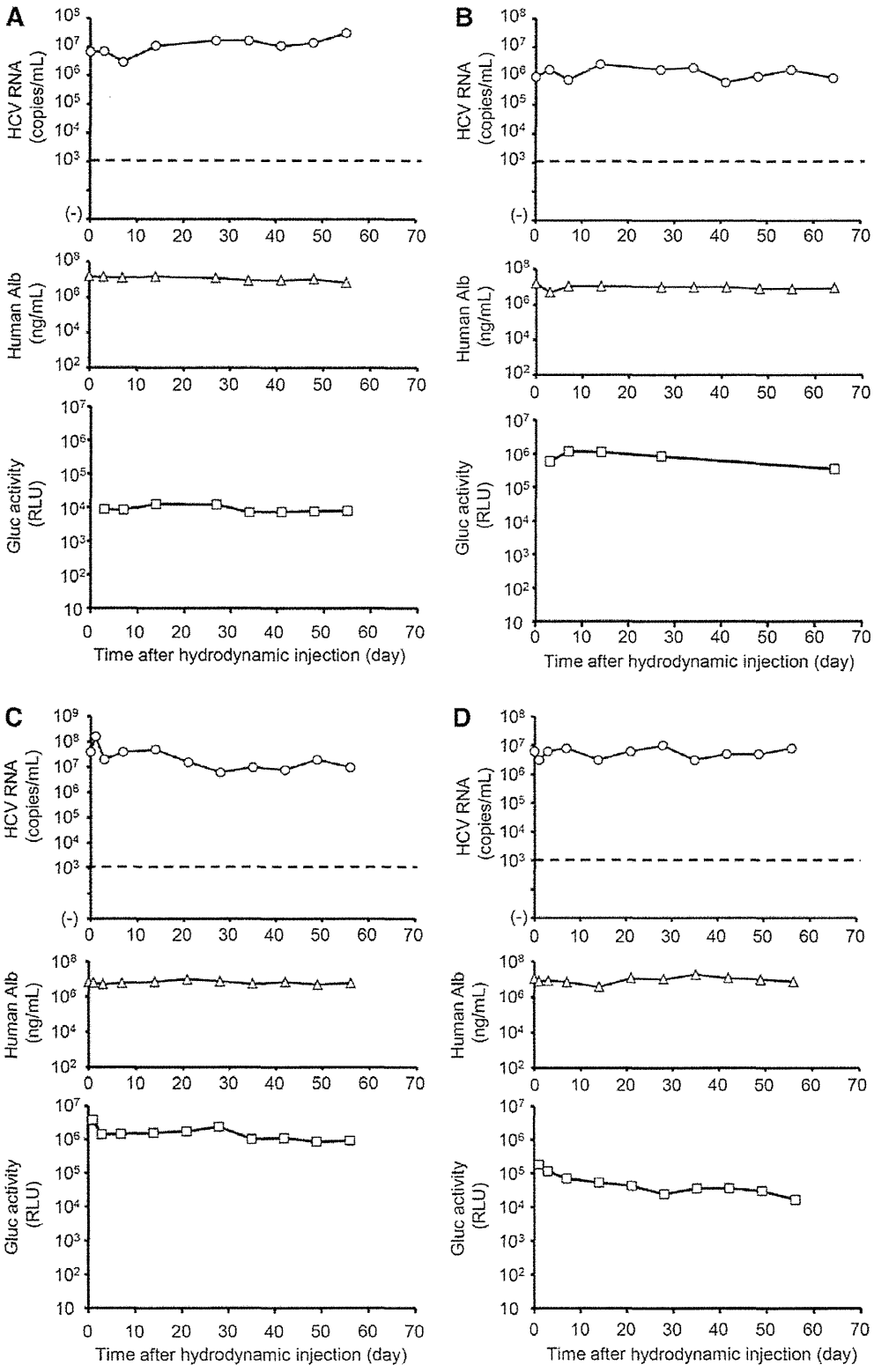

FIG. 4. Effects of pCpG-gLuc (control plasmid DNA) injection in HCV 1b-infected mice. Mice were injected intravenously with HCV-positive serum from patient-A. HCV-infected chimeric mice were injected with 250 μg of pCpG-gLuc. Data for individual mice are shown in panels (A–D). Serum concentration of HCV RNA copy number (○), HSA (△) and gLuc activity (□) is shown. The horizontal dashed line represents the detection limit (10^3 copies/mL). "(-)" on the vertical axis indicates negative for HCV by nested PCR.

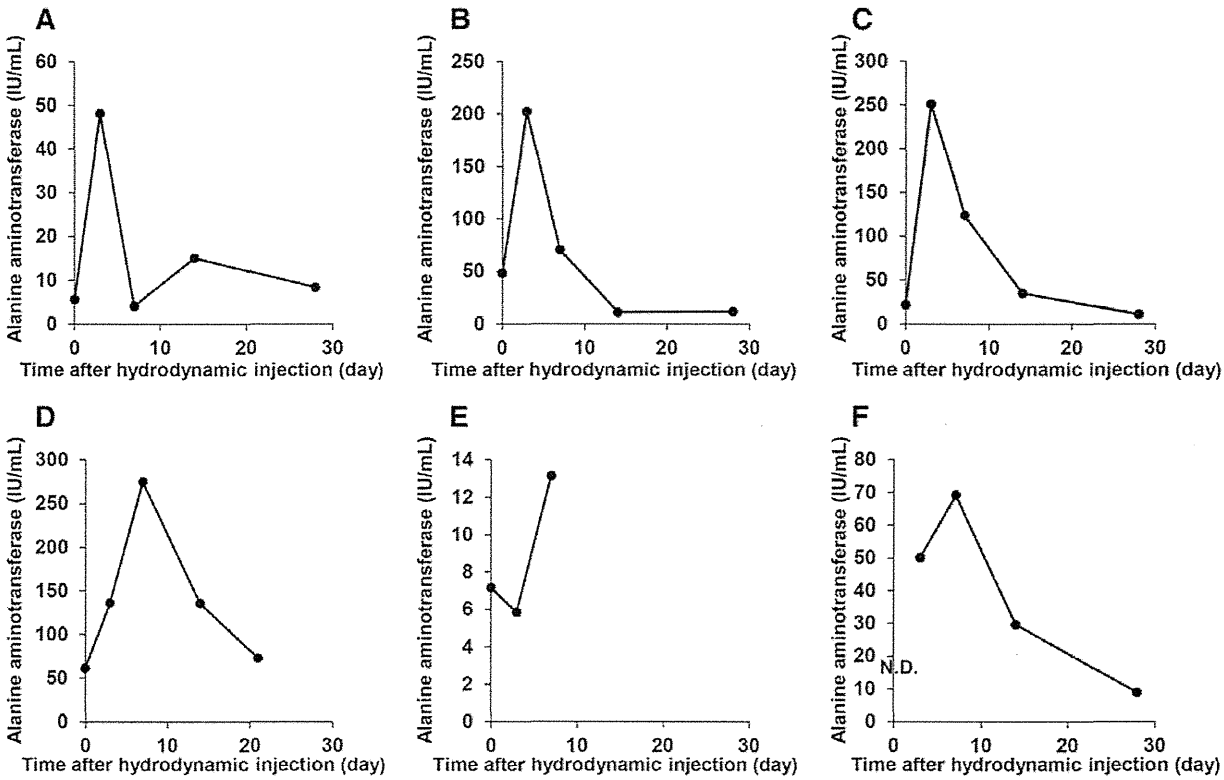

FIG. 5. Serum ALT levels in chimeric mice receiving hydrodynamic injections. Serum ALT levels in chimeric mice receiving pCpG-huIFNγ were measured. Panels **(A–F)** correspond to pCpG #1–6, respectively. N.D. indicates that serum ALT activity was not determined because of the exhaustion of serum sample. ALT, alanine aminotransferase.

(Shultz *et al.*, 1995). It is possible that introduction of IFN-γ produced anti-HCV effects by activation of resident NK cells. In the present study, flow cytometry analysis showed that activated NK cells were present in the liver 3 days after pCpG-huIFNγ treatment (Supplementary Fig. S3). However, the NK cell populations in the pCpG-huIFNγ-treated mouse livers were similar to those in pCpG-gLuc-treated mice. These results indicate that the anti-HCV effect of IFN-γ in this study seems to be caused by direct inhibition of viral replication.

During the observation period in the present study, two out of six mice injected with pCpG-huIFNγ died and two mice died after pCMV-huIFNγ injection. This was not caused by IFN-γ expression because the level of expressed IFN-γ was relatively low in the dead mice compared with that of survived mice. Vanwolleghem *et al.* (2010) have reported that chimeric mice are weak, and approximately 50% of mice die spontaneously 6 weeks after transplantation. In fact, we also observed some chimeric mice receiving hydrodynamic injection without plasmid DNA spontaneously died in preliminary experiments. Therefore, these four mice were highly unlikely to have died from hydrodynamic injection of IFN-γ-expressing plasmid DNA.

As a high level of IFN-γ might produce toxicity, we measured the serum level of ALT in the chimeric mice. On day 3 after injection of pCpG-huIFNγ, transient increase in the serum level of ALT was observed in the mice that expressed a relatively high level of IFN-γ (Fig. 5). However, 2 weeks after the gene transfer, the serum level of ALT returned to the level comparable with that obtained before the gene transfer. In the case of pCpG-gLuc injection, no significant change was observed (data not shown). Although these results suggest that a high level of IFN-γ expression in the liver may cause slight hepatotoxicity for a short period, which was not detected by histological analysis (Fig. 6C and D), the effect would be transient and limited. The serum level of HSA, an indicator of human hepatocyte number in chimeric mouse livers, did not markedly change after gene transfer of IFN-γ, and a sustained and constant transgene expression from pCpG vectors from the hepatocytes in the chimeric mice was also observed, implying that transgene-expressing hepatocytes were not damaged by IFN-γ. Histochemical analysis by hematoxylin–eosin staining of the liver sections also suggested that there was no significant damage in human hepatocytes even after long-term exposure to the human cytokine, IFN-γ (Fig. 6G and H). Taken together, these results suggest that sustained IFN-γ treatment would be tolerable in terms of hepatic toxicity induced by IFN-γ.

In the development of IFN-γ gene therapy as the treatment of patients with chronic HCV infection, the method of gene delivery presents a major challenge. In the present study, we found that human hepatocytes in the mouse liver can be transfected by hydrodynamic injection with a large variation in the transfection efficiency among the mice tested. Although the reasons for this variable transfection efficiency in human liver chimeric mice are not clear, some speculations

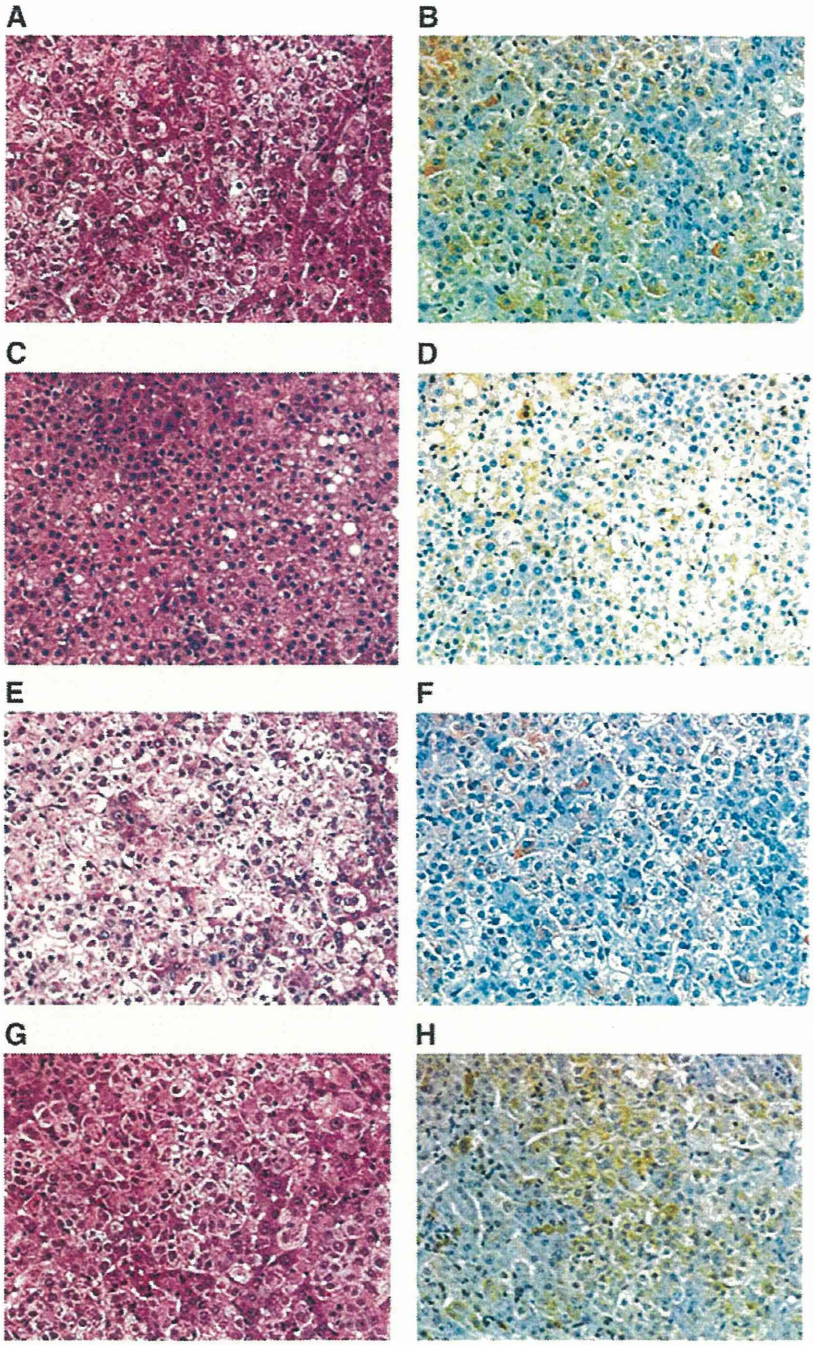

FIG. 6. Histological analysis of the liver of the pCpG-huIFNγ-treated chimeric mice. The livers of HCV-infected chimeric mice that had been untreated **(A** and **B)** or that had been injected with plasmid vectors were collected at 3 days after pCpG-huIFNγ administration **(C** and **D)**. In a separate set of experiments, the livers of HCV-infected chimeric mice were collected at 56 days after the administration of pCpG-gLuc **(E** and **F)** or pCpG-huIFNγ (pCpG #3; **G** and **H)**. The liver sections were histologically examined either by hematoxylin–eosin staining **(A, C, E,** and **G)** or immunohistochemical staining with antihuman serum albumin antibody **(B, D, F,** and **H)**. Original magnification, $\times 100$. Color images available online at www.liebertpub.com/humc

are possible. As poorly vascularized regions were observed on occasion in the liver of the chimeric mice, this might be the reason for the poor transfection efficiency and variability in transfection efficiency between mice (Sato *et al.*, 2008). Although the difference in the repopulation rate of human hepatocytes may affect the transgene gene expression level, an apparent correlation between transgene expression level and repopulation rate was not observed. In addition, there was no apparent effect of HCV level in the liver on IFN-γ transgene expression level. A recent study demonstrated that significant transgene expression is obtained in human liver segments after hydrodynamic injection; however, a very fast injection speed was required compared with that for mice (Herrero *et al.*, 2012). In addition, special attention should be paid when hydrodynamic gene transfer is performed in cirrhotic livers, because the transgene expression level obtained by this method has been shown to be lower in cirrhotic liver in rats (Yeikilis *et al.*, 2006). Under these circumstances, gene transfer to organs other than the liver, such as skeletal muscle, could be an option.

In conclusion, the present study has demonstrated that long-term elimination of human HCV in human hepatocyte chimeric mice with high titers of HCV RNA of genotype 1b can be achieved by IFN-γ exposure after systemic administration of pCpG-huIFNγ. These results indicate that continuous supply of IFN-γ, such as through IFN-γ gene transfer, appears to be a promising antiviral treatment for chronic HCV.

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Author Disclosure Statement

No competing financial interests exist.

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Efficient delivery of immunostimulatory DNA to mouse and human immune cells through the construction of polypod-like structured DNA

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Abstract

Investigation of mouse macrophage-like RAW264.7 cells showed that the immunostimulatory activity of CpG DNA is increased by formation of polypod-like structured DNA (polypodna), an assembly consisting of three or more oligodeoxynucleotides. To apply CpG polypodna to immunotherapy, its activity was examined in murine dendritic DC2.4 cells, splenic macrophages, and bone marrow-derived dendritic cells (BMDCs). In all cell types, increasing the pod number increased the cellular uptake of DNA and cytokine release. No significant release of cytokines was observed in macrophages lacking Toll-like receptor 9. Similar results were obtained after intradermal injection of polypodna. The polypodna preparations produced significantly higher amounts of interferon α in human peripheral blood mononuclear cells (PBMCs) compared with single-stranded DNA. The conditioned medium of hexapodna-treated human PBMCs effectively inhibited the activity of a hepatitis C virus subgenomic replicon reporter system. These results indicate that polypodna preparations are useful as an immunostimulator.

Key words: CpG DNA; Toll-like receptor 9; Dendritic cell; Peripheral blood mononuclear cell

Stimulation of innate immune cells leads to secretion of proinflammatory cytokines, which, in turn, causes inflammation. Such inflammation is likely essential for the induction of efficient immune responses to a variety of pathogens.[1] Ligands for Toll-like receptors (TLRs) have therapeutic potential, because TLR ligation initiates a signaling pathway that activates nuclear factor κB, which is a master transcription factor controlling the expression of proinflammatory cytokines.

One of the most-studied TLR ligands is DNA containing unmethylated cytosine–phosphate–guanine dinucleotides (CpG motifs), the ligand for TLR9.[2,3] This specific DNA is abundant in viral, bacterial and mitochondrial DNA, and it is categorized as a pathogen- or damage-associated molecular pattern.[4] TLR9 ligation induces the release of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-12 and interferon (IFN)-α.[5–8] Although natural phosphodiester CpG DNA is a ligand for TLR9, its susceptibility to enzymatic degradation limits its therapeutic applications. Therefore, phosphorothioate-stabilized CpG DNA has been used to induce cytokine production.[9,10] This stabilized DNA, however, is associated with adverse reactions, such as renal damage.[11] Therefore, other approaches for increasing the activity of CpG DNA are required to enhance its therapeutic potency.

Recent advances in DNA nanotechnology have resulted in the development of uniquely structured DNA assemblies. CpG DNA has been linked with or incorporated into these assembled DNA systems, such as Y- or X-shaped DNA,[12,13] dendrimer-like DNA,[14]

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DNA tetrahedra[15] and DNA origami.[16] We have recently developed a series of polypod-like structured DNAs, or polypodna, which is a DNA assembly consisting of three or more oligodeoxynucleotides (ODNs), and found that the immunostimulatory activity of CpG DNA is a function of the structural characteristics of the polypodna. For example, both pod number and size are important parameters that determine the cellular uptake of polypodna preparations in mouse macrophage-like RAW264.7 cells and the following cytokine production.[17]

Despite these advances, additional studies of CpG DNA-containing polypodna in cells other than RAW264.7 are required, since the cellular response to CpG DNA is highly cell type- and species-specific.[18,19] Human macrophages do not express TLR9,[20] and the major human cells responding to CpG DNA are considered to be plasmacytoid dendritic cells.[21] In addition, we have demonstrated that primary macrophages respond differently to CpG DNA than RAW264.7 cells.[19]

Based on these considerations, this study was designed to examine the immunostimulatory activity of polypodna in murine immune cells that play important roles in the response to CpG DNA. We also examined the immunostimulatory activity of polypodna in vivo after intradermal injection into mice. Finally, human peripheral blood mononuclear cells (PBMCs) were also used to investigate whether polypodna preparations effectively activate human immune cells.

Methods

Chemicals

Roswell Park Memorial Institute (RPMI) medium and Dulbecco's modified Eagle's medium (DMEM) powder were obtained from Nissui Pharmaceutical, Co., Ltd. (Tokyo, Japan). DMEM (liquid) was purchased from Life Technologies (Carlsbad, CA) and used only for LucNeo#2 cells. Opti-modified Eagle's medium (Opti-MEM) and Iscove's modified Dulbecco's medium (IMDM) and MEM non-essential amino acids (NEAA) were purchased from Life Technologies. Fetal bovine serum (FBS) was obtained from Biowest (Nuaille, France), monothioglycerol and cytochalasin B were obtained from Wako Pure Chemical (Osaka, Japan), G418 was purchased from Nacalai Tesque (Kyoto, Japan), and chloroquine diphosphate salt, dansylcadaverine and lipopolysaccharide (LPS; L3014) were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Male C57BL/6 mice were purchased from Japan SLC, Inc (Shizuoka, Japan). TLR9 knockout ($TLR9^{-/-}$) mice with a C57BL/6 genetic background were purchased from Oriental Yeast Company (Tokyo, Japan). All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

Cell culture

The murine macrophage-like cell line RAW264.7 was cultured in RPMI medium supplemented with 10% heat-inactivated FBS, 0.2% sodium bicarbonate, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. The murine dendritic cell line DC2.4 (kindly provided by Dr. K. L. Rock, University of Massachusetts Medical School, Worcester, MA) was cultured in RPMI medium supplemented with 10% heat-inactivated FBS, 0.2% sodium bicarbonate, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.5 mM monothioglycerol and 0.1 mM NEAA. These cells were replated on 24-well, 48-well or 96-well culture plates at a density of 2.5×10^5 cells/well, 1×10^5 cells/well or 5×10^4 cells/well, respectively, and cultured for 24 h prior to use. LucNeo#2, an Huh-7 cell harboring self-replicating subgenomic hepatitis C virus (HCV) RNA replicons with a luciferase reporter,[22] was kindly provided by Drs. Hijikata and Shimotohno of Institute for Virus Research, Kyoto University, Japan. LucNeo#2 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 0.1 mM NEAA in the presence of 500 μg/mL G418.

Preparation of mouse bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were generated from 8- to 10-week-old male C57BL/6 mice as previously reported.[23] Briefly, bone marrow cells were isolated by flushing femurs and tibiae with RPMI using a 26-gauge needle. After filtration through a 40-μm cell strainer (BD Falcon, Franklin Lakes, NJ), bone marrow cells were resuspended in 0.86% ammonium chloride for 5 min to lyse the red blood cells. Then, the remaining cells were cultured for 8 days at a density of 5×10^6 cells/mL in RPMI supplemented with 10% heat-inactivated FBS, 0.2% sodium bicarbonate, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.5 mM monothioglycerol, and 100 ng/mL mouse recombinant Flt-3 ligand (Peprotech, Rocky Hill, NJ). Cells were grown in humidified air containing 5% CO_2 at 37 °C. On day 7 of culture, non-adherent cells were harvested and used as BMDCs. Cells were replated on 96-well culture plates at a density of 3×10^5 cells/well for the cytokine release experiment.

Isolation of mouse splenic macrophages

Spleens were removed aseptically from 8- to 10-week-old male C57BL/6 mice, divided with a spatula and filtered through a 40-μm cell strainer (BD Falcon) to obtain single-cell suspensions. Red blood cells were lysed by exposure to 0.86% ammonium chloride for 5 min. After washing three times with phosphate-buffered saline (PBS), splenocytes were suspended in RPMI medium supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 mg/mL), and 0.5 mM monothioglycerol, and plated in 10-cm culture dishes. After a 3-h incubation in humidified air containing 5% CO_2 at 37 °C, adherent macrophages were washed twice with RPMI-1640 medium to remove non-adherent cells and replated at a density of 4×10^5 cells/well on 48-well or 96-well culture plates.

Isolation of human peripheral blood mononuclear cells

All experiments using human cells were approved by the institutional review board at the Graduate School of Medicine, Kyoto University, and following the tenets of the Declaration of Helsinki. Human PBMCs were obtained from healthy donors

Table 1
The sequences of ODNs used for polypodna preparations for mouse cells.

ODN	Sequence (5′ → 3′)
mTri-1	TCGTCAACGTCTGTGCTCTCACGTTGACGCTGTCGA
mTri-2, mTet-2, mPen-2, mHex-2	TCGACAGCGTCAACGTGAAACGTGAAGCGTCTGCGA
mTri-3, mPen-3, mHex-3	TCGCAGACGCTTCACGTTGAGCACAGACGTTGACGA
mTet-1, mPen-1, mHex-1	TCGCTGACGTTGCAGACATCACGTTGACGCTGTCGA
mTet-3	TCGCAGACGCTTCACGTTGCAGACAGACGTTGACGA
mTet-4	TCGTCAACGTCTGTCTGCTGTCTGCAACGTCAGCGA
mPen-4, mHex-4	TCGTCAACGTCTGTGCTCGCAGCGTCTTAACGTCGA
mPen-5	TCGACGTTAAGACGCTGCTGTCTGCAACGTCAGCGA
mHex-5	TCGACGTTAAGACGCTGCAGACGTTCAGGACTACGA
mHex-6	TCGTAGTCCTGAACGTCTTGTCTGCAACGTCAGCGA
mGCTri-1	TGCTCAAGCTCTGTGCTCTCAGCTTGAGCCTGTGCA
mGCTri-2, mGCTet-2, mGCPen-2, mGCHex-2	TGCACAGGCTCAAGCTGAAAGCTGAAGGCTCTGGCA
mGCTri-3, mGCPen-3, mGCHex-3	TGCCAGAGCCTTCAGCTTGAGCACAGAGCTTGAGCA
mGCTet-1, mGCPen-1, mGCHex-1	TGCCTGAGCTTGCAGACATCAGCTTGAGCCTGTGCA
mGCTet-3	TGCCAGAGCCTTCAGCTTGCAGACAGAGCTTGAGCA
mGCTet-4	TGCTCAAGCTCTGTCTGCTGTCTGCAAGCTCAGGCA
mGCPen-4, mGCHex-4	TGCTCAAGCTCTGTGCTCGCAGGCTCTTAAGCTGCA
mGCPen-5	TGCAGCTTAAGAGCCTGCTGTCTGCAAGCTCAGGCA
mGCHex-5	TGCAGCTTAAGAGCCTGCAGAGCTTCAGGACTAGCA
mGCHex-6	TGCTAGTCCTGAAGCTCTTGTCTGCAAGCTCAGGCA
mGCHex-6	TGCTAGTCCTGAAGCTCTTGTCTGCAAGCTCAGGCA

All ODNs have a phosphodiester backbone.

Table 2
The sequences of ODNs used for polypodna preparations for human cells.

ODN	Sequence(5′ → 3′)
hTri-1, hTet-1, hPen-1, hHex-1	TCGTCGTTCCGTCGTTACACTGCTCTGGCGGTCGTT
hTri-2, hTet-2, hPen-2, hHex-2	AACGACCGCCAGAGCAGTCGTGTCGTACTACGACGA
hTri-3	TCGTCGTAGTACGACACGGTAACGACGGAACGACGA
hTet-3, hPen-3, hHex-3	TCGTCGTAGTACGACACGGTCGTAAGCCTGGTCGTA
hTet-4	TACGACCAGGCTTACGACGTAACGACGGAACGACGA
hPen-4, hHex-4	TACGACCAGGCTTACGACAGTCTAGCTGATCGACGA
hPen-5	TCGTCGATCAGCTAGACTGTAACGACGGAACGACGA
hHex-5	TCGTCGATCAGCTAGACTGCTGTCGATGCCAACGAC
hHex-6	GTCGTTGGCATCGACAGCGTAACGACGGAACGACGA
hGCTri-1, hGCTet-1, hGCPen-1, hGCHex-1	TGCTGCTTGCCTGCTTACACTGCTCTGGCGGTGCTT
hGCTri-2, hGCTet-2, hGCPen-2, hGCHex-2	AAGCACCGCCAGAGCAGTGCTGTGCTACTAGCAGCA
hGCTri-3	TGCTGCTAGTAGCACAGCGTAAGCAGGCAAGCAGCA
hGCTet-3, hGCPen-3, hGCHex-3	TGCTGCTAGTAGCACAGCGTGCTAAGCCTGGTGCTA
hGCTet-4	TAGCACCAGGCTTAGCACGTAAGCAGGCAAGCAGCA
hGCPen-4, hGCHex-4	TAGCACCAGGCTTAGCACAGTCTAGCTGATGCAGCA
hGCPen-5	TGCTGCATCAGCTAGACTGTAAGCAGGCAAGCAGCA
hGCHex-5	TGCTGCATCAGCTAGACTGCTGTGCATGCCAAGCAC
hGCHex-6	GTGCTTGGCATGCACAGCGTAAGCAGGCAAGCAGCA

All ODNs have a phosphodiester backbone.

after obtaining their written informed consent. In brief, PBMCs were isolated from peripheral blood using Ficoll–Plaque PLUS (GE Healthcare Piscataway, NJ) density gradient centrifugation according to the manufacturer's protocol. PBMCs collected were replated on 48-well culture plates at a density of 6×10^5 cells/well in Opti-MEM. There were considerable individual differences in the response of cells to CpG DNA, and only highly responsive cells were used for experiments.

Oligodeoxynucleotides

Oligodeoxynucleotides (ODNs) with a length of 36-mer were purchased from Integrated DNA Technologies, Inc (Coralville, IA). The sequences of the ODNs are listed in Tables 1 and 2. Each ODN was given a name, such as mTri-1, hTri-1 and so on, with "m" for mouse and "h" for human, Tri, Tetra, Pen, Hex for tripodna, tetrapodna, pentapodna and hexapodna, respectively, and the ODN number. The ODNs for CpG polypodna for mouse cells were identical to those used in a previous study.[17] For cellular uptake studies, mTri-2 labeled with Alexa Fluor-488 at the 5′ end was purchased from Japan BioService Co., Ltd. (Saitama, Japan).

Preparation of polypodna

Polypodna samples were prepared by mixing equimolar amounts of their components as previously reported.[17] In brief, ODNs dissolved in an annealing buffer (10 mM Tris–HCl, pH 8.0, 1 mM ethylenediaminetatraacetic acid, and 150 mM sodium chloride) were mixed at a final concentration of 0.1 mM for each ODN. The mixtures of ODNs were annealed by heating them to 95 °C and gently cooling to 4 °C using a thermal cycler. Then, the concentrations of DNA samples were normalized. For cellular uptake studies, Alexa Fluor 488-labeled mTri-2 was used instead of mTri-2. The products of polypodna preparations were analyzed by 6% polyacrylamide gel electrophoresis (PAGE) at room temperature at 200 V for 20 min and stained with ethidium bromide for visualization.

Cytokine release

Cells were incubated with single-stranded (ss) DNA (mTri-2 for mouse cells or hTri-2 for human PBMCs) or polypodna at the

indicated concentrations for 24 h. Then, culture supernatants were collected for enzyme-linked immunosorbent assay (ELISA) and kept at −80 °C until use. Appropriate cytokines that are highly released from each type of cell were selected as indicators of cellular stimulation. The levels of mouse IL-6, the p40 subunit of IL-12 (IL-12p40) and tumor necrosis factor (TNF)-α were determined using OptEIA™ sets (Pharmingen, San Diego, CA). The levels of human IFN-α were determined using the IFN-α–ELISA module set (Bender Med Systems, Vienna, Austria).

Cellular uptake of polypodna

DC2.4 cells (5×10^4 cells/well) or splenic macrophages (4×10^5 cells/well) on 96-well culture plates were incubated with 2 μg/mL Alexa Fluor 488-labeled DNA samples for 8 or 4 h, respectively, at 37 °C or 4 °C. Then, cells were washed twice with 200 μL PBS and harvested. Finally, the fluorescence intensity of the cells was determined by flow cytometry (FACS Calibur, BD Biosciences, Franklin Lakes, NJ) using CellQuest software (version 3.1, BD Biosciences), and the mean fluorescent intensity (MFI) was calculated as an indicator of cellular uptake. To compare the cellular uptake of ssDNA and polypodna preparations, a normalized MFI was calculated by multiplying the MFI obtained with the fraction of Alexa Fluor 488-labeled ODN in samples, because the amount of Alexa Fluor 488-labeled ODN in the DNA samples was not identical.

Effect of inhibitors on cytokine release and cellular uptake in DC2.4 cells

Chloroquine (an inhibitor of endosomal fusion and acidification), dansylcadaverine (an inhibitor of clathrin-mediated endocytosis), and cytochalasin B (an inhibitor of phagocytosis) were used to examine the mechanisms involved in the cellular uptake of polypodna preparations. Cells were preincubated for 30 min with 6.25 μM chloroquine, 25 μM dansylcadaverine, or 5 μM cytochalasin B, then incubated with CpG hexapodna or LPS in the presence of each inhibitor for 24 h. LPS, a ligand for Toll-like receptor 4, was used to confirm whether the DC2.4 cells treated with these inhibitors have the ability to release IL-6. IL-6 release and cellular uptake were examined as described above.

IL-12p40 production in mice

Under anesthesia with isoflurane, mice were injected with saline with or without ssDNA, CpG tripodna or CpG hexapodna into the dorsal skin at a dose of 100 μg DNA/mouse. At 1, 3, 6, and 9 h after injection, the blood was collected from the tail vein of mice and serum was collected by centrifugation and stored at −20 °C until analysis. The levels of IL-12p40 in the serum were determined by ELISA as described above.

Inhibition of HCV replication in an HCV subgenomic replicon system

LucNeo#2 cells were used to examine the inhibition of HCV replication by conditioned media of polypodna-treated human PBMCs, because the luciferase activity of the cells can be used as an indicator of HCV replication. Human PBMCs were incubated with ssDNA or CpG hexapodna at a concentration of 2 μg DNA/mL as described above. Then, the supernatant was collected as the conditioned medium. LucNeo#2 cells were replated at a concentration of 2×10^4 cells/well on 48-well culture plates and cultured for 24 h in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Then, the conditioned medium of PBMCs was diluted 20-fold with DMEM and added to LucNeo#2 cells, and the cells were harvested after a 24-h culture and lysed. HCV replication was estimated by measuring the luciferase activity of the cell lysates in a luminometer Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany).

Statistical analysis

Differences were statistically evaluated by one-way analysis of variance followed by a Tukey–Kramer multiple comparisons test for multiple comparison or by Student's *t* test for comparison between two groups. *P*-values less than 0.05 were considered statistically significant.

Results

Cytokine release and uptake after addition of polypodna to mouse dendritic DC2.4 cells

Figure 1 shows the sequence and planar representation of CpG polypodna consisting of three, four, five, or six ODNs for mouse cells. Each ODN was identified with different colors. Figure 2, *A* shows the IL-6 concentrations in the culture media of DC2.4 cells after the addition of ssDNA or polypodna preparations containing CpG motifs. All polypodna preparations examined, i.e., tri-, tetra-, penta- and hexapodna, induced higher levels of IL-6 than ssDNA, although the same amount of DNA was added to the cells. The amount was almost proportional to the amount of DNA added and was the highest after addition of hexapodna, followed by pentapodna, tetrapodna, and tripodna, in this order. Similar results were obtained when the levels of TNF-α in the culture media were measured (data not shown). Figure 2, *B* shows the normalized mean fluorescent intensity (MFI) values of DC2.4 cells after 8 h-incubation with Alexa Fluor 488-labeled ssDNA or polypodna preparations at 37 °C. The higher the pod number, the greater the MFI values of the cells. The MFI values of cells incubated with DNA samples at 4 °C were much lower than those at 37 °C (data not shown).

Effect of inhibitors on IL-6 release and cellular uptake in DC2.4 cells

Figure 2, *C* shows the IL-6 concentrations in the culture media of DC2.4 cells after the addition of CpG hexapodna in the presence or absence of uptake inhibitors. All the inhibitors used significantly attenuated IL-6 release from the cells. Almost no IL-6 was released when chloroquine or cytochalasin B was added to the cells. When cells were treated with LPS, significant amounts of IL-6 were released, indicating that the cellular functions to release the cytokine were not impaired by the treatment. Figure 2, *D* shows the MFI values of cells treated with each inhibitor followed by with Alexa Fluor 488-labeled hexapodna. Chloroquine had relatively little effect on the cellular uptake of Alexa Fluor 488-labeled hexapodna,

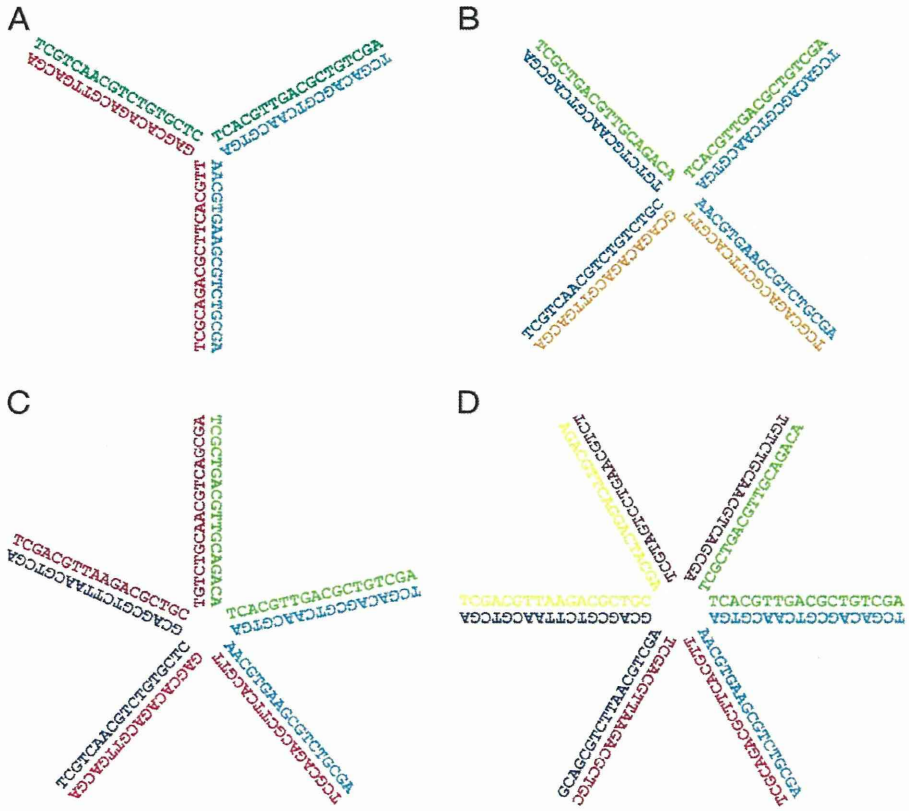

Figure 1. Sequence and planar representation of CpG polypodna for mouse cells. **(A)** CpG tripodna, **(B)** CpG tetrapodna, **(C)** CpG pentapodna, and **(D)** CpG hexapodna. Each ODN was identified with different colors.

whereas dansylcadaverine or cytochalasin B significantly reduced the uptake.

IL-12 release from mouse splenic macrophages

To investigate the responsiveness of primary immune cells to polypodna preparations, mouse splenic macrophages were used. Because splenic macrophages release higher amounts of IL-12 than IL-6,[5] IL-12p40 was measured as an indicator of the activation of splenic macrophages. Figure 3, *A* shows IL-12p40 concentrations in the culture media of splenic macrophages of C57/BL6 mice. The concentrations of IL-12p40 rose as the pod number increased. To confirm the importance of CpG motifs on cytokine release from splenic macrophages, DNA samples containing GpC sequences instead of CpG were added to splenic macrophages (Figure 3, *B*). No significant amounts of IL-12p40 were released when DNA-containing GpC sequences were added. DNA sensors other than TLR9 have also been reported to be involved in the DNA-mediated production of cytokines from immune cells.[24] To determine if alternative sensors were involved in the responses, we examined cytokine release in splenic macrophages isolated from TLR9$^{-/-}$ mice. As shown in Figure 3, *C*, no significant release of IL-12p40 was observed from splenic macrophages of TLR9$^{-/-}$ mice irrespective of the structure of CpG DNA.

Uptake of polypodna in mouse splenic macrophages

The differences in the release of IL-12p40 from splenic macrophages between wild-type and TLR9$^{-/-}$ mice or between CpG and GpC samples might be due to the differences in the cellular uptake of DNA samples. Therefore, the uptake of Alexa Fluor 488-labeled hexapodna by splenic macrophages was examined (Figure 3, *D*). There was no significant difference in the uptake of Alexa Fluor 488-labeled CpG hexapodna between wild-type and TLR9$^{-/-}$ macrophages. In addition, the uptake of Alexa Fluor 488-labeled GpC hexapodna by wild-type macrophages was comparable with that of Alexa Fluor 488-labeled CpG hexapodna.

IL-6 release from mouse BMDCs

Figure 4 shows the IL-6 concentrations in the culture media of BMDCs of C57/BL6 mice after the addition of ssDNA or polypodna preparations. BMDCs released large amounts of IL-6 depending on the pod number. Again, no detectable IL-6 was released from BMDCs of TLR9$^{-/-}$ mice (data not shown).

IL-12p40 production by polypodna after intradermal injection in mice

ssDNA, CpG tripodna, or CpG hexapodna was injected into mice by intradermal injection to examine IL-12p40 production by these preparations in vivo. Figure 5 shows the time course of the serum IL-12p40 levels of mice after injection of saline (vehicle) or DNA samples. No significant increase in the IL-12p40 level was observed in mice receiving saline or ssDNA. In contrast, the level significantly increased in mice receiving CpG tripodna or CpG hexapodna.