Hepatitis C Virus–Like Particles Combined With Novel Adjuvant Systems Enhance Virus-Specific Immune Responses

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We have previously described the generation of hepatitis C virus–like particles (HCV-LPs) in insect cells and shown that immunization with HCV-LPs elicited both humoral and cellular immune responses in mice. To further characterize the HCV-LPs as a vaccine candidate, we evaluated the effects of adjuvant AS01B (monophosphoryl lipid A [MPL] and QS21), CpG 10105, and the combination of the 2 adjuvants on the immunogenicity of HCV-LPs in AAD mice (transgenic for HLA-A2.1). All HCV-LP–immunized mice (with or without adjuvant) developed high titers of anti-HCV E1/E2 antibodies after 4 injections intramuscularly. However, antibody titers in mice immunized with HCV-LP plus AS01B, plus CpG 10105, or plus the combination of AS01B and CpG 10105 were 4, 3, and 10 times higher, respectively, than that of HCV-LP alone. Isotype analysis of the induced anti-envelope antibodies showed that HCV-LP alone induced a predominant immunoglobulin (Ig) G1 response. In contrast, when the 2 adjuvants AS01B and CpG 10105 were combined, the response became predominantly IgG2a whereas HCV-LP plus AS01B or CpG 10105 gave a mixed IgG1 and IgG2a response, indicating that AS01B and CpG 10105 promote a more T-helper type 1 (Th1) response and that combining the 2 adjuvants results in an additive or synergistic interaction. These observations were further confirmed by the results of CD4$^+$ enzyme-linked immunospot assay for interferon (IFN)-γ and interleukin (IL)-4 and intracellular cytokine staining of IFN-γ producing CD8$^+$ cells. In conclusion, HCV-LP is a promising vaccine candidate against HCV infection and the adjuvants used are potent immune enhancers for this approach. (HEPATOLOGY 2003;37:52-59.)

Hepatitis C virus (HCV) is a major causative agent of acute and chronic hepatitis, with 170 million persons infected worldwide.[1] Prospective studies have shown that 75% of cases of acute HCV can progress to chronic infection, of which 10% to 20% will develop complications of chronic liver disease such as liver cirrhosis and 1% to 5% will develop hepatocellular carcinoma.[2] However, therapy for chronically infected patients has been mostly dependent on interferon (IFN)-based regimens which, despite the improvement in treatment response with combination therapy, are at best effective in approximately 50% of infected persons.[3,4]

To date, there is no effective vaccine against HCV infection. Efforts to develop an HCV vaccine are complicated by the extensive genetic and possible antigenic diversity among HCV strains, the absence of a robust immunity after natural infection,[5] and the lack of tissue culture systems and small animal models. Accumulating evidence obtained from human and chimpanzee studies[6-9] suggests that strong HCV-specific cytotoxic T-cell (CTL) responses against structural and nonstructural proteins are likely to be important in viral clearance and possible protection. Thus, an ideal HCV vaccine may need to induce strong humoral responses against the envelope proteins and to prime broad, HCV-specific T-helper and CTL responses.[5] Of the cellular immune responses, the induction of T-helper type 1 (Th1) response, which has been linked to viral clearance in HCV[10-12] and other viral infections,[13] is probably important for an effective HCV vaccine.

Virus-like particles are attractive as a recombinant protein vaccine, because they mimic closely the properties of

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; CTL, cytotoxic T-cell; Th1, T-helper type 1; HCV-LP, hepatitis C virus–like particles; MPL, monophosphoryl lipid A; PBS, phosphate-buffered saline; Ig, immunoglobulin; IL, interleukin; SFU, spot-forming units.

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naive virions.[14] We have reported the synthesis of HCV-like particles (HCV-LPs) using a recombinant baculovirus that contains the complementary DNA of the HCV structural proteins (core/E1/E2). These HCV-LPs have biophysical, ultrastructural, and antigenic properties similar to the putative virions.[14] We recently reported that BALB/c mice[15] and HLA-A 2.1 transgenic (AAD) mice[16] immunized with HCV-LPs generate strong and broad humoral and cellular immune responses against HCV structural proteins. Furthermore, adoptive transfer of lymphocytes from HCV-LP–immunized mice to naive mice provided protection against recombinant HCV-vaccinia challenge in AAD mice.[16]

To further characterize the HCV-LPs as a vaccine candidate and to maximize its ability to elicit strong humoral and cellular immune responses of the Th1 type, we evaluated the effects of 2 vaccine adjuvants on the immunogenicity of HCV-LPs in AAD mice. CpG 10105 (provided by Coley Pharmaceutical Group, Wellesley, MA) is an immunostimulatory oligodeoxynucleotide optimized for immune stimulation of many species, including mice and humans. AS01B contains monophosphoryl lipid A (MPL) and QS21, a naturally occurring saponin molecule. These adjuvants have been shown to enhance the speed, vigor, and persistence of the immune responses by improving antigen presentation to T cells and the interaction between immunogen and macrophage[17,18] and/or preferentially stimulating Th1 response by modulation of the cytokine network in the local microenvironment.[19,20]

Materials and Methods

Production of HCV-LPs. Construction of recombinant baculovirus bvHCV.Sp7$^-$ containing the complementary DNA for the HCV structural proteins (genotype 1b J strain) has been described.[21] bvGUS generated from pFastBacGUS containing the coding sequence of the enzyme β-glucuronidase was used as the control baculovirus. HCV-LPs were partially purified by sucrose gradient centrifugation as described previously.[22]

Adjuvants. AS01B (MPL and QS21) was a gift from GlaxoSmithKline. CpG 10105 was kindly provided by Coley Pharmaceutical Group.

Immunization of Mice. AAD mice (6-8 weeks old) expressing the transgene with the $\alpha1$ and $\alpha2$ domains from the human HLA-A2.1 and the $\alpha3$ domain of murine H-2D^d in the C57BL/6 background[23] were obtained from Victor Engelhard (University of Virginia). Five groups of 4 mice each were immunized 4 times at 3-week intervals with 20 μg of p7$^-$ HCV-LPs into each quadriceps muscle in a total volume of 100 μL based on previously described immunization protocols[15,16]: group 1, HCV-LP alone + phosphate-buffered saline (PBS); group 2, HCV-LP + AS01B (50 μL); group 3, HCV-LP + 50 μg CpG 10105; group 4, HCV-LP + combination of AS01B (50 μL) and 50 μg CpG 10105; group 5, bvGUS (control preparation) + AS01B (50 μL). All animal experiments were conducted according to criteria published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Anti-HCV Envelope Antibodies Assay. Blood samples before immunization and 2 weeks after each immunization were collected from the tail vein and analyzed for HCV E1/E2 antibodies by enzyme-linked immunosorbent assay as described previously.[15] Isotype-specific secondary anti-mouse antibodies (American Qualex Antibodies, San Clemente, CA) were used for immunoglobulin (Ig) G subclass determination.

Enzyme-Linked Immunospot for IFN-γ and Interleukin-4. Ninety-six–well nitrocellulose-bottomed plates (Millititer; Millipore, Bedford, MA) were coated with 100 μL of anti–IFN-γ (R4-6A2) or anti–interleukin (IL)-4 (BVD4-1D11) antibodies (PharMingen, San Diego, CA) at 2 μg/mL in PBS and incubated overnight at 4°C. After removing unbound antibodies by 4 washes with PBS, plates were blocked for 1 hour at room temperature with 100 μL PBS/well containing 1% enzyme-grade bovine serum albumin. After washing twice with PBS, plates were blocked for 1 hour at room temperature with 100 μL RPMI 1640 medium containing 5% heat-inactivated fetal calf serum. A total of 100 μL splenocytes (3×10^5) in RPMI 1640 medium containing 10% fetal calf serum and 100 μL of 2 μg/mL antigen (core or E1/E2 protein) in RPMI 1640 medium without fetal calf serum were added to each well and incubated at 37°C with 5% CO_2 to allow production and capture of released cytokine. After 30 hours of incubation, plates were washed 3 times with PBS and 4 times with PBS containing 0.05% Tween 20. Biotinylated anti–IFN-γ (XMG1.2) or anti–IL-4 (BVD6-24G2) antibodies at 0.25 μg/mL (PharMingen) were added and incubated overnight at 4°C. The plates were washed 4 times with PBS containing 0.05% Tween 20 and incubated for 2 hours with 100 μL peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Unbound antibodies were removed by 4 washes with PBS. Finally, 100 μL of substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad Laboratories, Richmond, VA) was added and incubated until blue spots appeared (approximately 30 minutes). The reaction was stopped by rinsing the wells with distilled water. Plates were counted by KS Elispot-Axioplan 2I (Zeiss, Thornwood, NY) and results expressed as spot-forming units (SFU) per 3×10^5 cells. Splenocytes alone without addition of antigen were used as negative control and with addition of 10 μg/mL phytohemagglutinin (Sigma-Aldrich, St. Louis, MO) as positive control. All determinations were run in duplicate. The number of HCV-specific spots was determined by subtracting the number of spots in the absence of antigen (negative control) from the number of spots in the presence of antigen.

Intracellular Cytokine Staining. Splenocytes (3×10^7) from immunized mice were resuspended in 5 mL RPMI 1640 medium containing 10% fetal calf serum in 6-well plates. These responder cells were then stimulated with 10% rat T-STIM without concanavalin A (Becton Dickinson, Bedford, MA) and with 10 μg/mL of HCV core (amino acids 131-140 ADLMGYIPLV) or E2 (amino acids 614-622 RLWHYPCTI) peptide[24] for 5 days in a humidified incubator at 37°C with 5% CO_2. The stimulated cells (2×10^6) were distributed into 5-mL polystyrene round-bottom tubes and resuspended in 200 μL fresh RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were further stimulated with 10 μg/mL HCV core or E2 peptide or 1 μmol/L ionomycin and 20 ng/mL phorbol myristate acetate as positive controls or without peptide as negative controls. After an additional 2 hours of incubation at 37°C, 0.2 μL of 10 μg/mL brefeldin A (Sigma-Aldrich) was then added and incubated for another 4 hours. Cells were washed once with PBS and stained with 2 μL fluorescein isothiocyanate anti-CD8 (PharMingen) at 4°C for 1 hour. After washing once with PBS, the cells were fixed with 1% paraformaldehyde for 20 minutes at room temperature. After washing twice with PBS, cells were stained with R-phyeserythrin (PE) conjugated anti-mouse IFN-γ (PharMingen) or control PE-conjugated rat IgG1 isotype (PharMingen) in PBS containing 0.3% saponin (Calbiochem, San Diego, CA) overnight at 4°C. Cells were washed once with PBS and analyzed by fluorescence-activated cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, CA) using CellQuest software (Becton Dickinson). The percentage of HCV-core and -E2 specific IFN-γ^+CD8$^+$ cells was determined by subtracting the percentage of IFN-γ^+CD8$^+$ cells in the absence of HCV peptide from that in the presence of HCV peptide.

Statistical Analysis. Comparisons between groups of mice were analyzed using Student's *t* test. All tests were 2-tailed, and differences were considered significant when $P \leq .05$.

Results

Humoral Immune Response. Anti-E1/E2–specific humoral immune responses in mice immunized with HCV-LP alone or with various adjuvanted HCV-LP formulations were analyzed by enzyme-linked immunosorbent assay.[15] As a negative control, sera from mice immunized with sucrose gradient preparation of bvGUS-infected insect cells plus AS01B were tested in parallel. Serum samples collected before immunization and 2 weeks after each immunization were tested in duplicate.

All HCV-LP–immunized mice (with or without adjuvant) developed detectable anti-E1/E2 antibodies after the third immunization and titers increased after the fourth immunization. In contrast, animals immunized with the control preparation (bvGUS) + AS01B were negative for anti-E1/E2 antibodies at all time points (Fig. 1A). However, there was no significant difference in the timing of antibody development between HCV-LP alone and HCV-LP plus adjuvant(s). End-point titration of the serum samples after the fourth immunization showed that the mean antibody titers in mice immunized with HCV-LP plus AS01B (1:8,000), CpG (1:6,400), or AS01B and CpG in combination (1:22,400) were approximately 4, 3, and 10 times greater, respectively, than that in the HCV-LP–alone group (1:2,000) (Fig. 1B and Table 1). These results indicate that the adjuvants AS01B and CpG 10105 boosted the strength of humoral responses in mice but did not affect the timing of anti-envelope antibody production.

Isotype analysis of the induced E1/E2 antibodies showed that HCV-LP alone induced a predominant IgG1 response (IgG2a/IgG1 ratio, 0.58). In contrast, when the HCV-LP was administered with AS01B or CpG, a greater IgG2a response was induced (IgG2a/IgG1 ratios of 1.69 and 1.15, respectively). Remarkably, when AS01B and CpG were combined, the response became predominantly IgG2a (IgG2a/IgG1 ratio, 3.3). These results indicate that AS01B or CpG 10105 alone is capable of inducing a more Th1 response and the combination of the 2 adjuvants further boosts a Th1-biased isotype switching.

CD4$^+$ T-Helper Cell Response. To quantitatively examine the T-helper responses against the HCV structural proteins in AAD mice immunized with either HCV-LP alone or HCV-LP and adjuvant formulations, we performed enzyme-linked immunospot assay for IFN-γ and IL-4. Splenocytes were harvested after the fourth immunization and stimulated with recombinant HCV core or E1/E2 proteins as described in Materials and Methods. The number of HCV-specific SFU was calculated by subtracting the number of spots in the absence of antigen from those in the presence of antigen. As shown in Fig. 2A, IFN-γ–positive SFU against the core protein were detected in all groups of mice immunized

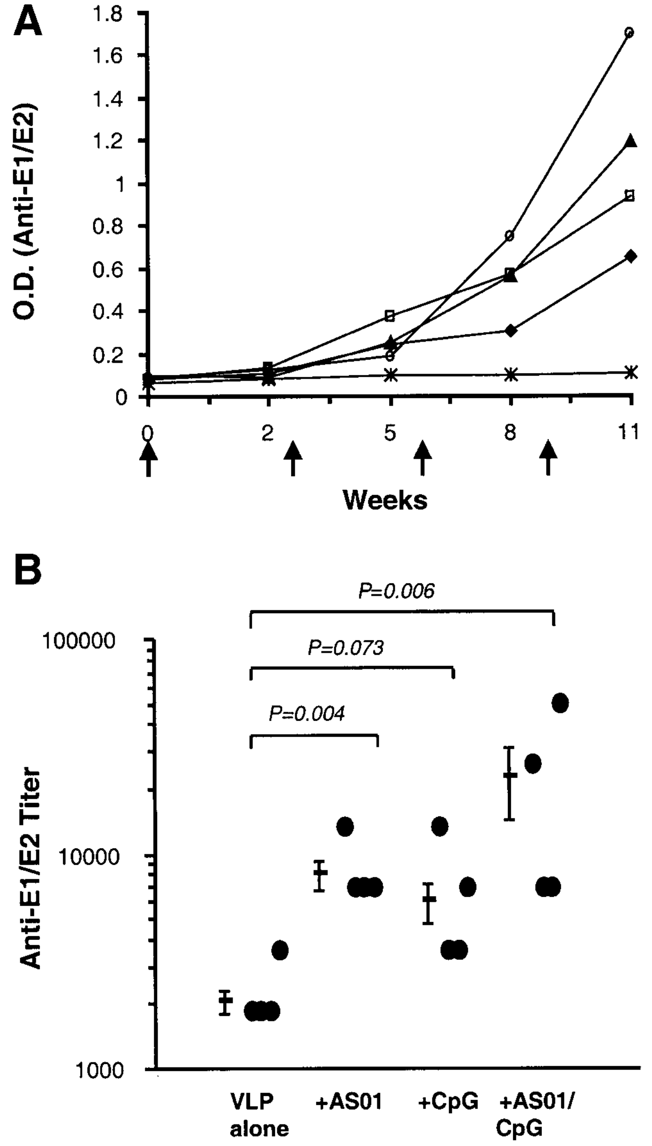

Fig. 1. Anti-E1/E2 antibody response in AAD mice immunized with HCV-LP alone or HCV-LP plus adjuvant(s). (A) Kinetics of humoral responses in HCV-LP- and HCV-LP/adjuvant(s)-immunized mice. The mice were immunized 4 times at 3-week intervals (time of immunization indicated by **arrows**) as described in Materials and Methods. Blood samples were collected before immunization and 2 weeks after each immunization. Anti-E1/E2 optical density (O.D.) of 405 nm represents the mean value of serum samples diluted 1:200 from 4 mice in each group. ◆, HCV-LP alone; □, HCV-LP and AS01B; ▲, HCV-LP and 50 μg CpG 10105; ○, HCV-LP and combination of AS01B and 50 μg CpG 10105; *, control bvGUS and AS01B. (B) Anti-E1/E2 titers in mice immunized with HCV-LP alone and HCV-LP/adjuvant(s). The 4 groups of HCV-LP–immunized mice are indicated as HCV-LP alone, +AS01B, +CpG, and +AS01/CpG, respectively. The anti-E1/E2 titers by end-point dilution in enzyme-linked immunosorbent assay are shown for each mouse. The mean titers ± SEM are shown as the bar on the left, and *P* values are shown above.

with HCV-LP alone or HCV-LP plus adjuvant(s), whereas no spots were detected in any of the control mice. However, SFU were significantly higher in all 3 groups receiving HCV-LP plus adjuvant(s) than that of HCV-LP alone ($P < .05$) (Fig. 2A). In contrast, significantly higher IFN-γ–positive SFU against E1/E2 protein were observed in mice immunized with HCV-LP in combination of AS01B and CpG compared with that of HCV-LP alone (Fig. 2B). The group of mice that received HCV-LP plus AS01B also showed higher IFN-γ–positive SFU against E1/E2 protein in 2 of 4 mice compared with that of HCV-LP alone (Fig. 2B), although the difference did not reach statistical significance ($P = .065$). In addition, HCV-LP plus CpG alone did not show any increase in SFU compared with that of HCV-LP alone (Fig. 2B). Furthermore, all groups of HCV-LP–immunized mice (with or without adjuvant) showed no or weakly positive IL-4 response against core protein and some positive response against E1/E2 protein. There were no significant differences in the number of IL-4–positive SFU among these groups (Fig. 2C and 2D). IFN-γ response seen in the HCV-LP–immunized animals suggested that HCV-LP immunization induced a Th1 response against the core but more of a Th0 response against the E1/E2 protein in AAD mice. Similar to the results of anti-E1/E2 isotype analysis, the addition of AS01B and CpG 10105 singly or in combination seemed to enhance the Th1 response.

CD8$^+$ CTL Response. To quantitatively examine and compare the CTL responses between mice immunized with HCV-LP alone and HCV-LP plus adjuvant(s), we performed intracellular cytokine staining of CD8$^+$ T cells. The splenic cells were stained for IFN-γ^+CD8$^+$ cells after *in vitro* stimulation with HLA-A2.1 restricted core or E2 peptide. As shown in Fig. 3, the percentage of HCV-core and -E2 specific IFN-γ^+CD8$^+$ cells in HCV-LP–immunized mice (with or without adjuvant) was significantly higher than that in the control group ($P < .05$), indicating that CD8$^+$ CTL responses were specific to HCV structural proteins. In mice immunized with HCV-LP alone, the percentages of HCV core- and E2-specific IFN-γ^+CD8$^+$ cells were 11.8% and 8.5%, respectively (Fig. 3). The percentage of HCV core-specific IFN-γ^+CD8$^+$ cells was higher in mice immunized with HCV-LP plus adjuvant(s) (16.3% in AS01B, 16.4% in CpG, and 18% in combination of AS01B and CpG) than that in HCV-LP alone. However, statistical significance was only observed in mice immunized with HCV-LP plus the combination of AS01B and CpG ($P = .04$) compared with that of HCV-LP alone (Fig. 3). In contrast, the percentage of HCV E2-specific IFN-γ^+CD8$^+$ cells in mice immunized with HCV-LP plus adjuvant(s) was not significantly different from that in HCV-LP alone (Fig. 3). These results indicate that HCV-LP could induce HCV-specific IFN-γ^+CD8$^+$ cells against both core and

Table 1. Effects of Adjuvant(s) on Immunogenicity of HCV-LP

	HCV-LP Alone	HCV-LP + AS01	HCV-LP + CpG	HCV-LP +AS01/CpG
Anti-E1/E2 antibody titer	1:2,000	1:8,000	1:6,400	1:22,400
IgG2a/IgG1 ratio	0.58	1.69	1.15	3.3
IFN-γ^+ CD4$^+$ enzyme-linked immunospot (SFU)				
Core	7.8	15.5	15.3	30.8
E1/E2	6.4	20.4	2.5	18.4
IFN-γ^+ CD8$^+$ cells (%)				
Core	11.8	16.3	16.4	18
E2	8.5	10.2	6.5	8.6

NOTE. Data represent the mean values of each group of mice.

E2 peptides. All 3 adjuvant formulations further boosted the induction of HCV-specific IFN-γ^+CD8$^+$ cells against the core peptide but apparently not against the E2 peptide. The reason for such preferential boosting of core-specific CD8$^+$ T cells by adjuvant(s) is not clear. It is possible that this may be the result of differential cell expansion during *ex vivo* stimulation. Alternatively, this may be due to the use of single CD8 epitopes that may not reflect the entire CD8$^+$ T-cell responses to HCV core or envelope protein.

Discussion

We previously reported the production of HCV-LPs containing HCV structural proteins (core/E1/E2) in insect cells using baculoviral expression.[21] These noninfec-

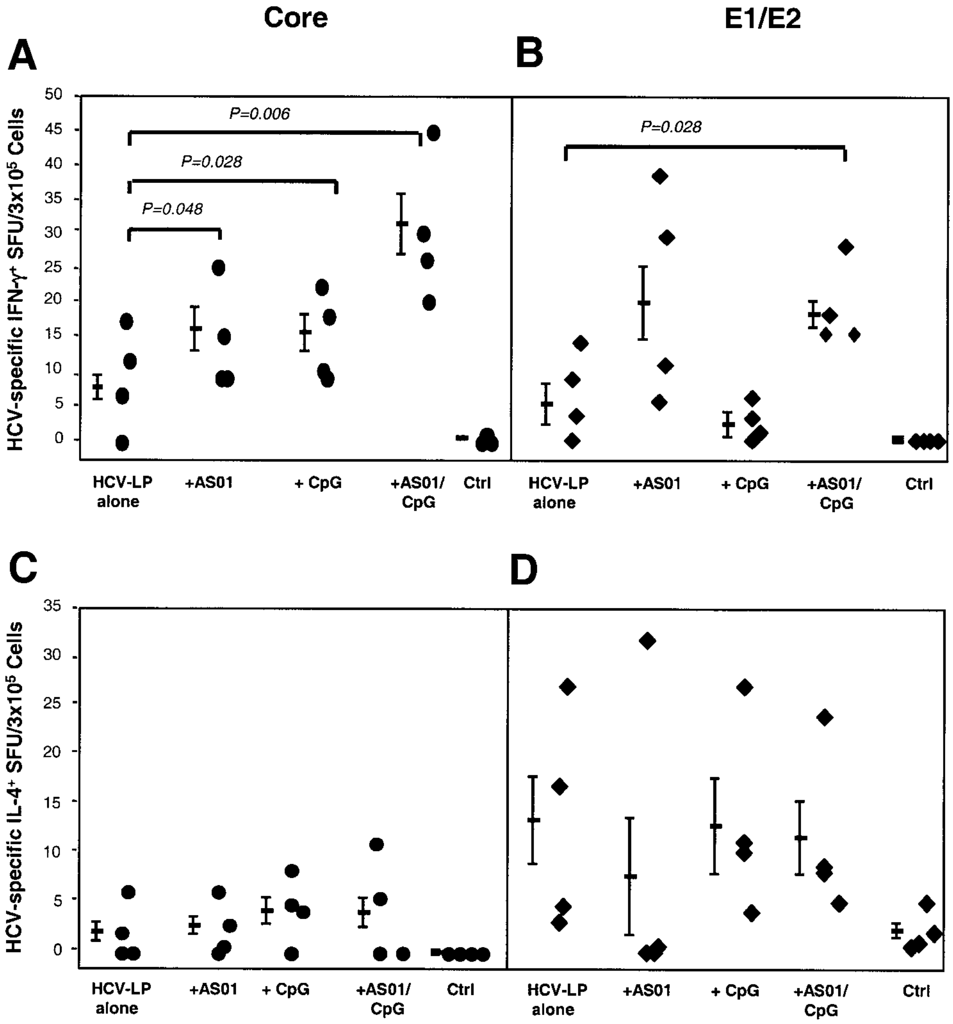

Fig. 2. CD4$^+$ T-helper responses in HCV-LP–immunized mice. (A and B) IFN-γ enzyme-linked immunospot assay and (C and D) IL-4 enzyme-linked immunospot assay of splenocytes after stimulation with (A and C) recombinant HCV core or (B and D) E1/E2 protein. Mean values of spots from duplicate tests are shown for individual mice, and the mean ± SEM is indicated as the bar on the left. Significant *P* values between vaccinated groups (HCV-LP alone, HCV-LP + AS01, HCV-LP + CpG, HCV-LP + AS01/CpG) are shown. Control represents the group of mice immunized with control preparation.

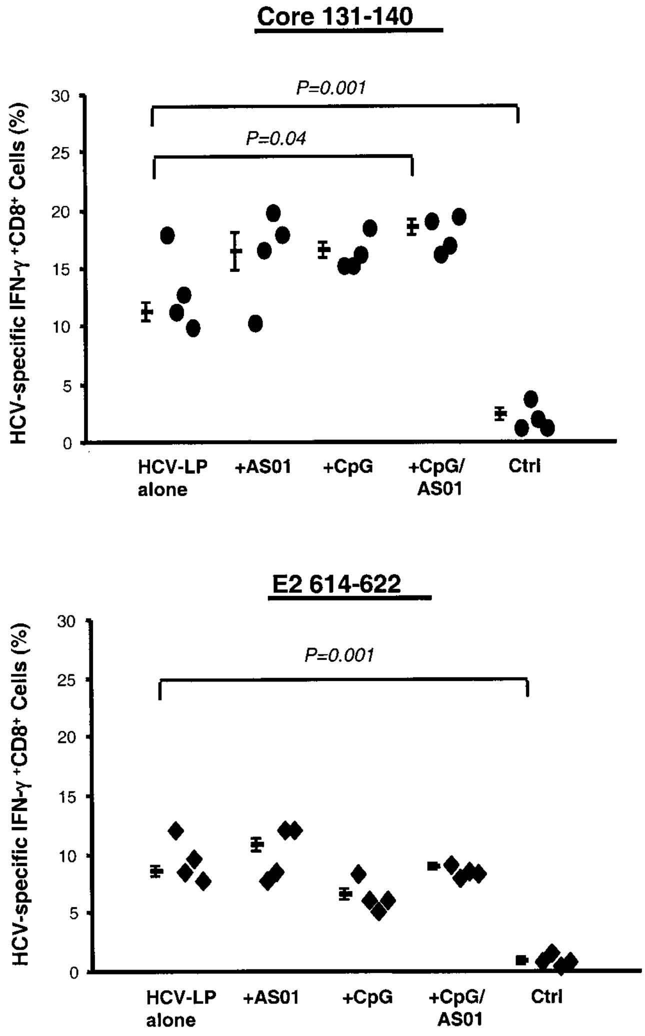

Fig. 3. $CD8^+$ CTL responses by intracellular cytokine staining in HCV-LP–immunized mice. Splenocytes were stimulated *in vitro* with HCV CTL peptide (core or E2) and then analyzed for intracellular IFN-γ staining. For background determination, splenocytes cultured for 5 days without HCV-specific peptide stimulation were tested in parallel with those stimulated with HCV CTL core or E2 peptide. The percentage of HCV-core and -E2 specific $IFN\text{-}\gamma^+CD8^+$ cells from each mouse is shown after subtracting the percentage of $IFN\text{-}\gamma^+CD8^+$ cells in the absence of HCV peptide from that in the presence of HCV peptide. The mean ± SEM for each group is shown as the bar on the left. Significant *P* values are shown.

tious 40- to 50-nm HCV-LPs were able to elicit humoral and cellular immune response against HCV structural proteins (core/E1/E2) in BALB/c mice[14,15] and AAD mice.[16] In this study, we evaluated the effects of adjuvants AS01B (MPL + QS21) and CpG 10105 as well as the combination of the 2 adjuvants on the immunogenicity of HCV-LPs in AAD mice. Our results show that all 3 vaccine adjuvant formulae were well tolerated except that AS01B was associated with a higher incidence of local reaction at the injection site. We showed herein that the immunogenicity of HCV-LP could be enhanced at both humoral and cellular levels by addition of adjuvants AS01B and CpG 10105 (Table 1). Furthermore, there may be a synergistic effect on the immunogenicity of HCV-LP when AS01B and CpG 10105 are used in combination.

We also observed that the adjuvant AS01B and/or CpG 10105 were able to promote a more Th1 response in HCV-LP–immunized AAD mice. Although MPL, QS21, and CpG may enhance immunogenicity of vaccines through different mechanisms, all have the ability to preferentially stimulate Th1 $CD4^+$ T-helper cells.[19,20,25,26] The Th1-type response is accompanied by secretion of IL-2, IFN-γ, and tumor necrosis factor-α, leading to activation of macrophages, CTL, and a high level of IgG2a antibodies in mice.[17] Th1-type response is highly desirable for vaccines targeting chronic viral diseases or infections caused by intracellular pathogens.[13] Analyses of the cytokine profiles of HCV-specific T cells showed that persons with a Th1 profile (antigen-dependent production of IL-2 and IFN-γ) are more likely to experience viral clearance.[10-12] At present, we do not know whether these adjuvants have direct effects on the HCV-LP itself or alter its antigenicity.

It is possible that a combination of adjuvants is necessary to achieve optimal effects, considering the complex series of events leading to an antigen-specific activation of the immune system. When used in combination, the adjuvant components should facilitate antigen presentation by professional antigen-presenting cells and lead to a potent induction of T-cell–mediated effector and immune memory mechanisms.[27] Indeed, many adjuvant combinations have been shown to give additive or synergistic effects on the humoral and cellular immune responses.[28,29] Weeratna et al.[29] suggested that CpG oligonucleuotides combined with almost any other adjuvant could induce a stronger Th1-type response than with CpG oligonucleotides alone. Therefore, it is not surprising that the combination of the 2 adjuvants AS01B and CpG 10105 resulted in an additive or a synergistic effect on the immunogenicity of HCV-LP in AAD mice.

The role of anti-E1/E2 antibodies in protection against HCV infection remains unclear. Although HCV envelope protein E2 is believed to contain important neutralizing epitopes, the recombinant E1/E2 proteins only protected chimpanzees against low-dose homologous challenge by HCV.[30,31] Nevertheless, self-limited infection occurred more frequently in the vaccinated than in the unvaccinated animals. HCV-LP may have the advantage of generating high levels of antibodies against full-length recombinant envelope proteins, possibly due to its close resemblance to the native form of the proteins. Indeed, papillomavirus- and rotavirus-like particles are potent in generating neutralizing antibodies *in vivo*.[32-34] The function of these anti-E1/E2 antibodies induced by HCV-LP in viral neutralization cannot be tested easily due to the lack of a suitable tissue culture system and a small animal model. Further studies would have to be conducted in a chimpanzee model to test the protective efficacy of HCV-LP.

HCV-LP has been shown previously[14-16] and again herein to generate cellular immune response in vaccinated animals. Strong cell-mediated immunity is essential for protection against many viral infections and is therefore desirable with almost all vaccines. Several human studies have presented evidence that a strong HCV-specific CD4$^+$ T-cell response is associated with resolution of acute hepatitis C[35] or a benign carrier state.[7,36,37] The importance of HCV-specific CD4$^+$ T cells in viral control is also suggested in patients with HCV recurrence after loss of virus-specific CD4$^+$ T-cell response in acute hepatitis C[38] or IFN therapy.[39] Therefore, our findings that HCV-LP can induce CD4$^+$ T-cell responses against core and E1/E2 proteins with robust production of IFN-γ are likely to be important for protection against HCV infection. Similarly, CD8$^+$ T cells are also likely to be crucial in viral clearance. Several clinical studies have shown an inverse correlation between levels of HCV-specific CTL activities and viral loads.[9,40] In a chimpanzee study, viral clearance during acute hepatitis C is more closely dependent on CD8$^+$ CTL activities than the antibodies.[41] Therefore, cellular immunity is likely to be crucial for viral clearance and disease control in HCV infection.

In this study, a seemingly lower frequency of HCV-specific CD4$^+$ T cells was detected compared with the frequency of IFN-γ^+CD8$^+$ T cells in HCV-LP–immunized animals. This likely reflects the difference in the assays used to quantitatively measure the cellular immune responses. Intracellular cytokine staining was performed with a prior 5-day stimulation with HCV-core or -E2 peptide, whereas enzyme-linked immunospot assay had only 30 hours of stimulation with HCV-core or -E1/E2 protein. During the 5-day stimulation, the HCV-specific T cells may undergo considerable expansion. This could explain the results in our study in which a relatively high frequency of IFN-γ–producing CD8$^+$ T cells was detected after HCV peptide stimulation whereas a much lower percentage of these T cells was actually present *in vivo*.

Our results indicate that HCV-LP is a promising vaccine candidate against HCV infection and that the adjuvants are potent immune enhancers for this approach. We are currently performing similar studies in nonhuman primates to test the immunogenicity and possible protective immunity of this vaccine formulation.

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