T cells and establishes persistent infection [7,8]. Therefore, activation of cellular immune responses to HCV might lead to improvement of the pathological condition caused by HCV.

The use of a DNA vaccine is an attractive approach for generating antigen-specific immunity to various pathogens because of its stability and simplicity of delivery. Many studies on DNA vaccines against HCV infection have been performed in mouse systems [9–22]. On the other hand, there have been a few studies in which the therapeutic effect of DNA vaccines was investigated in chronic HCV carrier model mice [23–25]. Furthermore, a conventional transgenic mouse model was used as a chronic HCV carrier status in those studies. Unfortunately, those transgenic mice were immunotolerant to their expressed HCV protein, and the immune status of the mice was therefore different from that of patients with chronic HCV infection.

To overcome the limitation of these mice, we used novel genetically modified ($CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$) mice that conditionally

* Corresponding author at: Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan. Tel.: +81 29 837 2053; fax: +81 29 837 2053.
*E-mail addresses:* yasutomi@nibio.go.jp, yasutomi@doc.medic.mie-u.ac.jp (Y. Yasutomi).

0264-410X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2013.10.037

Author's personal copy



express HCV cDNA, using Mx promoter-driven Cre recombinase with poly(I)·poly(C) induction. These mice expressed the HCV core protein consistently for at least 600 days and developed chronic active hepatitis, steatosis, lipid deposition, and hepatocellular carcinoma [26,27]. Since these pathological findings in the transgenic mice are very similar to those in humans with chronic HCV infection [27], it was thought that this mouse model of HCV would be useful for analyzing the immune responses to chronic hepatitis.

In this study, we constructed a DNA vaccine expressing an HCV structural protein (CN2) and non-structural protein (N25) and evaluated the efficacy of the vaccines as a candidate HCV vaccine in novel transgenic mice that conditionally express HCV cDNA.

## 2. Materials and methods

### 2.1. Mice

C57BL/6 mice were purchased from CLEA Japan. HCV-Tg mice CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ were previously described [27]. Mice used in this study ($n$ = 460) were 6-week-old males at the start of the experiment. HCV-Tg mice were injected intraperitoneally (i.p.) with 300 μg polyinosinic acid–polycytidylic acid (poly(I)·poly(c)) (GE Healthcare) three times at 48-h intervals to induce the expression of HCV protein 3 months before using the mice for experiments. Injection of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ or RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice with poly(I)·poly(C) induces IFN production and the expression of CN2-29 or RzCN5-15 gene products in hepatocytes, hematopoietic cells (mainly in Kupffer cells and lymphocytes), and spleens but not in most other tissues [26]. All animals were cared for according to ethical guidelines approved by the institutional Animal Care and Use Committee of the National Institute of Biomedical Innovation.

### 2.2. Cells, virus, peptide

EL-4 transformants that expressed E2 (EL-4/E2), NS2 (EL-4/NS2) or NS3/4A (EL-4/NS3/4A) of HCV protein and LC16m8, a highly attenuated strain of vaccinia virus (VV), and a recombinant vaccinia virus (rVV) that encoded mainly structural proteins (core/E1/E2/NS2; amino acids (aa) 1–1320) (rVV-CN2) were previously described [27].

HCV NS3$_{1629–1637}$ peptide (GAVQNEITL) was synthesized by Toray Research Center (Tokyo).

### 2.3. DNA immunization

For DNA immunization, two different plasmids, CN2 and N25 expressing the HCV core/E1/E2/NS2 (aa 1–1320) and E2/NS2/3/4/5 (aa 542–3010) polyproteins under the control of the CAG promoter were constructed (Fig. 1). The PCR product of HCV cDNA from a type 1b strain (R6) cDNA containing the plasmid vector pBMSF7C [27] as an *Xba*I/*Xho*I fragment was cloned into a CAG expression plasmid, pCAGGS [28]. All plasmid DNAs were purified with an endotoxin-free plasmid extraction kit (Qiagen).

Mice were intramuscularly injected with 100 μg of the plasmid DNA in 25 μl PBS, and then the site of inoculation was immediately given an electric pulse by an Electric Square Porator (T820; BTX) as previously described [29]. One group of mice was boosted with the same amount of DNA at 2 weeks for ELISPOT and cytotoxicity assay. Another group of mice were also boosted with the same amount of DNA at 2 and 4 weeks for histopathological examination.

### 2.4. ELISPOT assay

IFN-γ ELISPOT assay was performed according to the manufacturer's protocol (Mabtech). Briefly, total spleen cells

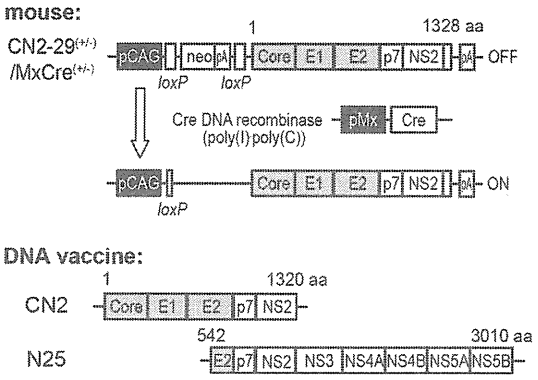



**Fig. 1.** Generation of HCV DNA vaccines. HCV gene structure in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and schematic diagram of plasmid constructs expressing an HCV structural protein (CN2) and a non-structural protein (N25). CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice conditionally express partial (nucleotides 294–3435, including the viral genes that encode the core, E1, E2, and NS2 proteins) of HCV genotype 1b cDNA. The expression of HCV proteins is regulated by the Cre/loxP switching expression system. pCAG, CAG promoter; pMx, Mx1 promoter; neo, neomycin-resistant gene; pA, poly A signal.

(1 × 10$^5$ cells/well), CD8$^+$ T cells or CD4$^+$ T cells (2 × 10$^5$ cells/well) were incubated with mitomycin C-treated EL-4 cells expressing HCV protein (1 × 10$^4$ cells/well) or with mitomycin C-treated syngenic splenocytes infected with an rVV carrying the cDNA of HCV Core-NS2 antigens (4 × 10$^4$ cells/well) or HCV NS3$_{1629–1637}$ peptide (0.1 μg/ml) at 37 °C for 48 h in a 96-well plate coated with anti-mouse IFN-γ mAb. Cells were removed and the plate was stained with biotinylated anti-mouse IFN-γ mAb, streptavidin-HRP, and a DAB Substrate kit for peroxidase (Vector Laboratories). Test wells were assayed in duplicate and antigen-specific T cells was calculated after subtracting the mean number of spots obtained in the absence of stimulation.

### 2.5. Generation of CTL effector cells and cytotoxicity assay

Spleen cells (1 × 10$^7$ cells) were co-cultured with mitomycin C-treated EL-4/NS2 cells (2 × 10$^6$ cells). The effector cells generated were harvested after 5 days of culture. $^{51}$Cr-labeled target cells (EL-4/NS2) were incubated for 4 h with effector cells. Specific lysis was calculated as previously described [29].

### 2.6. Quantification of HCV core proteins

HCV core protein concentrations were determined with ELISA kit (Ortho-Clinical Diagnostics) as previously described [27]. The HCV protein concentration in the tissue samples was divided by the total protein concentration and expressed as pg/mg of total protein.

### 2.7. Histopathological examination

Liver tissues were fixed in 10% phosphate-buffered formalin. Sections of paraffin-embedded tissue were cut at 4 μm in thickness and stained with hematoxylin and eosin (H&E).

### 2.8. Adoptive transfer of cells

For adoptive transfer experiments, spleen cells were isolated from C57BL/6 mice that had been immunized twice with N25 DNA vaccine. CD8$^+$, CD4$^+$, and CD8$^-$CD4$^-$ cells were prepared by using CD8a (Ly-2) and CD4 (L3T4) MicroBeads according to manufacturer's instructions (Miltenyi Biotec). Whole spleen cells (1 × 10$^8$ cells) or purified CD8$^+$, CD4$^+$ or CD8$^-$CD4$^-$ cells

Author's personal copy



($1 \times 10^7$ cells) were adoptively transferred into mice by i.p. injection. One week after the transfer, the recipient mice were sacrificed and tissues were analyzed.

### 2.9. DC immunization

Isolation and purification of $CD11c^+$ cells from spleens from mice were performed by using CD11c MicroBeads (Miltenyi Biotec). $CD11c^+$ cells were then pulsed with HCV $NS3_{1629-1637}$ peptide for 5 h, washed with RPMI-1640 medium three times, and injected into recipient mice via footpads ($2 \times 10^5$ cells). Two weeks after the transfer, spleen cells were isolated from the recipient mice and examined by the ELISPOT assay.

### 2.10. Statistics

Statistical significance ($P < 0.05$) was determined by 2-tailed Student's *t* test or ANOVA followed by Ryan's test.

## 3. Results

### 3.1. Immunization of C57BL/6 mice with DNA vaccine induces strong HCV-specific cellular immune responses

To analyze the cellular immune responses induced by the DNA vaccine, C57BL/6 mice were immunized twice with the DNA vaccine at a 2-week interval. Strong cellular immune responses to several HCV structural and non-structural proteins characterized by IFN-γ production (Fig. 2A) and cytotoxicity (Fig. 2B) were observed in CN2 or N25 DNA vaccine-immunized mice but not in mice injected with the empty plasmid DNA. We next assessed the activity of $CD8^+$ and $CD4^+$ T cells in mice immunized with the DNA vaccine. Purified $CD8^+$ or $CD4^+$ T cells from the spleen were re-stimulated in vitro with syngenic splenocytes infected with an rVV carrying the cDNA of HCV Core-NS2. Significant IFN-γ production in $CD8^+$ and $CD4^+$ T cells was observed in CN2 and N25 DNA-immunized mice but not in mice injected with the empty plasmid DNA, and the responses of $CD8^+$ T cells were much stronger than those of $CD4^+$ T cells (Fig. 2C).

### 3.2. Immunization with N25 DNA vaccine showed therapeutic effects in the liver of HCV transgenic mouse

We next assessed the expression of HCV protein in the liver after immunization with DNA vaccine using HCV-Tg mice. Three months after induction of HCV protein by poly(I)·poly(C) injection, $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice were immunized twice with the DNA vaccine at a 2-week interval. Immunization of mice with the N25 DNA vaccine resulted in reduced expression of HCV protein in the liver of $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice compared with the expression in mice injected with the empty plasmid DNA (Fig. 3A). Pathological changes in the liver after immunization with the DNA vaccine were also examined. Pathological changes, including swelling of hepatocytes and abnormal architecture of liver cell cords were observed in both empty plasmid DNA-immunized or CN2 DNA-immunized mice. However, these pathological changes in the liver were improved by the N25 DNA vaccine (Fig. 3B). These results suggested that the N25 DNA vaccine has a potential as a therapeutic vaccine for HCV infection.

### 3.3. $CD8^+$ and $CD4^+$ T cells are required for the decrease of HCV protein in the liver

We next searched for effector cells having the ability to reduce the expression of HCV protein in the N25 DNA-immunized mice. Whole spleen cells, $CD8^+$ T cells and $CD4^+$ T cells were obtained from spleens of C57BL/6 mice immunized with the N25 DNA vaccine. These cells were adoptively transferred into $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice that expressed HCV proteins. The adoptive transfer of unfractionated spleen cells, $CD8^+$ T cells or $CD4^+$ T cells decreased the expression HCV protein in the liver (Fig. 4). These results indicated that both $CD8^+$ and $CD4^+$ T cells were required for the decrease of HCV protein in the liver.

### 3.4. Immunization of $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice with DNA vaccine failed to induce strong HCV-specific cellular immune responses

Cellular immune responses induced by the DNA vaccine in $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice were also assessed. Unlike in WT (C57BL/6) mice, cellular immune responses to several HCV structural and non-structural proteins were reduced in the N25 DNA vaccine-immunized HCV-Tg ($CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$) mice (Fig. 5A). CD4 or CD8 T cell responses to HCV antigens were abolished in the CN2 DNA vaccine-immunized HCV-Tg mice. On the other hand, the N25 DNA vaccine elicited HCV-specific CD4 and CD8 T cell responses in HCV-Tg mice. The levels of CD4 T cell responses were equivalent to those in WT mice; however, CD8 T cell responses were weak compared with those in WT mice (Fig. 5B). These reductions of cellular immune responses were also observed in another strain of HCV-Tg ($RzCN5\text{-}15^{(+/-)}/MxCre^{(+/-)}$) mice that possessed the full-length cDNA of HCV (Fig. 5C and D).

### 3.5. Ability of DCs to induce HCV-specific CD8 T cells in HCV transgenic mice was not impaired

In the present study, the activity of effector cells induced by the DNA vaccine was thought to be suppressed in HCV-Tg mice. To explore these inhibitory effects on HCV-specific cellular immune responses by the DNA vaccine in HCV-Tg mice, adoptive transfer of effector cells was performed. Spleen cells were isolated from C57BL/6 mice that had been immunized with the N25 DNA vaccine. When these cells were transferred into C57BL/6 or HCV-Tg mice, nearly equal levels of CTL activities were detected in the recipient mice (Fig. 6A), suggesting that immunosuppressive mechanisms of IFN-γ production by mature CTLs did not exist in HCV-Tg mice.

Dendritic cells (DCs) play a critical role in the induction of immune responses by DNA vaccination [30]. Moreover, several studies have demonstrated that HCV impaired the function of DCs [31–34]. To assess DC function in HCV-Tg mice, DCs were freshly purified from spleens of WT and HCV-Tg ($RzCN5\text{-}15^{(+/-)}/MxCre^{(+/-)}$) mice, loaded with NS3 peptide, and transferred into WT and HCV-Tg mice. Two weeks later, the functional status of CD8 T cells in the recipient mice was evaluated. DC functions to induce HCV-specific CD8 T cell responses were not different in WT and HCV-Tg mice; however, NS3-specifc CD8 T cell responses in HCV-Tg mice that had been injected with DCs of either WT or HCV-Tg mice were much weaker than those in WT mice (Fig. 6B). These results indicated that the ability of DCs to induce HCV-specific CD8 T cells in HCV-Tg mice was not impaired.

## 4. Discussion

It has been reported that DNA vaccines elicited strong and long-lasting humoral and cell-mediated immune responses against pathogenic agents such as HBV, HIV, tuberculosis and malaria and that they had many advantages over traditional vaccines

Author's personal copy



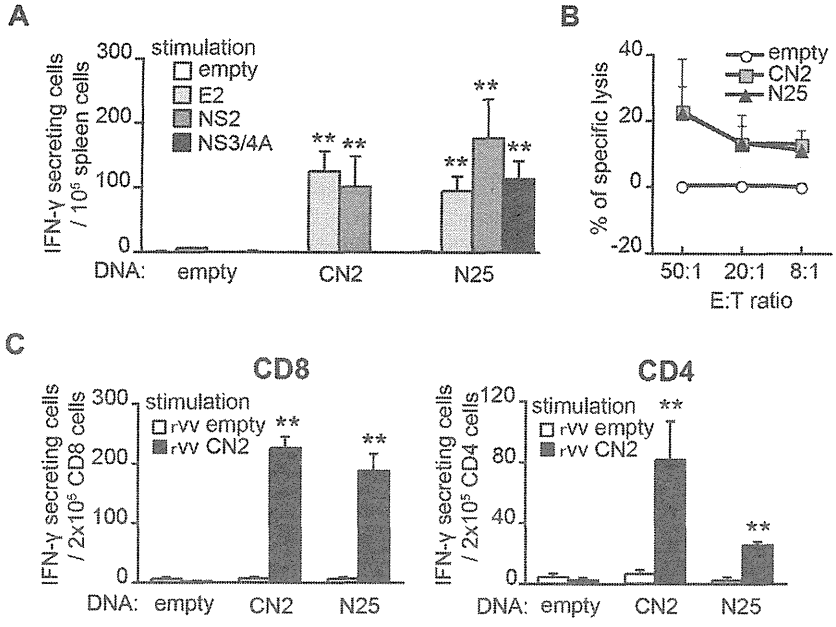



**Fig. 2.** Immunization of C57BL/6 mice with DNA vaccine induced strong HCV-specific cellular immune responses. C57BL/6 mice were immunized twice with the DNA vaccine at an interval of 2 weeks. At 2 weeks after the final immunization, spleen cells were isolated and examined by the IFN-γ ELISPOT assay using EL-4 cells expressing each of the HCV proteins (A), and $^{51}$Cr release assay using EL-4 target cells expressing HCV NS2 protein at indicated ratios (B). CD8 and CD4 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using syngenic splenocytes infected with a recombinant vaccinia virus (rVV) carrying the cDNA of HCV Core-NS2 (rVV CN2) (C). Data are shown as means ± SEM, $n = 3$ (A and B); $n = 5$ (C). Data shown in A–C are representative of at least 3 repeated experiments. $^{**}P < 0.01$ vs stimulated with empty vector-transfected cells.

that use live-attenuated or killed pathogens, proteins, or synthetic peptides [35]. Moreover, DNA vaccine against HIV and malaria showed high levels of safety and good tolerability profile in human clinical trials [36,37]. DNA vaccines can induce cytoplasmic expression of encoded antigens (Ags) that more closely resemble native conformation of pathogens than can immunization with proteins. A DNA vaccine induced immunity against encoded Ags, whereas cytoplasmic expression using a viral vector delivery system induced immune responses to not only vaccine Ags but also vector organisms [38]. Infection with a vaccine vector virus might be an obstacle by induction of unneeded immune responses to vectors as side effects [38]. A DNA vaccine might be

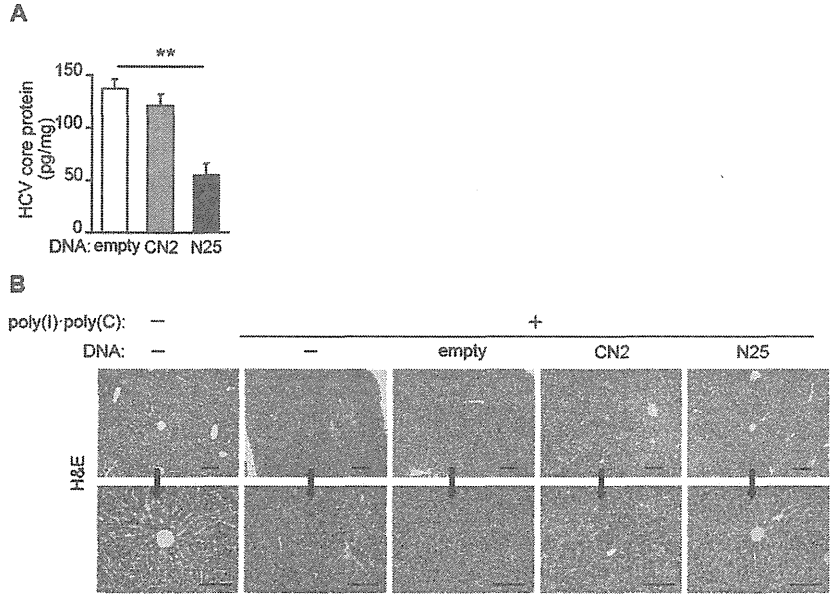



**Fig. 3.** Immunization with N25 DNA vaccine showed therapeutic effects in the liver of HCV transgenic mice. (A) Three months after induction of HCV protein by poly(I)·poly(C) injection, CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice were immunized twice with the DNA vaccine at an interval of 2 weeks. At 2 weeks after the final immunization, livers were isolated and examined for HCV core protein expression. The core protein expression in each experimental group is shown. Data are shown as means ± SEM, $n = 5$ each. $^{**}P < 0.01$. (B) Liver sections from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice before (–) and after poly(I)·poly(C) injection (+). Three months after induction of HCV protein, mice were immunized three times with the DNA vaccine at intervals of 2 weeks. At 4 weeks after the final immunization, livers were isolated and stained with hematoxylin and eosin (H&E). Scale bars: 100 μm. Data shown in A and B are representative of at least 3 repeated experiments.

Author's personal copy



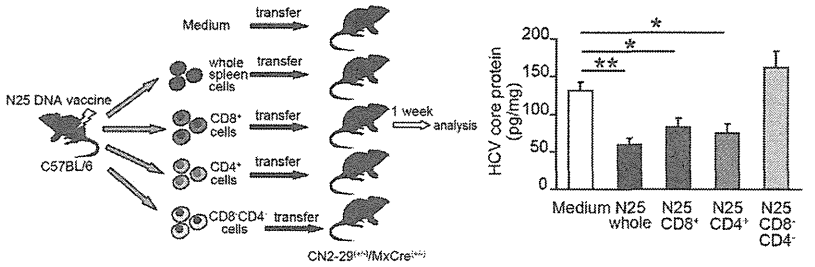



**Fig. 4.** CD8$^+$ and CD4$^+$ T cells are required for the decrease of HCV protein in the liver. Whole spleen cells, purified CD8$^+$ T cells or CD4$^+$ T cells from C57BL/6 mice that had been immunized twice with the N25 DNA vaccine at an interval of 2 weeks were adoptively transferred into CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, and livers were isolated from the recipient mice 1 week later. The core protein expression in each experimental group is shown. Data are shown as means ± SEM, $n = 5$ each. Data are representative of 3 repeated experiments. $^{**}P < 0.01$; $^{*}P < 0.05$.

one of the best candidates for a therapeutic vaccine for infectious disease.

In the case of viral persistence resulting in chronic disease, HCV-specific CD4 and CD8 T cell responses appeared to be diminished [39]. Various hypotheses have been proposed to explain the dysfunctional T cell response in HCV infection, including viral escape mutations, exhaustion of the T cell compartment, induction of regulatory T cells and impaired DC function [40]. DCs play an important role in triggering the primary antiviral immune responses. Therefore, modulation of the function of DCs has been suggested as one of the mechanisms used by persistent viruses to evade the immune system. Several studies have demonstrated impairment of DC function in HCV-infected individuals [32,34]. On the other hand, some studies have shown that HCV proteins did not impair DC function [41–44]. In the present study, the ability of DCs to induce HCV-specific CD8$^+$ T cells in HCV transgenic mice was not impaired (Fig. 6B), therefore, another mechanism that suppressed the generation of HCV-specific CD8 T cells might exist in this mouse model.

HCV conventional transgenic mice have been used as surrogate models for chronic HCV infection in humans. In a previous study, when FVB/n Tg mice expressing HCV structural proteins (core, E1 and E2) and WT FVB/n mice were intramuscularly immunized with plasmid DNA encoding core/E1/E2, CTL activities against E2 were detected in WT mice but not in Tg mice [23], and either CD4 or CD8 T cell responses against the envelope proteins appeared to be

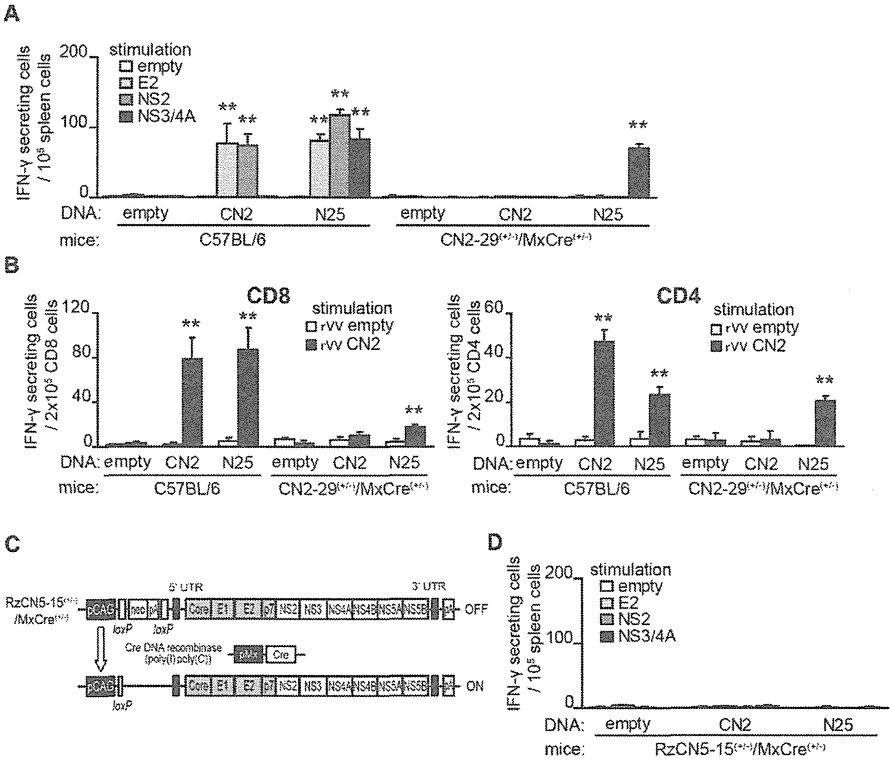



**Fig. 5.** Immunization of HCV-Tg mice with DNA vaccine failed to induce strong HCV-specific cellular immune responses. CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice were immunized with the DNA vaccine as in the experiment for which results are shown in Fig. 3A. (A and D) Spleen cells were isolated and examined by the IFN-γ ELISPOT assay using EL-4 cells expressing each of the HCV proteins. (B) CD8 and CD4 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using syngenic splenocytes infected with a recombinant vaccinia virus (rVV) carrying the cDNA of HCV Core-NS2 (rVV CN2). (C) HCV gene structure in RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice. RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice conditionally express full length of HCV genotype 1b cDNA. Data are shown as means ± SEM, $n = 3$ (A, B and D). Data shown in A, B and D are representative of at least 3 repeated experiments. $^{**}P < 0.01$ vs stimulated with empty vector-transfected cells.

Author's personal copy



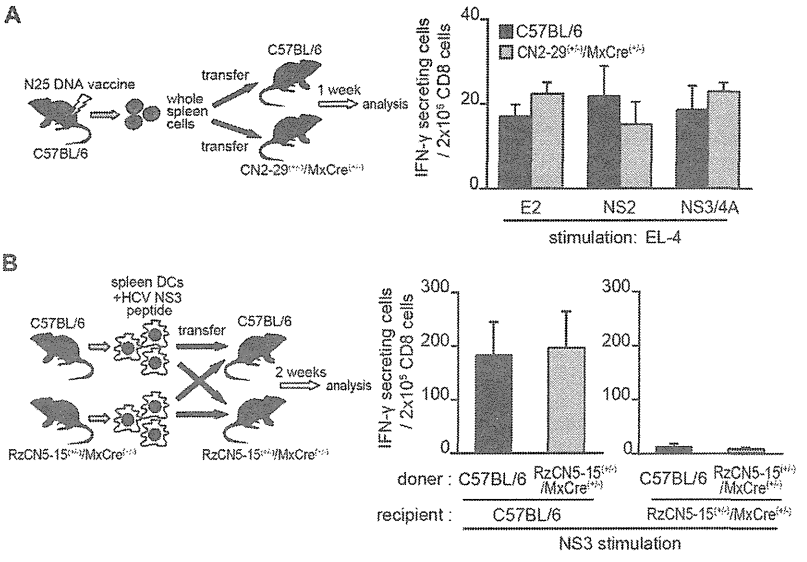



**Fig. 6.** Ability of DCs to induce HCV-specific CD8⁺ T cells in HCV transgenic mice was not impaired. (A) Whole spleen cells ($1 \times 10^8$ cells) from C57BL/6 mice that had been immunized twice with the N25 DNA vaccine at an interval of 2 weeks were adoptively transferred into C57BL/6 or CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, and spleen cells were isolated from the recipient mice 1 week later. CD8 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using EL-4 cells expressing each of the HCV proteins. (B) HCV NS3 peptide-pulsed spleen DCs ($2 \times 10^5$ cells) derived from C57BL/6 or RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice were adoptively transferred to C57BL/6 or RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice. Two weeks later, spleen cells were isolated from the recipient mice. CD8 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using HCV NS3 peptide. Data are shown as means ± SEM, $n = 3$ each. Data shown in A and B are representative of at least 3 repeated experiments.

immunologically tolerant and could not overcome this tolerance by DNA immunization in the Tg mice [23]. These observation are consistent with our findings that cellular immune responses to several HCV structural and non-structural proteins were abolished in the CN2 DNA vaccine-immunized HCV-Tg (CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$) mice, unlike in WT mice (Fig. 5A and B). On the other hand, the N25 DNA vaccine induced HCV-specific CD4 and CD8 T cell responses in HCV-Tg mice, and the level of CD4 T cell responses were equivalent to those in WT mice. However, the activities of CD8 T cell responses were not high compared with those in WT mice (Fig. 5B). This difference in the efficacy of CN2 and that of N25 may be caused by the difference of the expression site of HCV construct. The CN2 DNA construct contained the HCV core protein-encoding region. Several studies have demonstrated that the HCV core protein has the immunomodulatory function of suppressing host immune responses [45]. It has been reported that HCV core protein could suppress host immune responses by inhibiting antiviral CTL activity in mice infected with recombinant vaccinia virus expressing the core protein [7], and it has also been reported that Tg mice in which the HCV core protein was expressed in T cells under the control of the CD2 promoter showed significantly reduced T cell responses, including the production of IFN-γ and IL-2, compared to those in non-Tg mice [46]. The N25 DNA construct did not contain the HCV core protein-encoding region, and the N25 DNA vaccine may therefore not be susceptible to these immunosuppressive factors derived from DNA vaccine's own self.

Immunization of HCV-Tg mice with N25 DNA vaccine resulted in improvement in pathological changes in the liver. Sekiguchi et al. [27] reported that immunization with recombinant vaccinia virus strain (rVV-N25), which encoded the same non-structural HCV proteins as those encoded by N25 DNA vaccine in this study, alleviated the symptoms of pathological changes in the liver of HCV-Tg mice. They showed that TNF-α and IL-6 are responsible for the pathological symptoms in HCV-Tg mice and that immunization with rVV-N25 rapidly suppressed the inflammatory responses. The mechanism of action of N25 DNA vaccine may be similar to that of rVV-N25, though further examination is required.

In the present study, using novel HCV conditional transgenic mice to overcome the problem of immune tolerance in HCV conventional transgenic mice, the efficacy of a candidate HCV vaccine was evaluated. The use of DNA vaccines, especially the N25 DNA vaccine, expressing a non-structural protein gene resulted in reduced expression of HCV protein and improved pathological changes in the liver. Our findings may provide new avenues toward the development of an alternative approach for the treatment of individuals chronically infected with HCV, although further studies are needed.

## Acknowledgments

*Financial support:* This work was supported by Health Science Research Grants from the Ministry of Health, Labor and Welfare of Japan and the Ministry of Education, Culture, Sports, Science and Technology of Japan. *Conflict of interest statement*: None declared.

## References

[1] Alter MJ. Epidemiology of hepatitis C in the West. Semin Liver Dis 1995;15:5–14.
[2] Grakoui A, Shoukry NH, Woollard DJ, Han JH, Hanson HL, Ghrayeb J, et al. HCV persistence and immune evasion in the absence of memory T cell help. Science 2003;302:659–62.
[3] Diepolder HM, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, et al. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 1995;346:1006–7.
[4] Chang KM, Thimme R, Melpolder JJ, Oldach D, Pemberton J, Moorhead-Loudis J, et al. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. Hepatology 2001;33:267–76.
[5] Koziel MJ, Dudley D, Wong JT, Dienstag J, Houghton M, Ralston R, et al. Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis. J Immunol 1992;149:3339–44.
[6] Rehermann B, Chang KM, McHutchison JG, Kokka R, Houghton M, Chisari FV. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection. J Clin Invest 1996;98:1432–40.
[7] Large MK, Kittlesen DJ, Hahn YS. Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. J Immunol 1999;162:931–8.
[8] Koziel MJ. Cellular immune responses against hepatitis C virus. Clin Infect Dis 2005;41(Suppl. 1):S25–31.

Author's personal copy



[9] Lagging LM, Meyer K, Hoft D, Houghton M, Belshe RB, Ray R. Immune responses to plasmid DNA encoding the hepatitis C virus core protein. J Virol 1995;69:5859–63.
[10] Pancholi P, Liu Q, Tricoche N, Zhang P, Perkus ME, Prince AM. DNA prime-canarypox boost with polycistronic hepatitis C virus (HCV) genes generates potent immune responses to HCV structural and nonstructural proteins. J Infect Dis 2000;182:18–27.
[11] Youn JW, Park SH, Cho JH, Sung YC. Optimal induction of T-cell responses against hepatitis C virus E2 by antigen engineering in DNA immunization. J Virol 2003;77:11596–602.
[12] Li YP, Kang HN, Babiuk LA, Liu Q. Elicitation of strong immune responses by a DNA vaccine expressing a secreted form of hepatitis C virus envelope protein E2 in murine and porcine animal models. World J Gastroenterol 2006;12:7126–35.
[13] 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;26:6225–31.
[14] Arashkia A, Roohvand F, Memarnejadian A, Aghasadeghi MR, Rafati S. Construction of HCV-polytope vaccine candidates harbouring immune-enhancer sequences and primary evaluation of their immunogenicity in BALB/c mice. Virus Genes 2010;40:44–52.
[15] Masalova OV, Lesnova EI, Pichugin AV, Melnikova TM, Grabovetsky VV, Petrakova NV, et al. The successful immune response against hepatitis C nonstructural protein 5A (NS5A) requires heterologous DNA/protein immunization. Vaccine 2010;28:1987–96.
[16] Encke J, zu Putlitz J, Geissler M, Wands JR. Genetic immunization generates cellular and humoral immune responses against the nonstructural proteins of the hepatitis C virus in a murine model. J Immunol 1998;161:4917–23.
[17] Arichi T, Saito T, Major ME, Belyakov IM, Shirai M, Engelhard VH, et al. Prophylactic DNA vaccine for hepatitis C virus (HCV) infection: HCV-specific cytotoxic T lymphocyte induction and protection from HCV-recombinant vaccinia infection in an HLA-A2.1 transgenic mouse model. Proc Natl Acad Sci U S A 2000;97:297–302.
[18] Frelin L, Alheim M, Chen A, Soderholm J, Rozell B, Barnfield C, et al. Low dose and gene gun immunization with a hepatitis C virus nonstructural (NS) 3 DNA-based vaccine containing NS4A inhibit NS3/4A-expressing tumors in vivo. Gene Ther 2003;10:686–99.
[19] Simon BE, Cornell KA, Clark TR, Chou S, Rosen HR, Barry RA. DNA vaccination protects mice against challenge with *Listeria monocytogenes* expressing the hepatitis C virus NS3 protein. Infect Immun 2003;71:6372–80.
[20] Ahlén G, Nystrom J, Pult I, Frelin L, Hultgren C, Sallberg M. In vivo clearance of hepatitis C virus nonstructural 3/4A-expressing hepatocytes by DNA vaccine-primed cytotoxic T lymphocytes. J Infect Dis 2005;192:2112–6.
[21] Encke J, Bernardin J, Geib J, Barbakadze G, Bujdoso R, Stremmel W. Genetic vaccination with Flt3-L and GM-CSF as adjuvants: enhancement of cellular and humoral immune responses that results in protective immunity in a murine model of hepatitis C virus infection. World J Gastroenterol 2006;12:7118–25.
[22] Lang Kuhs KA, Toporovski R, Ginsberg AA, Shedlock DJ, Weiner DB. Induction of intrahepatic HCV NS4B, NS5A and NS5B-specific cellular immune responses following peripheral immunization. PLoS ONE 2012;7:e52165.
[23] Satoi J, Murata K, Lechmann M, Manickan E, Zhang Z, Wedemeyer H, et al. Genetic immunization of wild-type and hepatitis C virus transgenic mice reveals a hierarchy of cellular immune response and tolerance induction against hepatitis C virus structural proteins. J Virol 2001;75:12121–7.
[24] Encke J, Geissler M, Stremmel W, Wands JR. DNA-based immunization breaks tolerance in a hepatitis C virus transgenic mouse model. Hum Vaccine 2006;2:78–83.
[25] Holmström F, Pasetto A, Nahr V, Brass A, Kriegs M, Hildt E, et al. A synthetic codon-optimized hepatitis C virus nonstructural 5A DNA vaccine primes polyfunctional CD8+ T cell responses in wild-type and NS5A-transgenic mice. J Immunol 2013;190:1113–24.
[26] Kimura K, Kohara M. An experimental mouse model for hepatitis C virus. Exp Anim 2011;60:93–100.
[27] 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:e51656.
[28] Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 1991;108:193–9.
[29] Nishimura Y, Kamei A, Uno-Furuta S, Tamaki S, Kim G, Adachi Y, et al. A single immunization with a plasmid encoding hepatitis C virus (HCV) structural proteins under the elongation factor 1-alpha promoter elicits HCV-specific cytotoxic T-lymphocytes (CTL). Vaccine 1999;18:675–80.
[30] You Z, Huang X, Hester J, Toh HC, Chen SY. Targeting dendritic cells to enhance DNA vaccine potency. Cancer Res 2001;61:3704–11.
[31] Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology 2001;120:512–24.
[32] Auffermann-Gretzinger S, Keeffe EB, Levy S. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. Blood 2001;97:3171–6.
[33] Kanto T, Hayashi N, Takehara T, Tatsumi T, Kuzushita N, Ito A, et al. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol 1999;162:5584–91.
[34] Kanto T, Inoue M, Miyatake H, Sato A, Sakakibara M, Yakushijin T, et al. Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection. J Infect Dis 2004;190:1919–26.
[35] Shedlock DJ, Weiner DB. DNA vaccination: antigen presentation and the induction of immunity. J Leukoc Biol 2000;68:793–806.
[36] Kibuuka H, Kimutai R, Maboko L, Sawe F, Schunk MS, Kroidl A, et al. A phase 1/2 study of a multiclade HIV-1 DNA plasmid prime and recombinant adenovirus serotype 5 boost vaccine in HIV-Uninfected East Africans (RV 172). J Infect Dis 2010;201:600–7.
[37] Moorthy VS, Imoukhuede EB, Milligan P, Bojang K, Keating S, Kaye P, et al. A randomised, double-blind, controlled vaccine efficacy trial of DNA/MVA ME-TRAP against malaria infection in Gambian adults. PLoS Med 2004;1:e33.
[38] Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Annu Rev Immunol 1997;15:617–48.
[39] Bowen DG, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature 2005;436:946–52.
[40] Losikoff PT, Self AA, Gregory SH. Dendritic cells, regulatory T cells and the pathogenesis of chronic hepatitis C. Virulence 2012;3.
[41] Longman RS, Talal AH, Jacobson IM, Albert ML, Rice CM. Presence of functional dendritic cells in patients chronically infected with hepatitis C virus. Blood 2004;103:1026–9.
[42] Piccioli D, Tavarini S, Nuti S, Colombatto P, Brunetto M, Bonino F, et al. Comparable functions of plasmacytoid and monocyte-derived dendritic cells in chronic hepatitis C patients and healthy donors. J Hepatol 2005;42:61–7.
[43] Longman RS, Talal AH, Jacobson IM, Rice CM, Albert ML. Normal functional capacity in circulating myeloid and plasmacytoid dendritic cells in patients with chronic hepatitis C. J Infect Dis 2005;192:497–503.
[44] Larsson M, Babcock E, Grakoui A, Shoukry N, Lauer G, Rice C, et al. Lack of phenotypic and functional impairment in dendritic cells from chimpanzees chronically infected with hepatitis C virus. J Virol 2004;78:6151–61.
[45] Dustin LB, Rice CM. Flying under the radar: the immunobiology of hepatitis C. Annu Rev Immunol 2007;25:71–99.
[46] Soguero C, Joo M, Chianese-Bullock KA, Nguyen DT, Tung K, Hepatitis Hahn YS. C virus core protein leads to immune suppression and liver damage in a transgenic murine model. J Virol 2002;76:9345–54.

OPEN ACCESS Freely available online

PLOS ONE

# Intranasally Administered Antigen 85B Gene Vaccine in Non-Replicating Human *Parainfluenza* Type 2 Virus Vector Ameliorates Mouse Atopic Dermatitis

**Hiroshi Kitagawa[1☯], Mitsuo Kawano[2☯], Keiichi Yamanaka[1☯], Masato Kakeda[1], Kenshiro Tsuda[1], Hiroyasu Inada[3], Misao Yoneda[4], Tadashi Sakaguchi[2], Akina Nigi[2], Koumei Nishimura[2], Hiroshi Komada[4], Masato Tsurudome[2], Yasuhiro Yasutomi[5], Tetsuya Nosaka[2], Hitoshi Mizutani[1]***

**1** Department of Dermatology, Mie University, Graduate School of Medicine, Tsu, Mie, Japan, **2** Department of Microbiology and Molecular Genetics, Mie University, Graduate School of Medicine, Tsu, Mie, Japan, **3** Department of Pathology, Faculty of Pharmaceutical Science, Suzuka University of Medical Science, Suzuka, Mie, Japan, **4** Department of Clinical Nutrition, Graduate School of Suzuka University of Medical Science, Suzuka; Mie, Japan, **5** Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, Japan

## Abstract

Atopic dermatitis (AD) is a refractory and recurrent inflammatory skin disease. Various factors including heredity, environmental agent, innate and acquired immunity, and skin barrier function participate in the pathogenesis of AD. T-helper (Th) 2-dominant immunological milieu has been suggested in the acute phase of AD. Antigen 85B (Ag85B) is a 30-kDa secretory protein well conserved in *Mycobacterium* species. Ag85B has strong Th1-type cytokine inducing activity, and is expected to ameliorate Th2 condition in allergic disease. To perform Ag85B function in vivo, effective and less invasive vaccination method is required. Recently, we have established a novel functional virus vector; recombinant human *parainfluenza* type 2 virus vector (rhPIV2): highly expressive, replication-deficient, and very low-pathogenic vector. In this study, we investigated the efficacy of rhPIV2 engineered to express Ag85B (rhPIV2/Ag85B) in a mouse AD model induced by repeated oxazolone (OX) challenge. Ear swelling, dermal cell infiltrations and serum IgE level were significantly suppressed in the rhPIV2/Ag85B treated mouse group accompanied with elevated IFN-γ and IL-10 mRNA expressions, and suppressed IL-4, TNF-α and MIP-2 mRNA expressions. The treated mice showed no clinical symptom of croup or systemic adverse reactions. The respiratory tract epithelium captured rhPIV2 effectively without remarkable cytotoxic effects. These results suggested that rhPIV2/Ag85B might be a potent therapeutic tool to control allergic disorders.

**Citation:** Kitagawa H, Kawano M, Yamanaka K, Kakeda M, Tsuda K, et al. (2013) Intranasally Administered Antigen 85B Gene Vaccine in Non-Replicating Human *Parainfluenza* Type 2 Virus Vector Ameliorates Mouse Atopic Dermatitis. PLoS ONE 8(7): e66614. doi:10.1371/journal.pone.0066614

**Editor:** Andreas Zirlik, University Heart Center Freiburg, Germany

**Received** December 31, 2012; **Accepted** May 7, 2013; **Published** July 3, 2013

**Copyright:** © 2013 Kitagawa et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** K. Yamanaka (23591643) and H. Mizutani (24591647) received grants for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. Other authors did not receive any financial support. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: h-mizuta@clin.medic.mie-u.ac.jp

☯ These authors contributed equally to this work.

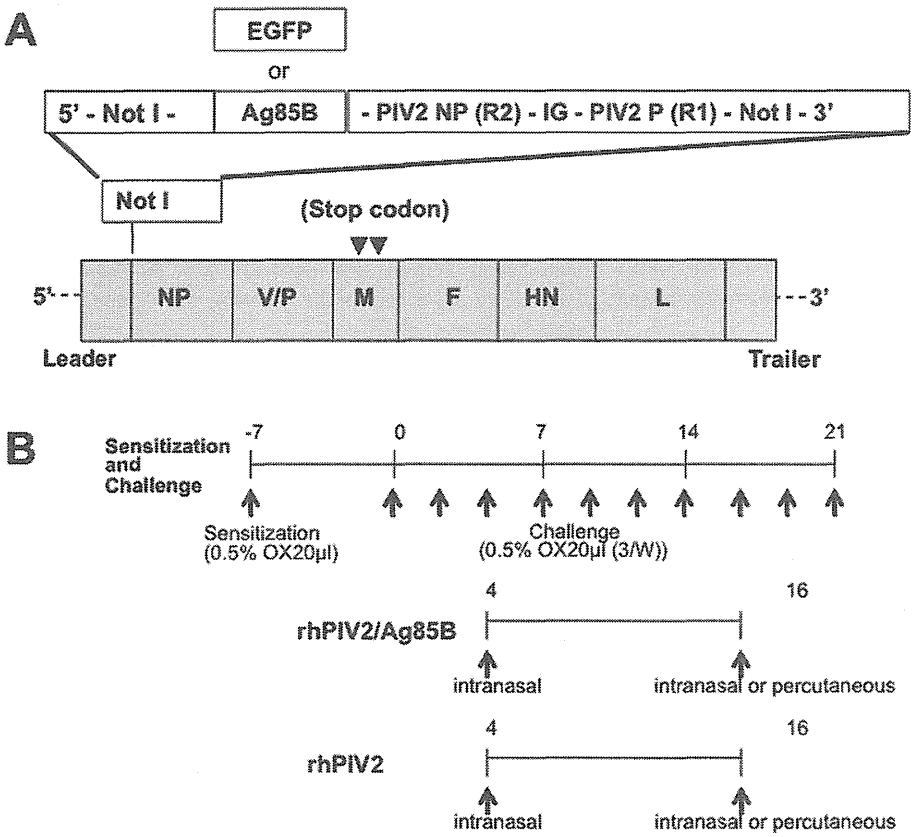
## Introduction

Atopic dermatitis (AD) is a refractory and recurrent inflammatory skin disease. Heredity, environmental agent, immunity, and skin barrier function participate in the pathogenesis of AD. AD symptoms are triggered by various non-specific or specific allergic reactions. The cytokine pattern of AD, especially in the acute phase skin lesion is Th2-type cytokine dominant [1]. The barrier disrupted skin in AD is easily permitted the percutaneous entry of environmental allergens that strongly promotes Th2 immunological responses [2]. Th2 cells as well as T regulatory cell (Treg) subsets play key roles in development of AD. Patients with AD have significantly increased numbers of peripheral blood Treg compared with healthy controls, which is correlated with disease activity in AD [3,4]. This suggests involvement of some self regulation system in immune responses in AD [5].

Repeated elicitation with hapten such as oxazolone (OX) on the ear of BALB/c mice develops immediate type responses with late phase reactions followed by delayed type hypersensitivity responses. This accompanied with balance shift of cytokines in the lesional skin from Th1 to Th2 type [6], and has been utilized as mouse AD.

Ag85B is 30-kDa major secretory protein well conserved in *Mycobacterium* species [7]. The studies for the tuberculosis vaccine revealed strong activities of Ag85B in priming naïve T cells for Th1 effector cells under the appropriate conditions, and induction of strong Th1-type immune responses in mice as well as in humans [8,9]. Recently we reported that plasmid DNA vaccination encoding Ag85B derived from M. *kansasii* inhibits immediate-type hypersensitivity responses with Treg induction in skin [10], and a combined vaccination with heat-killed BCG followed by Ag85B also suppressed skin eczematous reactions in AD model mice by inducing Treg [11].

Human parainfluenza type 2 virus (hPIV2) is one of the human respiratory pathogens and a member of the genus Rubulavirus of the family Paramyxoviridae in the order Mononegavirales,




C

| groups | Day4 | Day16 |
|---|---|---|
| PBS | PBS | PBS |
| Ag ear | rhPIV2/Ag85B | rhPIV2/Ag85B (percutaneous) |
| Ag nasl | rhPIV2/Ag85B | rhPIV2/Ag85B (intranasal) |
| PIV2 ear | rhPIV2 | rhPIV2 (percutaneous) |
| PIV2 nasal | rhPIV2 | rhPIV2 (intranasal) |

**Figure 1. Schematic diagram of constructs and strategy used in this study. A.** The constructs of recombinant hPIV2/EGFP and hPIV2/Ag85B. The EGFP or Ag85B gene open reading frame was engineered to be flanked by hPIV2-specific gene end of NP gene (R2), intergenic sequence (IG), and gene start (R1) transcriptional signal of V/P gene. It was inserted into a cloned cDNA of the hPIV2 antigenome at a Not I site that had been engineered to be at 5′-noncoding region of NP gene. A genomic nucleotide length divisible by six (the rule of six) was maintained. For generating of replication-deficient virus, two stop codons (▼) were introduced on the M gene. **B.** Schedule for the development of a hapten-induced atopic dermatitis model and vaccination of rhPIV2/Ag85B. Mice were initially sensitized with 20 μl of 0.5% OX solution to their right ear 7 days prior to the first challenge (day -7) and then 20 μl of 0.5% OX solution was repeatedly applied on the right ear 3 times per week from day 0 until day 21. Mice were inoculated intranasally with 20 μl ($5\times10^6$ $TCID_{50}$) of rhPIV2/Ag85B or rhPIV2 on day 4. rhPIV2 vector or phosphate buffered saline (PBS) were also applied as controls. On day 16, mice were vaccinated again intranasally or subcutaneously with PBS, rhPIV2 or rhPIV2/Ag85B. **C.** Summarized schedule of the experimental groups.

doi:10.1371/journal.pone.0066614.g001

possessing a non-segmented and negative-stranded RNA genome of 15,654 nucleotides. The genome of hPIV2 encodes 7 mRNAs [12–14] and has about 60-nt leader sequence at 3′ end and about 20-nt noncoding trailer sequence. The gene order is 3′ (leader)-NP-V/P-M-F-HN-L-(trailer)-5′. The coding proteins are the nucleocapsid (NP), the V (V) and phospho (P), the matrix (M), the fusion (F), the haemagglutinin-neuraminidase (HN), and the polymerase protein (L). The genomic RNA of the virus: viral RNA (vRNA) is encapsidated with the NP proteins, and the nucleocapsids are associated with the P and L proteins to form the

ribonucleoprotein complex. In paramyxovirus particles, vRNA is enclosed by the viral envelope composed of a cellular lipid bilayer and two envelope glycoproteins, HN and F, which are integral transmembrane proteins mediating virus attachment and cell fusion, respectively [15]. M protein underlies the lipid bilayer to ensure the structural integrity of the viral particles and is essential for interactions between the viral envelope and the RNP complex [15]. This association leads to the budding and release of viral particles from the cell surface [15].

Recently, as technology advances in reverse genetics [16], hPIVs offer several advantages as a vaccine vector. hPIVs efficiently infect the respiratory tract but don't spread far beyond it, which is an important safety factor. hPIV-based vectors have proven the effect in inducing local and systemic immunity against a number of foreign antigens [17]. hPIVs infect to various cell types and cause little cytopathic effects. Moreover, they replicate exclusively in the cytoplasm of infected cells, don't have a DNA phase during their life cycle and can thus avoid the possibility of integration of foreign genes into the host DNA genome [18].

In the present study, we utilized newly engineered rhPIV2: replication-deficient rhPIV2 vector. rhPIV2 lacks M gene that is an essential gene for virus particle formation by insertion of two stop codons. This alteration might support much safer application to animals than original proliferating virus vector. We first

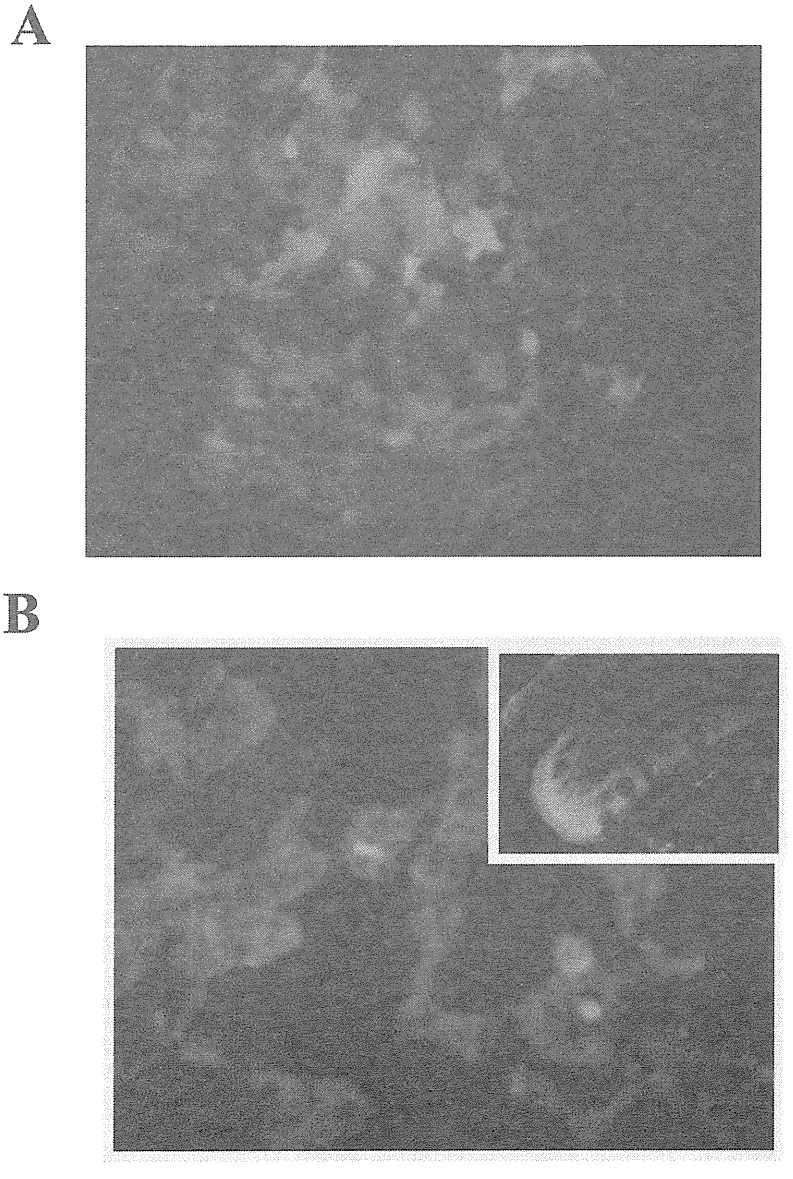



**Figure 2. Expression of EGFP from rhPIV2/EGFP. A.** HaCat cells were infected with rhPIV2/EGFP at an MOI of 0.5. Three days after, EGFP was clearly visualized using a fluorescence microscopy (x100). **B.** The rhPIV2/EGFP ($5\times10^6$ $TCID_{50}$) were administered to a wild type BALB/c mice intranasally EGFP was visualized clearly in the airway epithelial cells 4 days after administration (x200, upper right box, x400).
doi:10.1371/journal.pone.0066614.g002

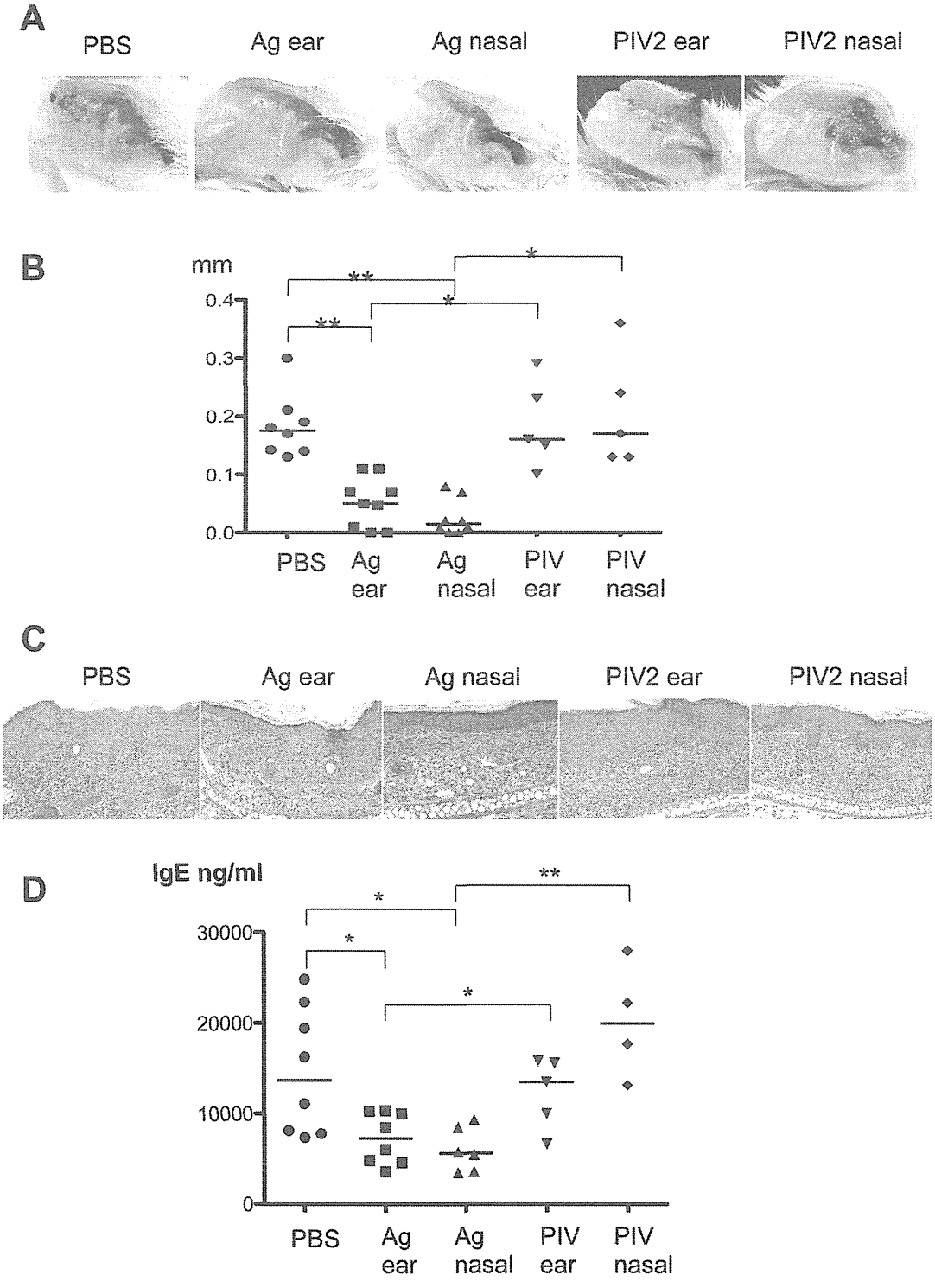



**Figure 3. Anti-inflammatory effects of vaccination with rhPIV2/Ag85B. A.** Clinical manifestation of the ear skin at 6 hours after OX challenge on day 21. The control groups (PBS, PIV2 ear, and PIV2 nasal on the panel) showed severe edema with erythema, however the intranasal and/or subcutaneous administration of the rhPIV2/Ag85B (Ag nasal and Ag ear on the panel, respectively) clearly reduced skin reactions in OX-sensitized mice. **B.** Ear thickness measured before and 6 hours after each OX application on day 21. The ear swelling was suppressed significantly in rhPIV2/Ag85B treated groups in two ways compared to those in the placebo treated groups. (*P<0.05, **P<0.01.) **C.** Histopathological changes of the ear skin obtained on day 21 in paraffin embedded sections stained with hematoxylin and eosin. The placebo treated groups (PBS, PIV2 ear and PIV2 nasal