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ORIGINAL ARTICLE

Codelivery of IL-7 Augments Multigenic HCV DNA Vaccine-induced Antibody as well as Broad T Cell Responses in Cynomolgus Monkeys

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Background: A crucial limitation of DNA vaccines is its weak immunogenicity, especially in terms of eliciting antibody responses in non-human primates or humans; therefore, it is essential to enhance immune responses to vaccination for the development of successful DNA vaccines for humans. **Methods:** Here, we approached this issue by evaluating interleukin-7 (IL-7) as a genetic adjuvant in cynomolgus monkeys immunized with multigenic HCV DNA vaccine. **Results:** Codelivery of human IL-7 (hIL-7)-encoding DNA appeared to increase DNA vaccine-induced antibody responses specific for HCV E2 protein, which plays a critical role in protecting from HCV infection. HCV-specific T cell responses were also significantly enhanced by codelivery of hIL-7 DNA. Interestingly, the augmentation of T cell responses by codelivery of hIL-7 DNA was shown to be due to the enhancement of both the breadth and magnitude of immune responses against dominant and subdominant epitopes. **Conclusion:** Taken together, these findings suggest that the hIL-7-expressing plasmid serves as a promising vaccine adjuvant capable of eliciting enhanced vaccine-induced antibody and broad T cell responses.
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INTRODUCTION

At least 170 million people worldwide are persistently infected with HCV, the most common reason for patients requiring a liver transplant. An estimated 2.3 to 4.7 million people are infected every year, but an effective vaccine is not yet available (1,2). Six different genotypes and a variety of quasispecies of HCV pose a major challenge for the development of an effective HCV vaccine. HCV-specific T cell responses have been shown to protect HCV-recovered chimpanzees upon homologous, heterologous and cross-genotype HCV rechallenge (3,4). A recent study on chimpanzees showed that vaccination with replication-defective adenoviral vector encoding HCV NS3-NS5B and booster vaccination with recombinant DNA plasmid effectively induced protective T cell immunity against challenge with a heterologous HCV (5). In addition to T cell response, neutralizing antibodies have been shown to be a key feature of effective vaccines against HCV infection (6,7). Thus, substantial effort has been focused on the induction of vigorous HCV-specific antibody as well as T cell immunity.

Multigenic DNA vaccination is one of the most effective ways to induce both antibody and broad T cell responses by intramuscular injection (8). DNA vaccines have used to generate protective immunity against various pathogens (9). Since the strength of the immune responses induced by DNA vaccines has been relatively weak compared with that of immune responses induced by conventional vaccines such as subunit vaccines, it is necessary to develop novel methods for circumventing this limitation, such as codelivery of novel cytokine adjuvants (10). Thus, immunostimulatory cytokines

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such as interleukin (IL)-2, IL-7, IL-12, IL-15 and IL-18 have been studied as genetic adjuvants (11).

IL-7 is a glycoprotein of 25 kDa and secreted by thymic and intestinal epithelial cells, bone marrow stromal elements and keratinocytes. It has been shown to be an essential growth factor for B and T lineage cells (12). Previous reports demonstrated that *in vivo* administration of IL-7 results in the increased numbers of B lineage cells and T cells with a preferential increase in $CD8^+$ T cells (13,14). It has also been reported that IL-7 can help promote the proliferation of T cells (15) and enhance the lytic activity of CTLs and lymphokine-activated killer cells (16).

Recent research into the biology of IL-7 suggests that it might serve as an effective vaccine adjuvant based on the following reasons. First, IL-7 receptor-α is expressed on the majority of resting, naive $CD8^+$ T cells. IL-7 signaling recruits T cells specific for low-affinity antigens into the proliferative pool in lymphopenic hosts (17,18). Additionally, like other common γ receptor chain (γc) cytokines, IL-7 prevents programmed cell death. Thus, IL-7 therapy may diminish the magnitude of cell contraction following antigen-specific activation (19). At present, accumulating evidence implicates the effectiveness of recombinant IL-7 proteins or IL-7-expreesing plasmids as a positive immune regulator of vaccine-induced T cell responses. It has been reported that recombinant IL-7 protein enhances the survival of *Mycobacterium tuberculosis*-infected mice by the activation of antigen-specific effector $CD8^+$ T cells (20). Furthermore, IL-7-expressing plasmids can enhance vaccine-induced CTL and/or Th2-type immune responses in mice injected with HSV-2 gD DNA vaccine (21). Co-formulation of IL-7-expressing plasmids in the HIV-1 DermaVir nanoparticle significantly induced Gag-specific central memory T cell responses but not effector memory T cell responses (22).

Although several reports state that administration of IL-7 as a vaccine adjuvant can enhance antigen-specific T cell responses in small animals, the role and action mechanism of IL-7 in augmenting antigen-specific antibody and T cell responses, respectively, are still unclear, especially in non-human primates (21-23). Thus, it is highly worthwhile to perform a detailed analysis regarding the immunomodulatory effects of IL-7 as a novel DNA vaccine adjuvant. The role of IL-7 in non-human primate models may provide valuable information, because most of the previous reports showing the adjuvant effects of IL-7 were performed in mice models (20,21,23,24).

Here, we evaluated the immunomodulatory effects of human IL-7 (hIL-7)-expressing DNA in cynomolgus monkeys injected with multigenic HCV DNA vaccine. We demonstrated for the first time that coinjection of hIL-7 DNA increases HCV DNA vaccine-induced antibody as well as broad T cell responses in non-human primates.

MATERIALS AND METHODS

Plasmids

HCV DNA vaccine contains three separate plasmids that express the core-NS2 (1～191 a.a. and 809～968 a.a.), E1E2 (192～729 a.a.) and NS34 (1,029～1,971 a.a.). All HCV genes derived from the Korean genotype 1b strain (gHCV) were codon-optimized and synthesized by GenScript (NJ, USA) (25). These synthesized genes were inserted into pGX27, which was fused with the signal sequences of human tissue plasminogen activator (tPa) (26). To construct pGX27-hIL-7, human IL-7 genes were codon-optimized and synthesized by GenScript and then cloned into pGX27 vector (Fig. 1A).

Immunization

Twelve naive cynomolgus monkeys were divided into 3 groups: 2 monkeys as the naive group; 5, group 1; and 5, group 2. A total 800 μg of the following plasmid DNA was intramuscularly administrated with *in vivo* electroporation to each group of monkeys 6 times at the indicated months (Fig. 1B). Group 1, pGX27-tpa-core-NS2 (200 μg)+pGX27-tpa-E1E2 (200 μg)+pGX27-tpa-NS34 (200 μg)+pGX27-mock (200 μg); Group 2, pGX27-tpa-core-NS2 (200 μg)+pGX27-tpa-E1E2 (200 μg)+pGX27-tpa-NS34 (200 μg)+pGX27-hIL-7 (200 μg).

Synthetic HCV peptides

A total of 156 overlapping peptides having 20 amino acids in length with 10 amino acid overlap was synthesized by Peptron Inc. (Daejeon, S. Korea): 12 peptides for core (43～172 a.a.), 18 peptides for E1 (192～381 a,a.), 31 peptides for E2 (384～713 a.a.), 15 peptides for NS2 (809～968 a.a.), 16 for NS3 protease (1,029～1,217 a.a.), 34 for NS3 helicase (1,208～1,647 a.a.) and 30 peptides for NS4 (1,657～1,966 a.a.). All peptide sequences used for stimulation in the IFN-γ ELISPOT assay were derived from the gHCV vaccine strain (genotype 1b) (27).

IFN-γ ELISPOT assay

The ELISPOT assay was performed according to the manu-

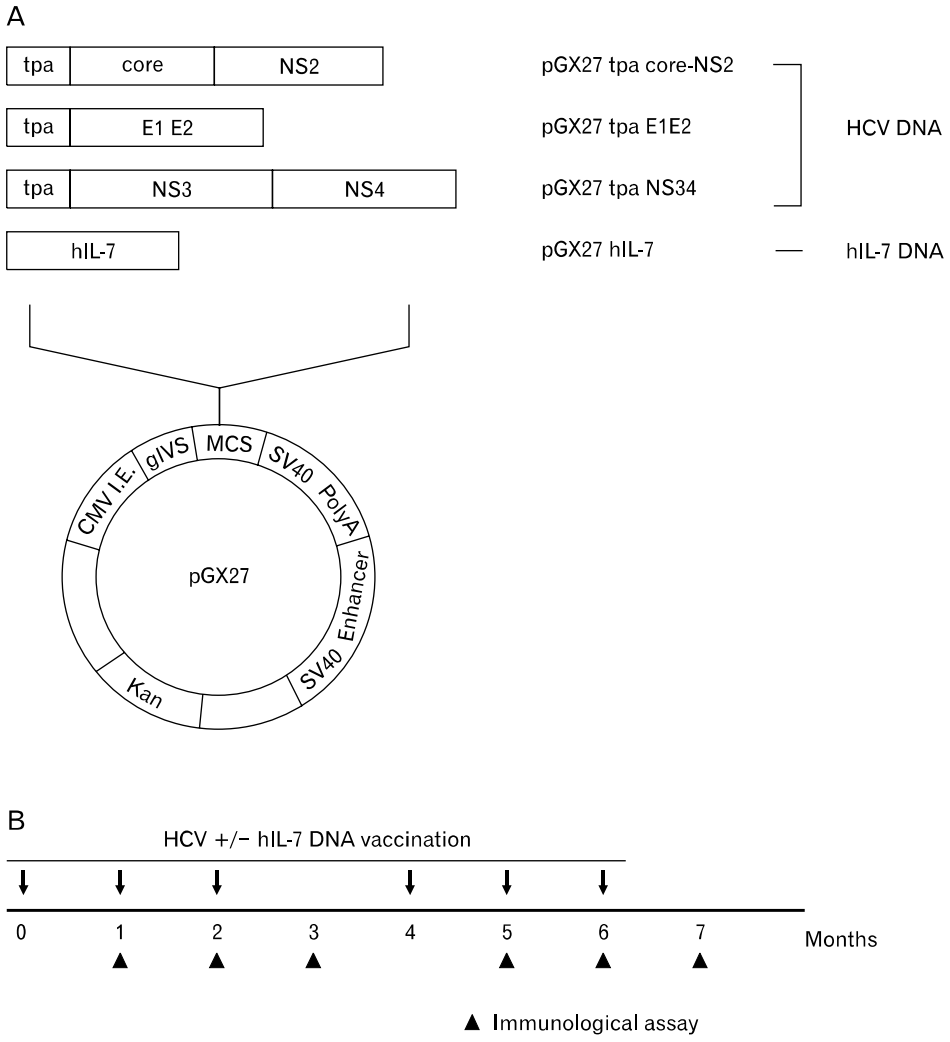

Figure 1. Schematic diagrams of DNA constructs and experimental schedules. (A) HCV DNA vaccines consisting of three separate plasmids encoding core-NS2, E1E2 and NS34 were prepared as described in Materials and Methods. As a vaccine adjuvant, the gene encoding hIL-7 was cloned into pGX27 vector. (B) HCV DNA vaccines with (group 2) or without (group 1) hIL-7 DNA were intramuscularly immunized with *in vivo* electroporation to each group of monkeys six times at the indicated months.

facturer's instructions in the IFN-γ ELISPOT kit with modifications (#CT126-PR20, U-CyTech, Netherlands). In brief, 3×10^5 peripheral blood mononuclear cells (PBMCs) from each monkey were plated onto 96-well plate in triplicate and were stimulated for 24 hours with the indicated peptide pool (1 μg/ml per each peptide). The number of IFN-γ secreting cells was enumerated using an ELISPOT image analyzer (AID GmbH, Germany). Results were expressed as spot-forming cells (SFCs) per 10^6 PBMCs.

To characterize peptides recognized by NS3-specific T cells in monkey PBMCs, the same numbers of PBMCs from each group 1 and group 2 monkey were pooled and then stimulated with each of the peptides form NS3 protease (1,029～1,217 a.a.) and NS3 helicase (1,208～1,647 a.a.) at a concentration of 10 μg/ml. Results were expressed as SFCs per 1×10^6 PBMCs. Cutoff values were set to have a higher SFCs than those of control (stimulated with irrelevant synthetic peptide, EDRNNSHSEEQNEKQ) plus 3 standard deviations.

ELISA

Plasma samples were diluted 1/50 and used for the determination of anti-E2 IgG responses by standard ELISA technique as described (6,25,28). Plates were coated with 50 μl (2 μg/ml) of hgh-E2t (384～718 a.a., derived from the gHCV strain) (25). Color was generated by adding TMB substrate, and optical density was measured at 450 nm using an ELISA reader (Bio-Tek instruments).

Statistical analysis

The difference in immune responses between groups was determined with *student's T test*. A p value of less than 0.05 was considered statistically significant.

RESULTS

Codelivery of hIL-7 enhances HCV-specific T cell responses in cynomolgus monkeys injected with multigenic HCV DNA vaccine

To investigate HCV-specific immune responses induced by multigenic HCV DNA vaccination in the presence or absence of hIL-7 DNA, twelve cynomolgus monkeys were divided into 3 groups: 2 naive monkeys; 5 monkeys immunized with HCV DNA vaccine (group 1); and 5 monkeys immunized with HCV DNA vaccine plus hIL-7 DNA (group 2). The adjuvant effect of hIL-7 DNA on the induction of HCV DNA vaccine-induced T cell responses was evaluated by IFN-γ ELISPOT assays for HCV core, E1, E2, NS2, NS3 and NS4 proteins using cryopreserved PBMCs at four weeks after each immunization (Fig. 1B). As expected, PBMCs from two naive monkeys during this study showed no HCV-specific IFN-γ production (Fig. 2A). Additionally, no significant T cell responses specific for HCV peptide pools were detected in HCV multigenic DNA-immunized group (group 1) even after the second vaccination. This observation is consistent with previous reports that, in contrast to small animals such as mice, twice vaccination with DNA is unlikely to induce antibody and T cell immune responses in non-human primates (29). However, these HCV-specific T cell responses became detectable after the third vaccination and then continuously increased by repeated DNA vaccination until the sixth vaccination. Codelivery of hIL-7 DNA significantly increased the total HCV-specific T cell responses ($p<0.05$; after the 4^{th} and the 6^{th} immunization), in comparison to multigenic HCV DNA alone. Moreover, coimmunization of hIL-7 DNA elicited detectable T cell responses induced by HCV DNA vaccine even after the second vaccination, and increased vaccine-induced T cell responses against all vaccine antigens such as core-NS2, E1E2 and NS34. This suggests that the hIL-7 induces earlier T cell responses elicited by codelivered HCV DNA vaccine and its adjuvant effect is not restricted to nature of certain viral antigens.

Increased T cell responses by codelivery of hIL-7 are due to enhancement of breadth and magnitude of immunity against dominant and subdominant epitopes

To determine the action mechanisms related to the enhancement of T cell responses by codelivery of hIL-7 in multigenic HCV DNA vaccination, we compared the HCV DNA vaccine alone group (group 1) and the hIL-7 DNA codelivered group (group 2) in terms of the breadth and magnitude of vaccine-induced T cell responses against each overlapping peptide spanning the entire HCV NS3 region by IFN-γ ELISPOT assay at four weeks after the sixth immunization (Fig. 2B). In group 1, we identified antigen-specific T cell responses specific for four (#1069, #1078, #1098 and #1178) and two (#1228 and #1588) peptides containing NS3 protease- and helicase-specific immunodominant epitopes, respectively. The T cell frequency was significantly increased in group 2 in regards to these immunodominant epitopes (#1069, #1078, #1098, #1178 and #1588) except for the #1228 peptide, which indicates the codelivery effect of hIL-7 DNA on overall enhancement of antigen-specific T cell immunity specific to the immunodominant epitopes. Interestingly, codelivery of hIL-7 DNA appeared to induce T cell responses specific to additional two (#1128 and #1138) and one (#1238) peptide from NS3 protease and helicase regions, respectively, which were not immunogenic in group 1. This indicates that codelivery of hIL-7 broadens DNA vaccine-induced T cell immunity by inducing HCV-specific T cell responses specific for subdominant epitopes. Taken together, we demonstrated that the enhanced HCV DNA vaccine-induced T cell responses observed in group 2 are due to the increase of breadth and magnitude of HCV-specific T cell responses specific to subdominant and dominant epitopes, respectively.

Codelivery of hIL-7 increases anti-E2 antibody responses in cynomolgus monkeys injected with multigenic HCV DNA vaccine

To investigate the adjuvant effect of hIL-7 DNA on the induction of antibody responses by HCV DNA vaccine, the anti-E2 antibody response was measured longitudinally using the plasma from naive and immunized monkeys, since the titer of anti-E2 antibody is closely associated with protection from HCV infection (6). As expected, 2 naive monkeys did not show any anti-E2 antibody response through this study (Fig. 3). The anti-E2 antibody response became detectable after the second immunization in both group 1 and group 2,

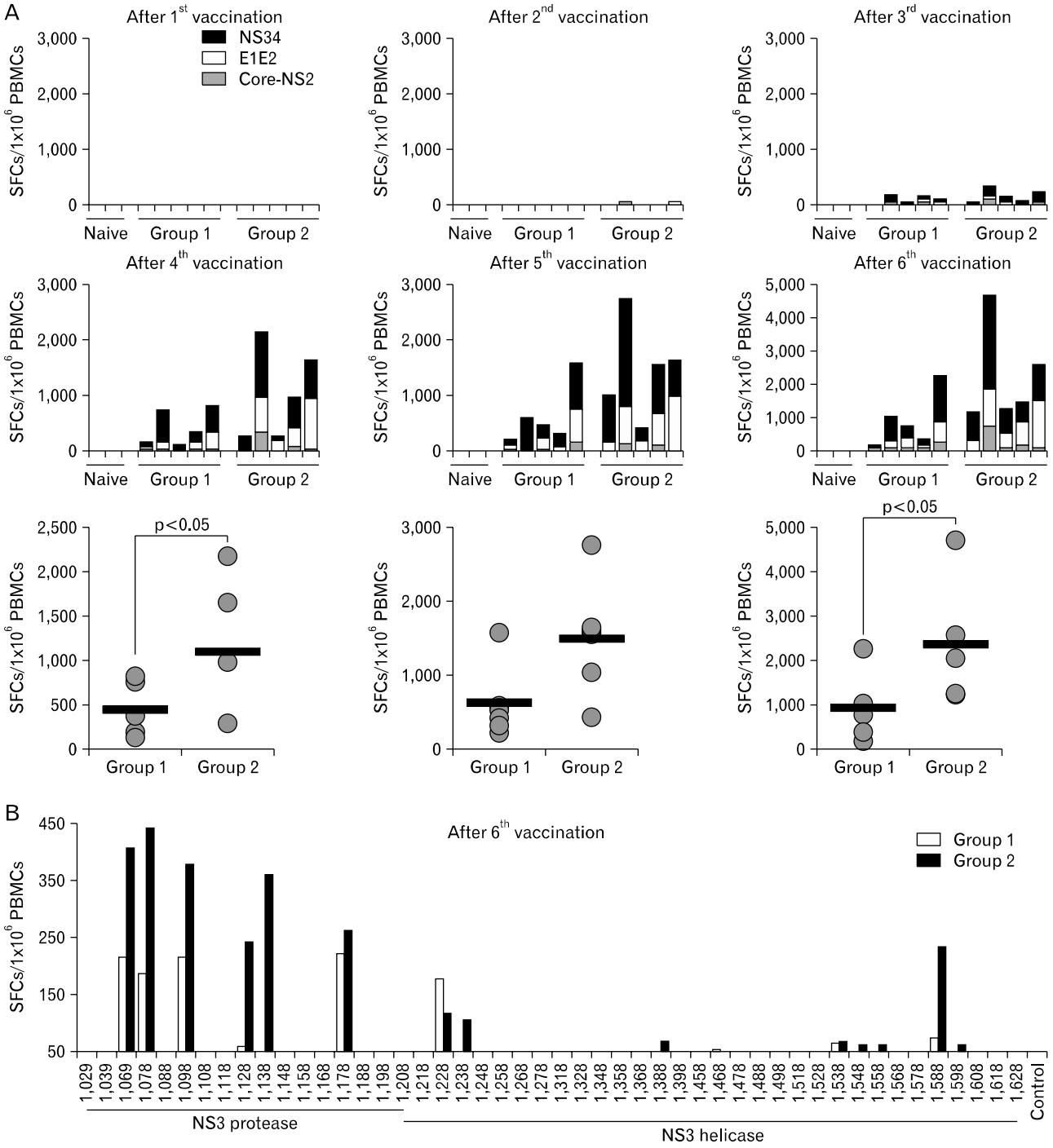

Figure 2. Effect of hIL-7 codelivery on multigenic HCV DNA vaccine-induced T cell responses. (A) For longitudinal analysis of HCV-specific T cell responses in cynomolgus monkeys, IFN-γ ELISPOT assays using PBMCs stimulated with peptide pools encompassing core-NS2, E1E2 and NS34 were performed. To evaluate the T cell adjuvant effects of hIL-7 after the 4th, 5th and 6th vaccination, the results were rearranged to show total HCV-specific T cell responses of each group. Responses are indicated as the number of IFN-γ-secreting cells per 1×10^6 PBMCs. (B) At 4 weeks after the 6th immunization, DNA vaccine-induced T cell responses against each 20-mer peptide spanning NS3 protease (1,029~1,217 a.a.) and NS3 helicase (1,208~1,647 a.a.) were examined by IFN-γ ELISPOT assay. Responses are indicated as the number of IFN-γ-secreting cells per 1×10^6 PBMCs.

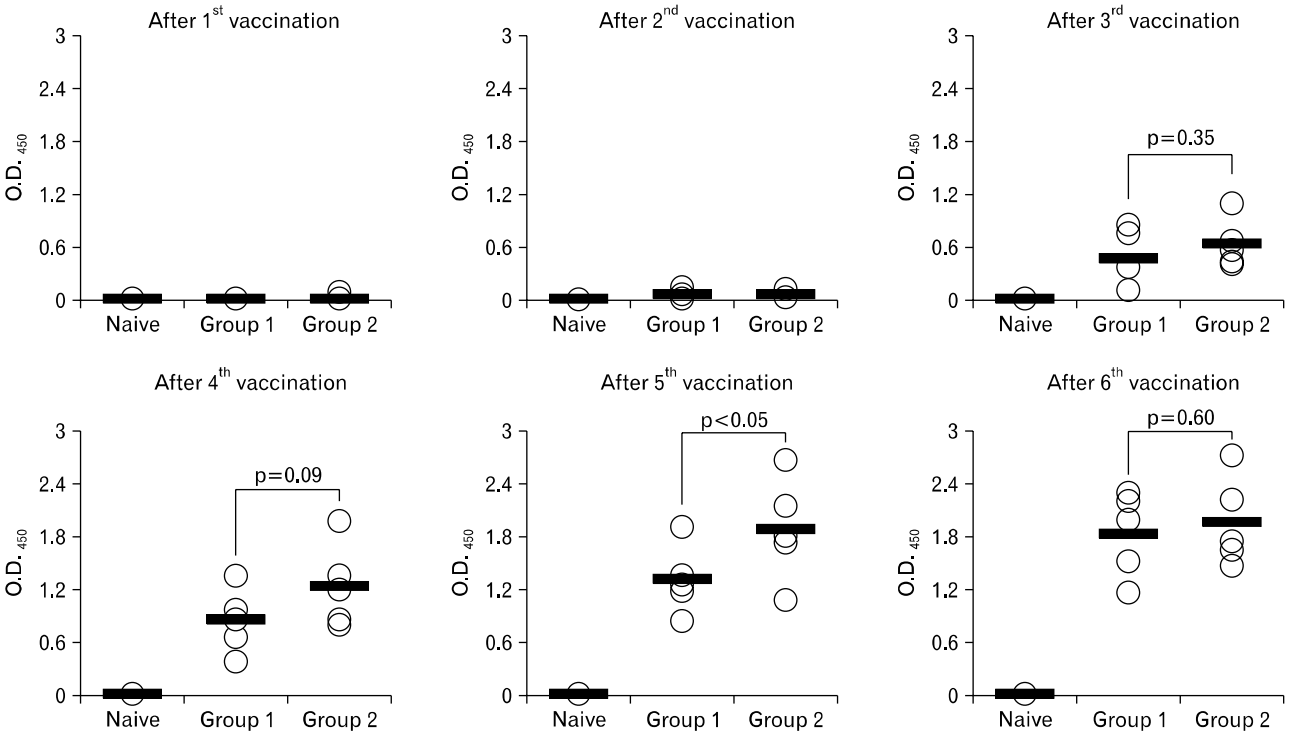

Figure 3. Effect of hIL-7 codelivery on HCV DNA vaccine-induced anti-E2 antibody responses. For longitudinal analysis of anti-E2 antibody response in cynomolgus monkeys, anti-E2 total IgG responses were determined by ELISA. Antibody responses were expressed as absorbance at 450 nm within the linear range.

which were significantly increased after the third immunization. These antibody responses were continuously increased by additional immunization until the sixth immunization. Codelivery of hIL-7 slightly enhanced vaccine-induced anti-E2 antibody responses after the third and the forth immunization (p=0.35 and p=0.09, respectively), and the enhancement of anti-E2 antibody responses by codelivery of hIL-7 DNA became statistically significant after the fifth immunization ($p<0.05$). However, the adjuvant effects of hIL-7 DNA were diminished after the sixth immunization (p=0.60).

DISCUSSION

We demonstrated for the first time that codelivery of hIL-7 DNA as a genetic adjuvant augmented multigenic DNA vaccine-induced T cell responses by enhancing both the magnitude and breadth of T cell responses in non-human primate models. Additionally, hIL-7 appeared to increase DNA vaccine-induced anti-E2 antibody response. Since IL-7 has been known to play a role in T cell homeostasis and survival, several studies have examined the adjuvant effects of IL-7 on T cell responses in small animal models (22,23,30). As previously reported, IL-7 augments the number of tumor-reactive T cells responding to subdominant tumor antigens in tumor-bearing hosts and lowers the threshold level for graft-versus-host disease (31). However, little is known about the adjuvant effect of IL-7 on the modulation of antibody as well as T cell responses in non-human primates. IL-7 is recognized as playing an important role in early B-cell development including survival, proliferation and maturation (32). Continuous *in vivo* administration of recombinant IL-7 significantly increased B-cell numbers by expanding pre-B cell compartment (33). In contrast to the administration of recombinant IL-7 protein, the injection of IL-7-expressing plasmid induces sustained *in vivo* expression of IL-7. Thus, it is possible that co-administration of IL-7 DNA, as demonstrated in this study, may possibly expand the pre-B-cell pool, which can further increase naive B-cell population that can differentiate into plasmablasts or plasma cells.

There are accumulating results that large animals such as

non-human primates are less immunogenic than small animal for DNA vaccine-induced immunity, which may be an intrinsic drawback of DNA vaccines. However, our results suggest that repeated DNA vaccination up to six times may overcome this limitation by eventually inducing strong antibody and T cell responses. Furthermore, we showed that codelivery of hIL-7 DNA augmented the breadth and magnitude of T cell responses after six repeated administration of HCV DNA vaccine. It was previously reported that both broad HCV-specific T cell response and high titers of anti-E2 antibody response have been shown to play a critical role in protection against HCV infection (3-7). Together, our results may provide valuable information for designing an effective HCV DNA vaccine to prevent vaccinee from HCV infection or play a significant role in viral clearance in chronically-infected individuals with HCV.

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CONFLICTS OF INTEREST

The authors declare no financial or commercial conflict of interest.

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