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Coupling to the surface of liposomes alters the immunogenicity of hepatitis C virus-derived peptides and confers sterile immunity

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ABSTRACT

We have previously demonstrated that antigens chemically coupled to the surface of liposomes consisting of unsaturated fatty acids were cross-presented by antigen presenting cells to cytotoxic T lymphocytes (CTLs). Liposomal form of immunodominant CTL epitope peptides derived from lymphocytic choriomeningitis virus exhibited highly efficient antiviral CTL responses in immunized mice. In this study, we coupled 15 highly conserved immunodominant CTL epitope peptides derived from hepatitis C virus (HCV) to the surface of liposomes. We also emulsified the peptides in incomplete Freund's adjuvant, and compared the immune responses of the two methods of presenting the peptides by cytotoxicity induction and interferon-gamma (IFN-γ) production by $CD8^+$ T cells of the immunized mice. We noticed significant variations of the immunogenicity of each peptide between the two antigen delivery systems. In addition, the immunogenicity profiles of the peptides were also different from those observed in the mice infected with recombinant adenoviruses expressing HCV proteins as previously reported. Induction of anti-viral immunity by liposomal peptides was tested by the challenge experiments using recombinant vaccinia viruses expressing corresponding HCV epitopes. One D^b-restricted and three HLA-A*0201-restricted HCV CTL epitope peptides on the surface of liposomes were found to confer complete protection to immunized mice with establishment of long-term memory. Interestingly, their protective efficacy seemed to correlate with the induction of IFN-γ producing cells rather than the cytotoxicity induction suggesting that the immunized mice were protected through non-cytolytic mechanisms. Thus, these liposomal peptides might be useful as HCV vaccines not only for prevention but also for therapeutic use.

1. Introduction

Hepatitis C virus (HCV) causes persistent infections in more than half of infected patients which often lead to the development of cirrhosis and hepatocellular carcinoma [1]. Pegylated interferon and ribavirin therapy, although beneficial in about half of treated patients, are expensive and associated with significant side effects [2]. In this clinical context there is an urgent need for the development of a therapeutic and/or prophylactic HCV vaccine [3]. It is well documented that major histocompatibility complex (MHC) class I-restricted, $CD8^+$ cytotoxic T lymphocytes (CTLs) play a major role in the immune control of various virus infections. In the case of HCV infection, spontaneous resolution of acute HCV infection was associated with vigorous HCV-specific CTL responses in chimpanzees [4] and humans [5,6]. Hence, HCV-specific CTLs are likely to be crucial to eradicate HCV.

We have previously demonstrated that antigens chemically coupled to the surface of liposomes consisting of unsaturated fatty acids were cross-presented by antigen presenting cells (APCs) to CTLs [7]. When we coupled liposomes to immunodominant CTL epitope peptides derived from lymphocytic choriomeningitis virus (LCMV) they exhibited highly efficient antiviral responses in immunized mice [8]. Single immunization of as low as 280 ng of liposomal peptide along with CpG conferred complete protection to the immunized mice which was also effective against a highly virulent mutant strain, clone 13. In addition, a long-term memory induction was achieved in the absence of $CD4^+$ T cell help. This form of CTL-based liposomal vaccine was also developed for SARS coronavirus [9] and influenza virus which was effective enough to protect mice from heterosubtypic influenza viruses [10].

Previously, we have evaluated 24 HLA-A*0201-restricted, HCV-derived epitopes by examining peptide-binding affinity for HLA-A*0201 molecules, the stability of peptide-HLA-A*0201 complexes, killing activities of CTLs and frequencies of intracellular interferon-gamma (IFN-γ)-positive $CD8^+$T cells induced in mice immunized with HCV protein-expressing recombinant adenoviruses [11]. On the basis of those results, 24 peptides tested were classified into six groups. In this study, we chose 14 out of the 24 peptides from the five groups and coupled them as well as one

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murine MHC-restricted HCV CTL epitope peptide on the surfaces of liposomes. We also emulsified these peptides in incomplete Freund's adjuvant (IFA) and compared their immunogenicity by analyzing the cytolytic activity and the number of IFN-γ-producing cells induced in the immunized mice. All of the liposomal peptides were tested for their protective efficacy by the challenge experiments.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Tokyo Laboratory Animals Science Co. Ltd. (Tokyo, Japan). CD4 knockout (KO) mice on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). Transgenic mice, HHD (a kind gift from Dr. F. A. Lemonnier, Pasteur Institute, Paris, France) express a transgenic HLA-A*0201 monochain in which human beta-2 microglobulin (β2m) is covalently linked to a chimeric heavy chain composed of HLA-A*0201 (α1 and α2, domains) and H-2D^b (α3, transmembrane, and cytoplasmic domains) [12]. Six- to 12-week-old mice were used for all experiments. The mice were housed in appropriate animal care facilities at Saitama Medical University (Saitama, Japan), and handled according to international guidelines. Experimental protocols were approved by the Animal Research Committee of Saitama Medical School.

2.2. Cell lines

A mouse lymphoma cell line EL-4 (H-2b) was obtained from the ATCC, and African green monkey-derived kidney cell lines CV-1 and BS-C-1 were provided by Dr. T. Shioda (Osaka University, Osaka, Japan). These cell lines were cultured in DMEM with 10% FCS (D-10). A mouse lymphoma cell line transfected with the HHD gene, RMA-HHD (H-2b) [12] was maintained in D-10 containing G418 (Sigma–Aldrich, St. Louis, MO) at a final concentration of 500 μg/ml.

2.3. Reagents

The peptides listed in Table 1 and a peptide HBVcore128–140 (TPPAYRPPNAPIL) [13] were synthesized by Operon Biotechnology (Tokyo, Japan). Synthetic CpG ODN (5002: TCCATGACGTTCTTGATGTT) was purchased from Hokkaido System Science (Sapporo, Japan) and phoshorothioate protected to avoid nuclease-dependent degradation.

Table 1
HCV-derived peptides used in this study.

Name	Restriction	Derivation	Residues	Sequence (Ref.)	Type[a]
A2-1	HLA-A*0201	Core	35–44	YLLPRRGPRL	IV
A2-2	HLA-A*0201	Core	132–140	DLMGYIPLV	I
A2-3	HLA-A*0201	Core	178–187	LLALLSCLTV	V
A2-4	HLA-A*0201	E1	257–266	QLRRHIDLLV	III
A2-5	HLA-A*0201	E2	686–694	ALSTGLIHL	I
A2-6	HLA-A*0201	E2	726–734	LLFLLLADA	III
A2-7	HLA-A*0201	NS3	1073–1081	CINGVCWTV	I
A2-8	HLA-A*0201	NS3	1406–1415	KLVALGINAV	II
A2-9	HLA-A*0201	NS3	1585–1593	YLVAYQATV	II
A2-10	HLA-A*0201	NS4A	1671–1680	VLAALAAYCL	I
A2-11	HLA-A*0201	NS4B	1807–1816	LLFNILGGWV	II
A2-12	HLA-A*0201	NS4B	1851–1859	ILAGYGAGV	II
A2-13	HLA-A*0201	NS4B	1920–1928	WMNRLIAFA	I
A2-14	HLA-A*0201	NS5A	1992–2000	VLSDFKTWL	IV
603	D^b	NS3	1629–1637	GAVQNEVTL	NA

Abbreviations: NA, not applicable.

[a] Classification of HCV-derived CTL epitopes determined in our previous study [11]. Type I: High or medium in the affinity, high or medium in the lysis, and high or medium in the intracellular cytokine staining (ICS) for IFN-γ. Type II: High or medium in the affinity, high or medium in the lysis, and low or ND in the ICS. Type III: Low in the affinity, and high in the lysis. Type IV: High in the affinity, low or ND in the lysis, and medium in the ICS. Type V: High or medium in the affinity, low or ND in the lysis, and low or ND in the ICS. Type VI: Low in the affinity, ND in the lysis and ICS. ND, not detected.

2.4. Coupling of peptides to liposomes

Oleoyl liposomes consisted of dioleoyl phosphatidyl choline, dioleoyl phosphatidyl ethanolamine, dioleoyl phosphatidyl glycerol acid, and cholesterol in a 4:3:2:7 M ratio. Each peptide was coupled to the surface of liposomes via disuccinimidyl suberate as described previously [7]. The final preparations contained 0.7 mg or less of the peptide and 10 mg of the liposome per ml.

2.5. Viruses

Generation of a recombinant vaccinia virus expressing the HCV core (VV-core) was described [14]. Recombinant vaccinia viruses expressing the HCV C-E1 (VV-C-E1), E2 (VV-E2), NS3 (VV-NS3), NS4 (VV-NS4) or NS5 (VV-NS5) were generated as described [15]. In brief, the HCV C-E1, E2, NS3, NS4 and NS5 genes corresponding to amino acid residues 1–380, 381–810, 1027–1657, 1658–1972 and 1959–2872, respectively, were amplified from the plasmid pBRTM/HCV1–3011con which contains the entire DNA sequence derived from the HCV H77 clone [16] (kindly provided by Dr. Charles M. Rice, The Rockefeller University, New York, NY) by PCR and inserted into the transfer vector, pNZ68K2. Then, recombinant vaccinia viruses were generated by homologous recombination between wild-type vaccinia virus (VV-wt) (WR strain) and the transfer vector, purified in C143 cells, and propagated in CV1 cells.

2.6. Immunization

Each mouse was subcutaneously (s.c.) immunized with either a liposomal peptide of the indicated amount together with 5 μg of CpG in 50 μl of PBS, or a peptide (50 μg) emulsified in 250 μl of IFA with the peptide HBVcore128–140 (100 μg).

2.7. ^{51}Cr-release assay

^{51}Cr-release assays were carried out as described [8]. In brief, spleen cells of immunized mice were cultured for seven days with irradiated (40 Gy) syngeneic naive spleen cells pre-pulsed with 10 μM of a relevant peptide, and employed as effector cells in standard ^{51}Cr-release assays. Target cells were pulsed with or without 10 μM of each peptide for 2 h, and then labeled with $Na_2^{51}CrO_4$ for 1 h. The labeled target cells were plated with effector cells at an effector to target (*E*/*T*) ratio of 100. After a 4-h incubation, the radioactivity of the supernatant was counted. Percent lysis was calculated as $[(cpm_{sample} - cpm_{spontaneous})/(cpm_{maximum} - cpm_{spontaneous})] \times 100$. Maximum ^{51}Cr release was determined from supernatants of cells that were lysed by addition of 2% Nonidet P-40. Spontaneous release was measured in supernatants from target cells incubated without effector cells. Percent specific lysis was calculated by subtracting the percent lysis with unpulsed targets from that with peptide-pulsed targets.

2.8. IFN-γ ELISPOT assay

Detection of IFN-γ secreting cells was performed by using a mouse IFN-γ enzyme-linked immunospot (ELISPOT) set (BD Pharmingen, San Diego, CA) as described [8].

2.9. Viral challenge

After immunization, mice were challenged i.p. with 2×10^6 pfu of VV-core, VV-NS3, or VV-NS4, and five days later, mice were sacrificed, and the titer of the homogenate of two ovaries from each mouse was determined on BS-C-1 cells as described [14]. All titrations were performed in duplicates, and the average PFU per mouse was calculated.

2.10. Statistical analysis

Statistical analyses were performed with Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Coupling of a D^b-restricted HCV CTL epitope peptide to the surface of liposomes

We observed that a murine MHC (D^b)-restricted HCV CTL epitope 603 [17] showed much higher immunogenicity than human HLA-A*0201-restricted HCV CTL epitopes in (C57BL/6 × HHD) F1 mice when they were infected with a recombinant adenovirus expressing HCV NS3 (unpublished data). Therefore, at first, we chose peptide 603 and made two vaccines: a surface-linked liposomal peptide (Lip-603) and a peptide emulsified in IFA with a MHC class II pan T helper peptide, HBVcore128–140 [13] (P-603). The immunogenicities of these two forms of peptide 603 were compared by analyzing spleen cells from the immunized mice for their cytotoxicity and IFN-γ production by ^{51}Cr-realse assays (Fig. 1A) and ELISPOT assays (Fig. 1B), respectively. We noticed that Lip-603 induced significant and higher responses in both assays than P-603.

When the immunized mice were challenged by a recombinant vaccinia virus, VV-NS3, only Lip-603 was found to induce sterilizing immunity to mice whereas P-603 did not show significant effects (Fig. 1C). The sterilizing immunity conferred by Lip-603 could be observed even at 12 weeks after immunization (Fig. 1D) and long-term memory was confirmed by strong recall responses observed at 12 weeks postimmunization by ELISPOT (IFN-γ) assays (Fig. S1). Memory induction by Lip-603 was also observed in CD4 KO mice (Fig. 1D) suggesting that it was carried out without $CD4^+$ T cell help as had previously been shown for liposomal LCMV GP33 (Lip-GP33) [8] and ovalbumin257–276 [18] peptides. The dose–response ELISPOT (IFN-γ) assay revealed that as low as 56 ng of Lip-603 could induce significant IFN-γ responses (Fig. 1E). This highly efficient induction of IFN-γ-producing cells by Lip-603 was very similar to that observed for Lip-GP33 [8].

3.2. Analyses of immunogenicity of HLA-A*0201-restricted HCV CTL epitope peptides

Among 24 HLA-A*0201-restricted HCV CTL epitope peptides which had been characterized for their immunogenicity and classified into six groups in our previous study [11], 14 peptides (A2-1 to A2-14) (Table 1) were chosen and coupled to the surfaces of liposomes (Lip-A2-1 to Lip-A2-14) or emulsified in IFA (P-A2-1 to P-A2-14). We immunized (s.c.) HHD mouse with each peptide twice, and one week after the second immunization, spleen cells were tested by ^{51}Cr-realse assays (Fig. 2A) and ELISPOT assays (Fig. 2B). The type of each peptide (I–V, Table 1) determined by the immunogenicity in mice that endogenously expressed HCV proteins during infection with recombinant adenoviruses [11] is shown in Fig. 2 for comparison. Considerable variability in the immunogenicity was observed between the peptides and also between the modes of antigen delivery. The accordance of the three assay results for each peptide was observed only for A2-1, A2-3, and A2-14 (Fig. 2A and B).

Induction of anti-viral activity was tested for all of the 14 liposomal peptides by challenge experiments with recombinant vaccinia viruses expressing corresponding epitopes. Among them, only Lip-A2-8, Lip-A2-11, and Lip-A2-12 exhibited complete protection that was reproducible in at least three independent experiments. The others showed insignificant, partial, or non-reproducible protection (Fig. 2C). Notably, all of the three liposomal peptides that conferred perfect protection, L-A2-8, L-A2-11 and L-A2-12, belonged to the highest inducers of IFN-γ against $CD8^+$ T cells (Fig. 2B). Lip-A2-5, which induced high cytolytic activity but poor IFN-γ responses by $CD8^+$ T cells, showed only partial and non-reproducible protection.

Lip-A2-8, Lip-A2-11 and Lip-A2-12, which showed excellent anti-viral effector $CD8^+$ T cell induction, were also analyzed for the ability of long-term memory induction. The memory at 12 weeks postimmunization induced by the three strong inducer of IFN-γ completely protected mice from VV-NS3 or VV-NS4 challenge, while the poor inducer of IFN-γ, Lip-A2-5, did not exhibit significant titer reductions against VV-E2 (Fig. 3A). The memory induction was confirmed by recall responses by ELISPOT (IFN-γ) assays (Fig. 3B), which correlated well with the results of the challenge experiments (Fig. 3A).

4. Discussion

In this report, we demonstrate that HCV-derived antigenic peptides coupled to the surface of liposomes serve as efficient vaccine vehicles for the induction of anti-viral immunity mediated by $CD8^+$ T cells as had previously been shown for LCMV-derived peptide GP33 [8]. Immunization with Lip-603 induced significant levels of cytotoxicity and IFN-γ-producing cells and sterile immunity in the immunized mice (Fig. 1A–C). The dose–response ELISPOT assays revealed that Lip-603 could induce significant responses at less than 56 ng of the peptide (Fig. 1E) which was similar to that observed for Lip-GP33 [8]. Long-term memory induction was also confirmed at 12 weeks after immunization, and it seemed to be independent of $CD4^+$ T cell help (Fig. 1D, Fig. S1).

However, this study showed clearly that both the qualitative and quantitative immune responses induced by peptides coupled on the surface of liposomes may differ from that of the same peptides emulsified with a MHC class II pan T helper peptide in IFA. P-603 induced lower responses in ^{51}Cr-release (Fig. 1A) and ELISPOT assays (Fig. 1B) compared to Lip-603, and did not show significant protection in the challenge experiments (Fig. 1C). HLA-A*0201-restricted HCV epitope peptides also showed considerable differences in immunogenicity between the liposomal and emulsion forms (Fig. 2A and B). A2-5 peptide in IFA induced both cytolytic and IFN-γ responses whereas the same peptide on the surface of liposomes predominantly induced cytolytic activity. On the other hand, A2-11 and A2-12 showed opposite results: P-A2-11 and P-A2-12 mainly induced cytolytic responses, and L-A2-11 and L-A2-12 induced strong IFN-γ responses rather than cytolytic responses. A2-8 on liposomes showed strong responses in both assays whereas that in IFA induced only cytolytic responses. A2-7 was found to be a strong inducer of cytolytic activity when emulsified in IFA, but on the surface of liposomes, induced neither cytolytic nor IFN-γ responses.

When these HLA-A*0201-restricted HCV epitope peptides on the surface of liposomes were tested for their efficacy to induce protection against virus challenges, only A2-8, A2-11, and A2-12 exhibited perfect and reproducible protection (Fig. 2C). Interestingly, these three liposomal peptides were the strongest inducer

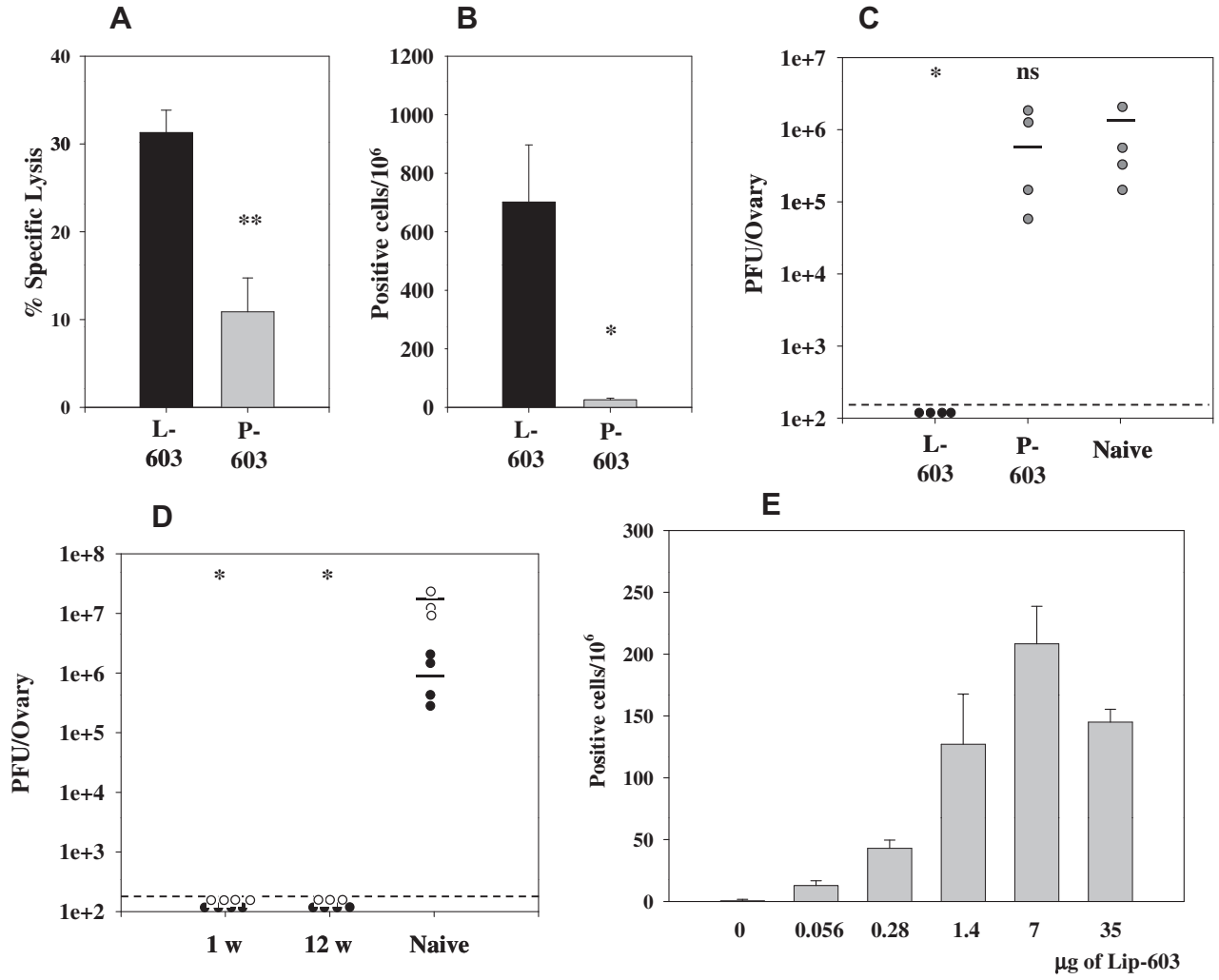

Fig. 1. Comparison of the immunogenicities of Lip-603 and P-603. Each C57BL/6 mouse received (s.c.) 20 μl of Lip-603 diluted in 50 μl of PBS containing 5 μg of CpG or 250 μl of IFA emulsion containing 50 μg of peptide 603 and 100 μg of peptide HBV128–132. The mouse received the second immunization at day 11. Spleen cells were prepared seven days after the last immunization for 51Cr release assays (A) and ELISPOT assays (B). (A) 51Cr release assays were performed at *E*/*T* ratio of 100 using EL-4 cells pulsed with 603 peptide as targets. Spontaneous release was 11%–16%. (B) IFN-γ-producing cells responding to 603 peptide were detected by ELISPOT assays as described in Section 2. (C) The immunized and naïve mice were challenged at one week after the last immunization with 2 × 106 PFU of VV-NS3 (i.p.) and the virus titers in the ovaries were quantitated by plaque assays on BS-C-1 cells at day 5 postchallenge. Virus titers are indicated for each animal in the study. A dotted line represents the lower limit of detection (2 × 102 PFU/Ovary). (D) Challenge experiments were performed at one and 12 weeks for C57BL/6 mice (closed circles) and CD4 KO mice (open circles) immunized with Lip-603. (E) Dose–response experiments. Serial fivefold dilutions of Lip-603 starting at 50 μl (<35 μg of peptide) were prepared in 50 μl of PBS containing 5 μg of CpG and injected s.c. to each C57BL/6 mouse. Seven days later, ELISPOT (IFN-γ) assays were performed for spleen cells from immunized mice. Data are representative of two independent and reproducible experiments. Results are shown as the means of 3 to 4 mice per group ± standard errors of the means. *, $p < 0.05$; **, $p < 0.01$; ns, not statistically significant.

of IFN-γ responses (Fig. 2B). In contrast, Lip-A2-5 was found to induce only non-reproducible protection with a titer reduction of less than two logs although it was a strong inducer of cytolytic activity. These lines of evidence suggest that the anti-viral activity induced by liposomal peptides is mediated by non-cytolytic mechanisms related to IFN-γ responses. Similar observations were obtained in our previous study [8]. LCMV-derived D^b-restricted epitope peptide NP396, on the liposomes, induced strong IFN-γ responses but no cytolytic responses, and the mice were highly protected against LCMV challenge. Both LCMV and HCV have been reported to be susceptible to non-cytolytic anti-viral mechanisms [19–21]. Taken together, the peptides on the surface of liposomes may have an advantage in application for therapeutic use in that virus-infected cells may be eliminated without tissue damages. Further studies are needed to clarify this issue.

We speculate that the mechanisms underlying the induction of different immunogenicities of the peptides by coupling to the surface of liposomes are associated with the differences in the antigen presentation mechanisms. Peptides emulsified in IFA are thought to fit into the pockets of MHC class I molecules on APCs replacing the original endogenously synthesized peptides and presented to $CD8^+$ T cells [22]. On the other hand, we have shown that antigens chemically coupled to the surface of liposomes consisting of unsaturated fatty acids (oleoyl liposomes) are cross-presented by APCs [7]. Our further study has shown that oleoyl liposomes might be taken up by APCs via at least two pathways: fusion with and penetration of plasma membranes, and pinocytosis [23]. These unique antigen presentation mechanisms may be responsible for the unique immunogenicity and anti-viral potency of the peptide antigens. The 14 HLA-A*0201-restricted epitope peptides have been characterized for their immunogenicity in our previous study by analyzing the immune responses of mice immunized with recombinant adenoviruses that expressed HCV proteins [11]. The immunogenicity profiles in response to the adenovirus-vectored

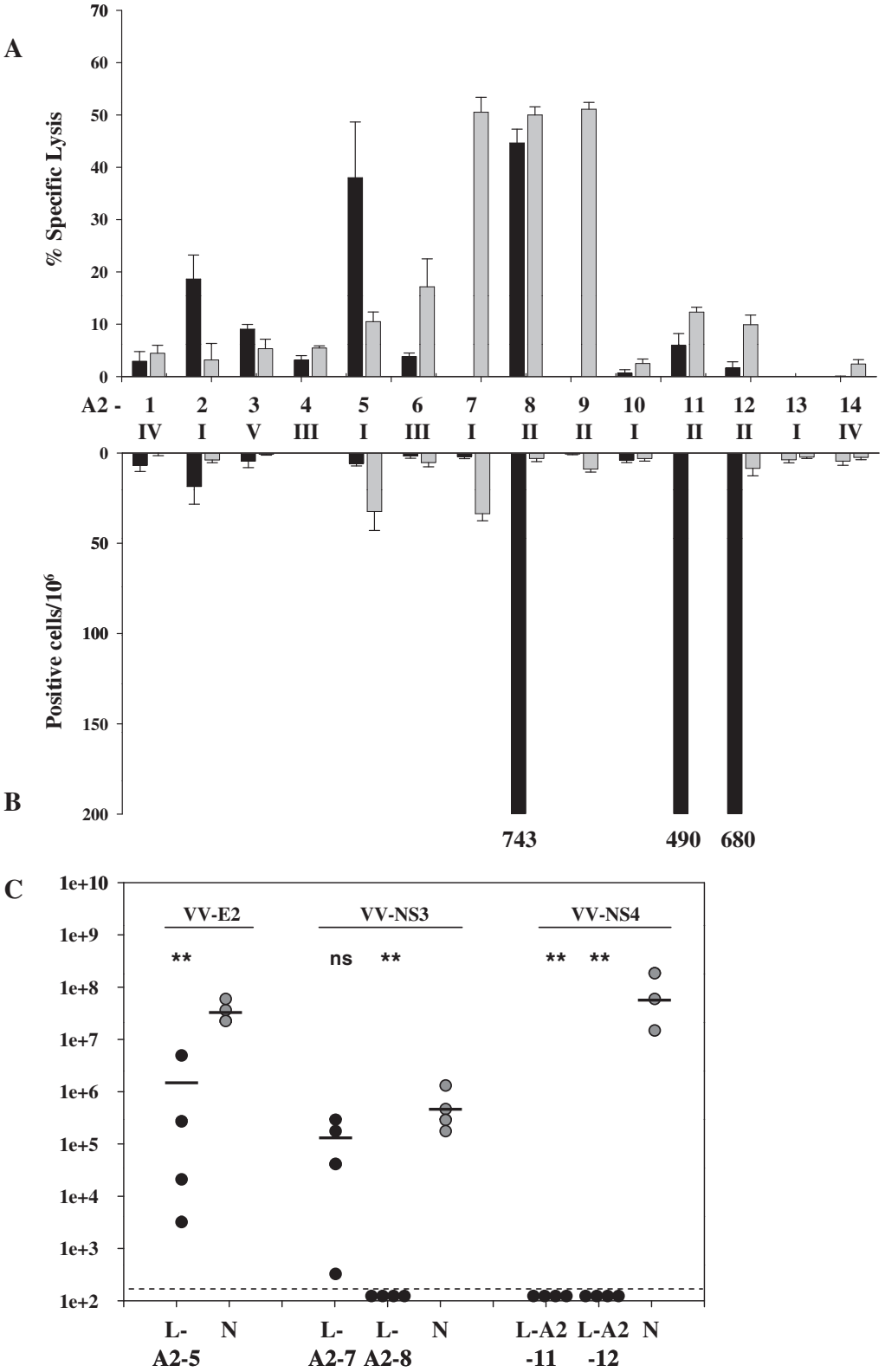

Fig. 2. Each HHD mouse was immunized (s.c.) with one of the 14 HLA-A2*0201-restricted HCV CTL epitope peptides (A2-1 to A2-14) in the form of liposome (20 μl with 5 μg of CpG) (black bars) or emulsion in IFA (50 μg with 100 μg of HBV128–132 peptide) (gray bars) twice with one to four weeks interval. At one week after the second immunization, spleen cells were taken and their cytotoxicity and production of IFN-γ in response to the corresponding peptide were determined by 51Cr-release assays (A) and ELISPOT assays (B). (A) 51Cr release assays were performed at *E*/*T* ratio of 50 using peptide-pulsed RMA-HHD cells as targets. Spontaneous release was 10%–20%. (B) IFN-γ-producing cells responding to a relevant peptide were detected by ELISPOT assays. The group number (I–V) in which each epitope was classified in our previous study [11] (Table 1) is shown under each peptide number. (C) HHD mice immunized with liposomal peptides were challenged with recombinant vaccinia viruses expressing corresponding HCV epitopes. The virus titers in the ovaries were determined as in Fig. 1C, and the results of liposomal A2-5, 7, 8, 11, and 12 peptides are shown. Data are representative of at least three (A–C) independent and reproducible experiments. Results are shown as the means of 4 to 5 mice per group ± standard errors of the means (A and B). N, naïve mice; **, $p < 0.01$; ns, not statistically significant.

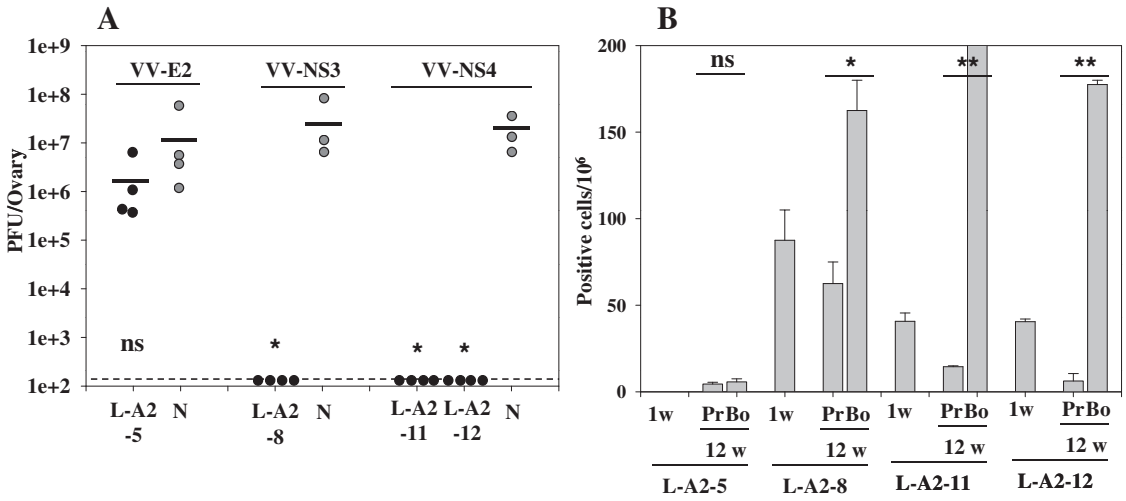

Fig. 3. Analyses of memory CD8+ T cell induction by Lip-A2-5, Lip-A2-8, A2-11, and Lip-A2-12. Each HHD mouse received 50 μl of liposomal peptide with 5 μg of CpG, and challenge experiments with recombinant vaccinia viruses (A) and ELISPOT assays (B) were performed. (A) Challenge experiments were performed by i.p. injections of 2×106 PFU of recombinant vaccinia viruses expressing corresponding HCV epitopes at 12 weeks postimmunization. N, naïve mice. (B) At one (1w) and 12 weeks postimmunization (Pr), IFN-γ-producing cells responding to the peptides were detected by ELISPOT assays of spleen cells. At 12th week, the mice were boosted by the same manner as the primary immunizations, and their recall responses were analyzed one week later (Bo).

immunization were quite different from any of those obtained from the two types of peptide vaccinations (Fig. 2A and B). In adenovirus vaccination, the peptides were endogenously synthesized and presented by MHC class I molecules on virus-infected cells. Therefore, the immunogenicity profile induced by this third antigen presentation mechanism may also support our hypothesis that these different immunization systems result in different antigen presentations and therefore different immune responses.

In conclusion, coupling peptide antigens on the surface of liposomes consisting of unsaturated fatty acids seems to give rise to unique immune response profiles to peptides. HCV peptide/liposomes may be useful as prophylactic and therapeutic vaccines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.028.

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