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2. 学会発表

1) SARSコロナウイルスのpolyprotein 1a由来HLA-A*0201拘束性CTLエピトープの同定と、そのペプチドを結合したリポソームによる細胞傷害性T細胞の誘導
高山俊輔、須田達也、種市麻衣子、赤塚俊隆、内田哲也、松井政則
第13回日本ワクチン学会　札幌　2009年9月

2) Efficient induction of SARS coronavirus-specific CTLs by immunization with surface-linked liposomal peptides derived from nucleocapsid and a non-structural polyprotein 1a
Shunsuke Kohyama, Tatsuya Suda, Maiko Taneichi, Tetsuya Uchida, and Masanori Matsui
第39回日本免疫学会　大阪　2009年12月

G. 知的財産権の出願・登録状況（予定を含む。）

1. 特許出願

1) 鳥インフルエンザワクチン（国際特許）
出願番号：PCT/JP2009/70053
出願日：2009年11月27日
発明者:内田哲也、種市麻衣子（国立感染研）、松井政則（埼玉医大）、梶野喜一（北海道大学）、小田洋（日油）

2) SARSコロナウイルスの細胞傷害性T細胞エピトープペプチド及びその用途（国際特許）
出願番号：PCT/JP2009/70043
出願日：2009年11月27日

内田哲也、種市麻衣子（国立感染研）、松井政則（埼玉医大）、小田洋（日油）

3) 鳥インフルエンザウイルスワクチン（国内特許、追加出願）

出願日：2009 年 12 月 21 日

発明者:内田哲也、種市麻衣子（国立感染研）、松井政則（埼玉医大）、梶野喜一（北海道大学）、小田洋（日油）

２．実用新案登録

該当無し

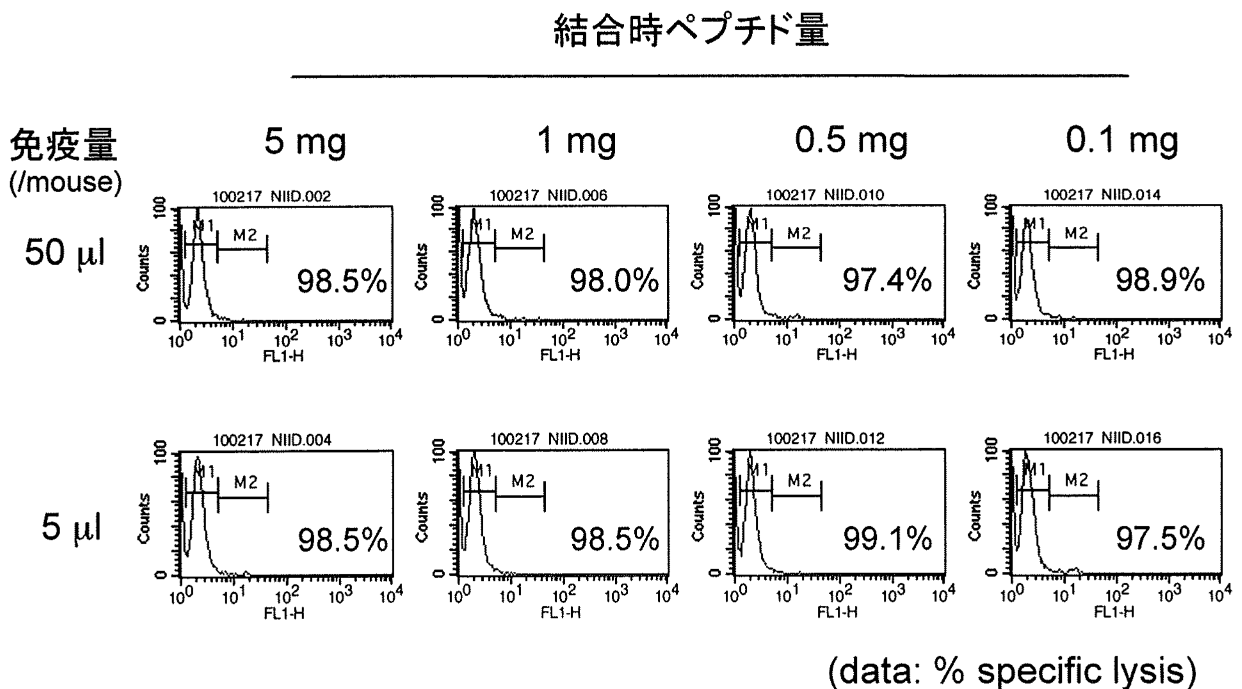
３．その他

該当無し

表-1: ペプチド-リポソーム結合効率

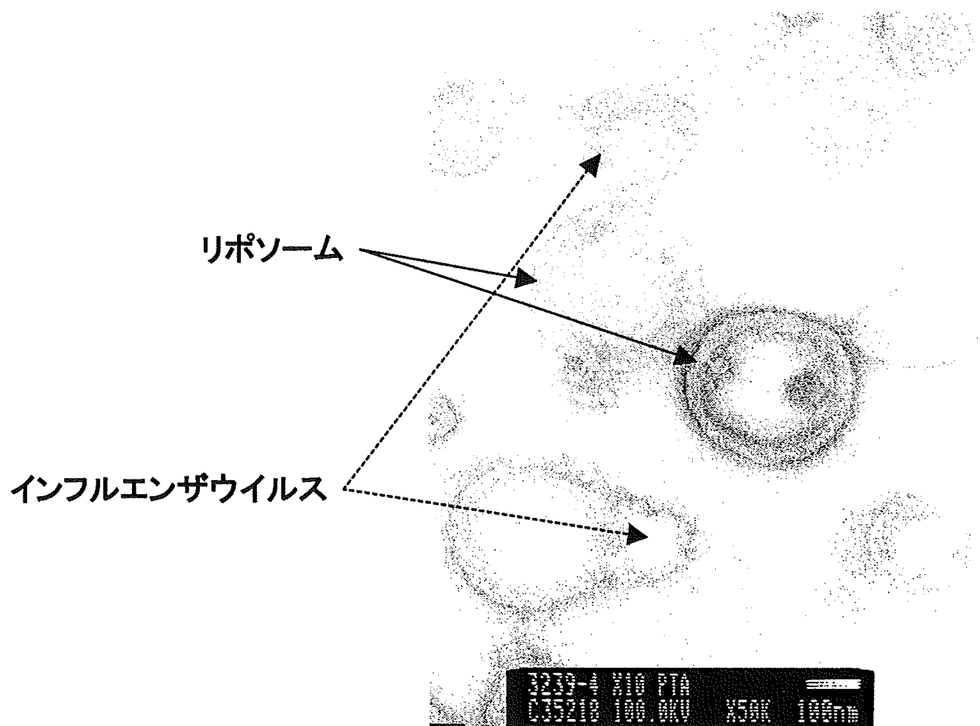
ペプチド由来	添加ペプチド量(mg)	リポソーム結合ペプチド量(μg/ml)	回収率
OVA	5	46.5	5.6
	1	26.6	16.6
	0.5	7.6	9.4
	0.1	1.7	10.8
Influenza virus	5	104.0	12.5
	1	65.0	40.6
	0.5	31.6	39.5
	0.1	4.8	30.1

図-1: ペプチド-リポソームによるCTL誘導

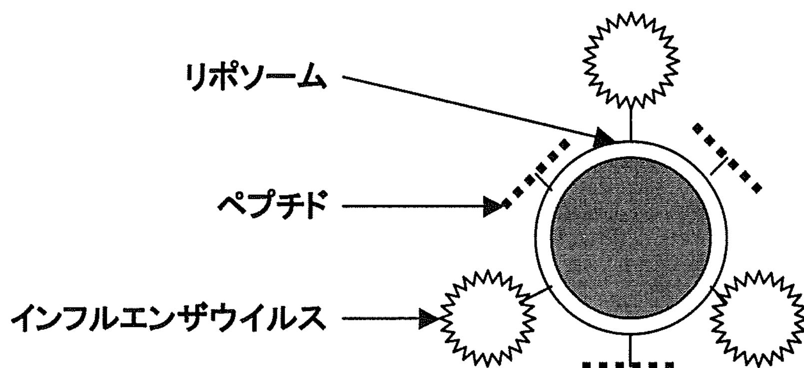


(data: % specific lysis)

図−2：ウイルス結合リポソームの電顕像



(ウイルス+ペプチド)-リポソームのイメージ



表−2：ウイルス結合リポソームによるCTL誘導

免疫	アジュバント	CTL誘導(% specific lysis)
ペプチド単独	なし	0.0
ペプチド−リポソーム	なし	0.0
ペプチド−リポソーム	CpG	98.7
(ペプチド+ウイルス全粒子)−リポソーム	なし	87.2

III.　研究成果の刊行に関する一覧表

書籍

該当なし

雑誌

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IV.　研究成果の刊行物・別刷

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Efficient induction of cytotoxic T lymphocytes specific for severe acute respiratory syndrome (SARS)-associated coronavirus by immunization with surface-linked liposomal peptides derived from a non-structural polyprotein 1a

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A B S T R A C T

Spike and nucleocapsid are structural proteins of severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) and major targets for cytotoxic T lymphocytes (CTLs). In contrast, non-structural proteins encoded by two-thirds of viral genome are poorly characterized for cell-mediated immunity. We previously demonstrated that nucleocapsid-derived peptides chemically coupled to the surface of liposomes effectively elicited SARS-CoV-specific CTLs in mice. Here, we attempted to identify HLA-A*0201-restricted CTL epitopes derived from a non-structural polyprotein 1a (pp1a) of SARS-CoV, and investigated whether liposomal peptides derived from pp1a were effective for CTL induction. Out of 30 peptides predicted on computational algorithms, nine peptides could significantly induce interferon gamma (IFN-γ)-producing $CD8^+$ T cells in mice. These peptides were coupled to the surface of liposomes, and inoculated into mice. Six liposomal peptides effectively induced IFN-γ-producing $CD8^+$ T cells and seven liposomal peptides including the six peptides primed CTLs showing *in vivo* killing activities. Further, CTLs induced by the seven liposomal peptides lysed an HLA-A*0201 positive cell line expressing naturally processed, pp1a-derived peptides. Of note, one of the liposomal peptides induced high numbers of long-lasting memory CTLs. These data suggest that surface-linked liposomal peptides derived from pp1a might offer an efficient CTL-based vaccine against SARS.

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1. Introduction

The outbreak of severe acute respiratory syndrome (SARS) in early 2003 led to thousands of infected patients and hundreds of deaths (Groneberg et al., 2003). SARS is caused by a novel coronavirus termed SARS-associated coronavirus (SARS-CoV) (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). This is a plus-stranded RNA virus with an approximately 30 kb long genome encoding replicase gene products and the structural proteins containing spike, envelop, membrane and nucleocapsid (Groneberg et al., 2005). Although the viral receptor has been identified (Li et al., 2003b), the pathogenesis of SARS remains poorly understood and the apparent latency of SARS-CoV in animal reservoirs continuously provides us a serious threat of reemergence.

Spike protein of SARS-CoV is a major target for neutralizing antibodies because this protein interacts with the cellular receptor (Li et al., 2003b). High titers of neutralizing antibodies to SARS-CoV were detected in sera of the recovered patients (Li et al., 2003a), and humoral immunity induced by a DNA vaccine contributed to the protection against SARS-CoV challenge in mice (Yang et al., 2004). These data strongly suggest that neutralizing antibodies play a central role in the clearance of SARS-CoV. On the other hand, a rapid loss of both $CD4^+$ and $CD8^+$ T cells was observed in patients suffering from severe SARS, and the cell counts gradually returned to normal ranges as the patients recovered (Tang et al., 2003). Furthermore, certain HLA class I alleles have been reported to correlate with SARS susceptibility (Lin et al., 2003; Ng et al., 2004), suggesting that cytotoxic T lymphocytes (CTLs) play an important role in the elimination of SARS-CoV as well. Several CTL epitopes have been identified from spike and nucleocapsid proteins of SARS-CoV (Wang et al., 2004a; Wang et al., 2004b; Chen et al., 2005; Tsao et al., 2006; Zhou et al., 2006; Ohno et al., 2009). However, CTL epitopes have not been found in non-structural proteins of SARS-CoV.

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In general, non-structural proteins are more conserved and synthesized earlier than structural proteins, and therefore, they could be preferable as an antigenic target for CTLs.

A synthetic peptide vaccine is a potential candidate for a CTL-based vaccine against pathogenic viruses because short peptides rarely cause undesirable responses including general toxicity, immunosuppression and autoimmunity. However, the immunogenicity of this type of vaccine is very weak. Liposomes have extensively been investigated as a delivery system for antigen (Alving et al., 1995). In most cases, it has been prepared by antigen entrapment within the aqueous lumen of liposomes. In contrast, we have previously demonstrated that an ovalbumin (OVA)-derived peptide, $OVA_{257-264}$ conjugated on the surface of liposomes induced $OVA_{257-264}$-specific CTLs in mice more effectively than did liposomes containing $OVA_{257-264}$ inside (Taneichi et al., 2006; Nagata et al., 2007). Furthermore, we have recently shown that surface-linked liposomal peptides derived from nucleocapsid of SARS-CoV effectively induced SARS-CoV-specific CTLs in mice (Ohno et al., 2009). These data suggest that liposomes would become an excellent adjuvant vehicle for a synthetic peptide vaccine when a peptide(s) is chemically coupled to the surface of liposomes.

In the current study, we attempted to identify HLA-A*0201-restricted CTL epitopes derived from a largest non-structural polyprotein 1a (pp1a) of SARS-CoV using computational algorithms and HLA-A*0201 transgenic mice. There are several reasons why we focused on this protein. First of all, pp1a protein is a regulatory protein, and hence, more conserved and synthesized earlier than spike and nucleocapsid proteins (Groneberg et al., 2005). Second, since pp1a is a largest protein composed of 4382 amino acids among SARS-CoV-associated proteins (Groneberg et al., 2005), it is more likely to find highly immunogenic, dominant epitopes in pp1a protein than in any other proteins of SARS-CoV. Peptides identified were then chemically conjugated on the surface of liposomes and evaluated for their abilities to induce SARS-CoV-specific CTLs.

2. Materials and methods

2.1. Prediction of CTL epitopes

To define potential HLA-A*0201-binding peptides derived from pp1a of SARS-CoV (Urbani strain) (GenBank accession number: AAP13439), we used two computer-based programs, SYFPEITHI (http://www.syfpeithi.de/) (Rammensee et al., 1999) and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) (Parker et al., 1994). As shown in Table 1, 30 peptides with superior scores were selected for predicted CTL epitopes. These peptides were synthesized by Operon Biotechnologies (Tokyo, Japan). In addition, three known epitopes derived from the SARS-CoV spike protein (Spike-978: LITGRLQSL; Spike-1167: RLNEVAKNL; Spike-1203: FIAGLIAIV) (Wang et al., 2004a; Wang et al., 2004b) were synthesized as well.

2.2. Cell lines

T2 (Salter et al., 1985) is a transporter associated with antigen processing (TAP)-deficient, human lymphoblastoid cell line expressing natural HLA-A*0201. C1R-A2 is a human B cell line, HMy2.C1R that was transfected with an HLA-A*0201 gene (Winter et al., 1991). T2 and C1R-A2 cells were maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS) (R-10) and R-10 containing 500 μg/ml G418 (Sigma–Aldrich), respectively.

2.3. Peptide binding assay

Peptide binding assay was performed as described by Ohno et al., 2006. Briefly, T2 cells were suspended in AIM V serum-free medium (Life Technologies, Rockville, MD) containing 100 nM human beta-2 microglobulin (β2m) (Sigma–Aldrich) and were incubated with each synthetic peptide at various concentrations overnight at 37 °C. Cells were then stained with the anti-HLA-A*0201 monoclonal antibody (mAb), BB7.2 (Parham and Brodsky, 1981), followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma–Aldrich). The mean fluorescence intensity (MFI) was measured by flow cytometry (FACScan, BD Biosciences, San Jose, CA). The concentration of each peptide that yields the half-maximal MFI of T2 cells pulsed with a control peptide derived from hepatitis C virus, NS3-1585 (Ohno et al., 2006) was calculated as the half-maximal binding level (BL_{50}). Experiments were performed three times, and data are given as mean values ± standard deviation (SD).

2.4. Mice

Mice express a transgenic HLA-A*0201 monochain, designated as HHD, in which human β2m is covalently linked to a chimeric heavy chain composed of HLA-A*0201 (α1 and α2 domains) and $H\text{-}2D^b$ (α3, transmembrane, and cytoplasmic domains) (Pascolo et al., 1997; Matsui et al., 2004). Eight- to twelve-week-old mice were used for all experiments. Mice were housed in appropriate animal care facilities at Saitama Medical University, Saitama, Japan, and handled according to international guidelines for experiments with animals.

2.5. Surface-coupled liposomal peptides

Surface-coupled liposomal peptides were prepared as described previously by Taneichi et al., 2006 via disuccinimidyl suberate (DSS). Briefly, 10 ml of anhydrous chloroform solution containing 0.136 mM dioleoyl phosphatidyl ethanolamine (DOPE) and 24 μl of triethylamine was mixed with 26.6 ml of anhydrous chloroform solution containing 0.681 mM DSS and stirred for 5 h at 40 °C. The solvent was evaporated, and 18 ml of a 2:1 mixture of ethyl acetate and tetrahydrofuran was added to dissolve the residue. Thirty six milliliters of 100 mM sodium phosphate (pH 5.5) and 90 ml of saturated NaCl aqueous solution were added to the solution, shaken for 1 min, and allowed to separate. The upper layer was washed with the same buffer again. After evaporation of the solvent, 3 ml of acetone was added to dissolve the residue. Ice-cold acetone (100 ml) was added in drops and kept on ice for 30 min to precipitate. Crystals were collected and dissolved in 5 ml of chloroform. After evaporation, 34.4 mg of DOPE-DSS was obtained. For preparation of unsaturated liposomes, DOPE-DSS (0.03 mM), dioleoyl phosphatidylcholine (0.18 mM), cholesterol (0.21 mM), and dioleoyl phosphatidyl glycerol (0.06 mM) were dissolved in 10 ml of chloroform/methanol. The solvent was then removed under reduced pressure and 5.8 ml of phosphate buffer (pH 7.2) was added to make a 4.8% lipid suspension. The vesicle dispersion was extruded through a 0.2 μm polycarbonate filter to adjust the liposome size. A 2 ml suspension of DSS-introduced liposomes and 0.5 ml of 5 mg/ml peptide solution were mixed and stirred for 3 days at 4 °C. The liposome-coupled and -uncoupled peptides were separated using CL-4B column chromatography. Liposomes used in the experiments were prepared under the regulation of Good Manufacturing Practice and involved no detectable endotoxin.

2.6. Immunization

For identification of CTL epitopes, mice were immunized intravenously (i.v.) with 2×10^7 syngeneic spleen cells pre-pulsed with 10 μM of each synthetic peptide. In the case of immunization with liposomal peptides, mice were subcutaneously (s.c.) immunized once or several times at one-week intervals with each of

Table 1
Predicted CTL epitopes for SARS-CoV pp1a protein.

Name	Position	Sequence	SYFPEITHI[a]	BIMAS[b]	BL_{50} (μM)[c]
pp1a-15	15-23	QLSLPVLQV	26	156	70.1 ± 10.9
pp1a-103	103-11	TLGVLVPHV	26	156	3.6 ± 0.9
pp1a-445	445-53	TLNEDLLEI	28	98	22.6 ± 14.4
pp1a-634	634-42	KLSAGVEFL	27	463	64.3 ± 8.1
pp1a-651	651-9	FLITGVFDI	27	640	8.1 ± 1.4
pp1a-1121	1121-9	ILLAPLLSA	26	72	48.1 ± 16.4
pp1a-1139	1139-47	SLQVCVQTV	28	160	5.2 ± 0.9
pp1a-1288	1288-96	MLSRALKKV	25	272	70.1 ± 8.7
pp1a-1652	1652-60	YLSSVLLAL	28	226	7.8 ± 1.9
pp1a-2187	2187-95	CLDAGINYV	27	352	3.8 ± 1.9
pp1a-2207	2207-15	AMWLLLLSI	27	144	378.1 ± 106.5
pp1a-2340	2340-48	WLMWFIISI	26	1552	1803.0 ± 439.6
pp1a-2546	2546-54	ILLLDQVLV	26	437	6.6 ± 2.0
pp1a-2754	2754-62	TLLCVLAAL	29	182	111.0 ± 30.2
pp1a-2755	2755-63	LLCVLAALV	25	118	167.6 ± 46.8
pp1a-2758	2758-66	VLAALVCYI	26	224	99.3 ± 6.4
pp1a-2990	2990-8	ALSGVFCGV	25	132	8.0 ± 3.2
pp1a-3444	3444-52	VLAWLYAAV	27	177	56.8 ± 12.7
pp1a-3459	3459-67	FLNRFTTTL	27	373	35.5 ± 8.0
pp1a-3560	3560-8	MLLTFLTSL	29	1174	61.1 ± 24.3
pp1a-3564	3564-72	FLTSLLILV	25	736	96.1 ± 14.9
pp1a-3616	3616-24	FLLPSLATV	33	2723	17.1 ± 14.2
pp1a-3687	3687-95	TLMNVITLV	25	592	15.0 ± 8.0
pp1a-3709	3709-17	SMWALVISV	28	959	3.2 ± 2.9
pp1a-3730	3730-8	FLARAIVFV	29	4047	20.1 ± 8.1
pp1a-3745	3745-53	LLFITGNTL	26	134	185.4 ± 36.9
pp1a-3816	3816-24	KLNIKLLGI	27	84	58.2 ± 16.8
pp1a-3848	3848-56	VLLSVLQQL	27	309	106.0 ± 24.8
pp1a-4071	4071-9	ALWEIQQVV	25	970	12.9 ± 10.5
pp1a-4219	4219-27	VLGSLAATV	26	118	79.1 ± 32.4

[a] Peptide binding scores to HLA-A2.1 were determined by the SYFPEITHI database (Rammensee et al., 1999) at http://www.syfpeithi.de/.
[b] Peptide binding scores to HLA-A2.1 were determined by the BIMAS database (Parker et al., 1994) at http://www-bimas.cit.nih.gov/molbio/hla_bind/.
[c] Data of peptide binding assays are shown as BL_{50}, indicating a concentration of each peptide that yields the half-maximal MFI of T2 cells pulsed with a control peptide, NS3-1585. Data are given as mean values ± SD of three independent experiments.

surface-coupled liposomal peptides (100 μg/mouse) together with CpG-ODN (5′-TCCATGACGTTCTGATGTT-3′, Hokkaido System Science, Sapporo, Japan) (5 μg/mouse) in 100 μl PBS in the footpad.

2.7. Intracellular cytokine staining (ICS)

ICS was performed as described by Matsui et al., 2005. Briefly, after one week following immunization, 2×10^6 spleen cells of immunized mice were incubated with 10 μM of a relevant peptide for 5 h at 37 °C in the presence of brefeldin A (GolgiPlug™, BD Biosciences). After blocking Fc receptors with the rat anti-mouse CD16/CD32 mAb (Fc Block™, BD Biosciences), cells were stained with FITC-conjugated rat anti-mouse CD8α mAb (BD Biosciences) for 30 min at 4 °C. Cells were then fixed, permeabilized, and stained with phycoerythrin (PE)-conjugated rat anti-mouse interferon-gamma (IFN-γ) mAb (BD Biosciences). After washing the cells, flow cytometric analyses were performed.

2.8. In vivo CTL assay

In vivo CTL assay was carried out as described before by Suvas et al., 2003. In brief, spleen cells from naive HHD mice were equally split into two populations. One population was pulsed with 10 μM of a relevant peptide and labeled with a high concentration (2.5 μM) of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR). The other population was unpulsed and labeled with a lower concentration (0.25 μM) of CFSE. An equal number (1×10^7) of cells from each population was mixed together and adoptively transferred i.v. into mice that had been immunized once with a liposomal peptide two weeks earlier. Twelve hours later, spleen cells were prepared and analyzed by flow cytometry. To calculate specific lysis, the following formula was used: % specific lysis = [1 − {(number of $CFSE^{low}$ cells in normal mice)/(number of $CFSE^{high}$ cells in normal mice)}/{(number of $CFSE^{low}$ cells in immunized mice)/(number of $CFSE^{high}$ cells in immunized mice)}] × 100.

2.9. Generation of C1R-A2 cells expressing multiple CTL epitopes

A multiepitope minigene that encodes multiple predicted epitopes and natural flanking amino acid sequences proximal to the N and C termini of each epitope (Fig. 1A) was synthesized by Invitrogen Japan K.K. (Tokyo, Japan). This minigene contains the Kozak sequence and the 3xFLAG-epitope-tag sequence upstream of the multiepitope sequence (Fig. 1A). The minigene was then cloned into the *Nhe* I and *Hind* III sites of a mammalian expression vector, pAcGFP1-Hyg-N1 (Clontech Laboratories Inc., Mountain View, CA). Since this vector encodes a green fluorescent protein (GFP) downstream of the multiple cloning site, the resultant pAcGFP1-pp1a plasmid expresses a GFP fusion protein containing pp1a-derived CTL epitopes under the control of the CMV promoter in mammalian cells. This plasmid was transfected into C1R-A2 cells by electroporation (Gene Pulser, Bio-Rad Laboratories Inc., Hercules, CA). After selection with hygromycin B (Invitrogen, Carlsbad, CA) at a final concentration of 1.5 mg/ml, the transfectant, C1R-A2-pp1a was confirmed for their GFP expression by flow cytometry (Fig. 1B).

2.10. ^{51}Cr-release assay

^{51}Cr-release assays were carried out as described before by Matsui et al., 2004. In brief, after two weeks following immunization, spleen cells of immunized mice were cultured with 10 μM of a relevant peptide for one week, and used as effector cells in standard ^{51}Cr-release assays. C1R-A2-pp1a cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$, and used for target cells.

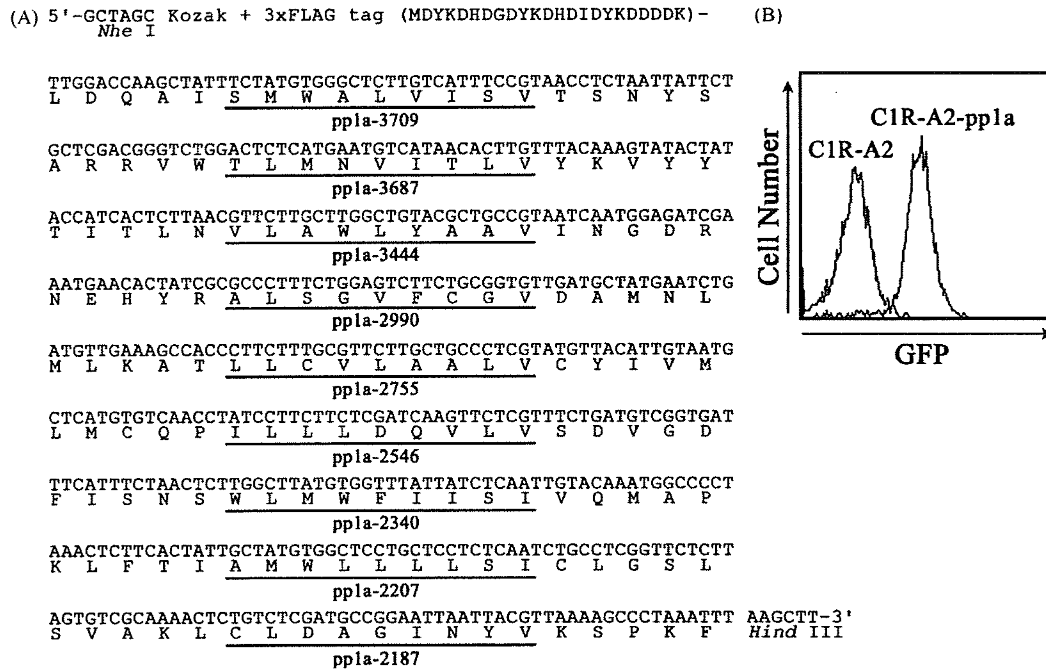

Fig. 1. (A) Nucleotide and amino acid sequences of nine predicted epitopess (pp1a-3709, pp1a-3687, pp1a-3444, pp1a-2990, pp1a-2755, pp1a-2546, pp1a-2340, pp1a-2207, pp1a-2187) with flanking amino acid residues encoded in a minigene. (B) Expression of a GFP fusion protein containing pp1a-derived epitopes in C1R-A2-pp1a cells. C1R-A2-pp1a cells transfected with the minigene were confirmed for their expression of a GFP fusion protein containing pp1a-derived epitopes in comparison with C1R-A2 cells by flow cytometry.

C1R-A2 cells transfected with pAcGFP1-Hyg-N1 vector were used as a negative target control. After a 4 h incubation, supernatant of each well was harvested and the radioactivity was counted. Results were calculated as the mean of a triplicate assay. Percent specific lysis was calculated according to the formula: % specific lysis = $[(cpm_{sample} - cpm_{spontaneous})/(cpm_{maximum} - cpm_{spontaneous})] \times 100$. Spontaneous release represents the radioactivity released by target cells in the absence of effectors, and maximum release represents the radioactivity released by target cells lysed with 5% Triton X-100.

2.11. Statistical analyses

Statistical comparisons between two groups were performed by the Student's t test. One-way ANOVA followed by post-hoc tests were used for statistical analyses between multiple groups in Fig. 2. All statistic analyses were performed using Graphpad Prism software. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Selection of potential HLA-A*0201-restricted CTL epitopes within SARS-CoV pp1a protein

The amino acid sequence of pp1a protein of SARS-CoV was searched for potential HLA-A*0201-restricted CTL epitopes by two computer-based programs, SYFPEITHI (Rammensee et al., 1999) and BIMAS (Parker et al., 1994). Based on the scores calculated, 30 nonameric peptides were selected and synthesized (Table 1). These peptides were then evaluated for their binding affinities to HLA-A*0201 molecules as described before by Ohno et al., 2006 (Table 1). Twenty-four out of the 30 peptides were high binders displaying BL_{50} values less than 100 μM, and four of them were medium binders displaying BL_{50} values ranging from 100 to 200 μM, suggesting that most epitopes should be properly predicted. In contrast, two peptides showed low affinity binding.

3.2. Induction of IFN-γ-producing CD8⁺ T cells by immunization with peptide-pulsed cells

To examine whether the predicted peptides could elicit peptide-specific CTLs *in vivo*, HHD mice were immunized i.v. with syngeneic spleen cells pre-pulsed with each of pp1a-derived peptides. One week after immunization, spleen cells of the immunized mice were prepared and stimulated *in vitro* with a relevant peptide for 5 h. $CD8^+$ T cells were then analyzed for their peptide-induced intracellular expression of IFN-γ. As shown in Fig. 2, significant numbers of IFN-γ-producing $CD8^+$ T cells ($p < 0.01$) were detected in mice immunized with syngeneic cells pulsed with each of nine pp1a-derived peptides including pp1a-2187, -2207, -2340, -2546, -2755, -2990, -3444, -3687, and -3709, suggesting that these nine peptides may be HLA-A*0201-restricted CTL epitopes derived from SARS-CoV pp1a protein.

3.3. Induction of SARS-CoV pp1a-specific CTLs by immunization with surface-linked liposomal peptides

Since the nine pp1a peptides were expected to be CTL epitopes (Fig. 2), these peptides were conjugated on the surface of liposomes. The resultant surface-linked liposomal peptides were then evaluated for their capabilities of CTL induction in mice. After HHD mice were immunized once with one of the nine liposomal peptides, spleen cells of them were prepared, stimulated with a relevant synthetic peptide, and stained for their expression of surface CD8 and intracellular IFN-γ. As shown in Fig. 3, six liposomal peptides including Lip-pp1a-2187 ($p < 0.05$), -2340 ($p < 0.05$), -2546 ($p < 0.05$), -2755 ($p < 0.05$), -2990 ($p < 0.01$), and -3709 ($p < 0.01$), were able to significantly induce IFN-γ-producing $CD8^+$ T cells compared with each negative control stimulated without a rel-

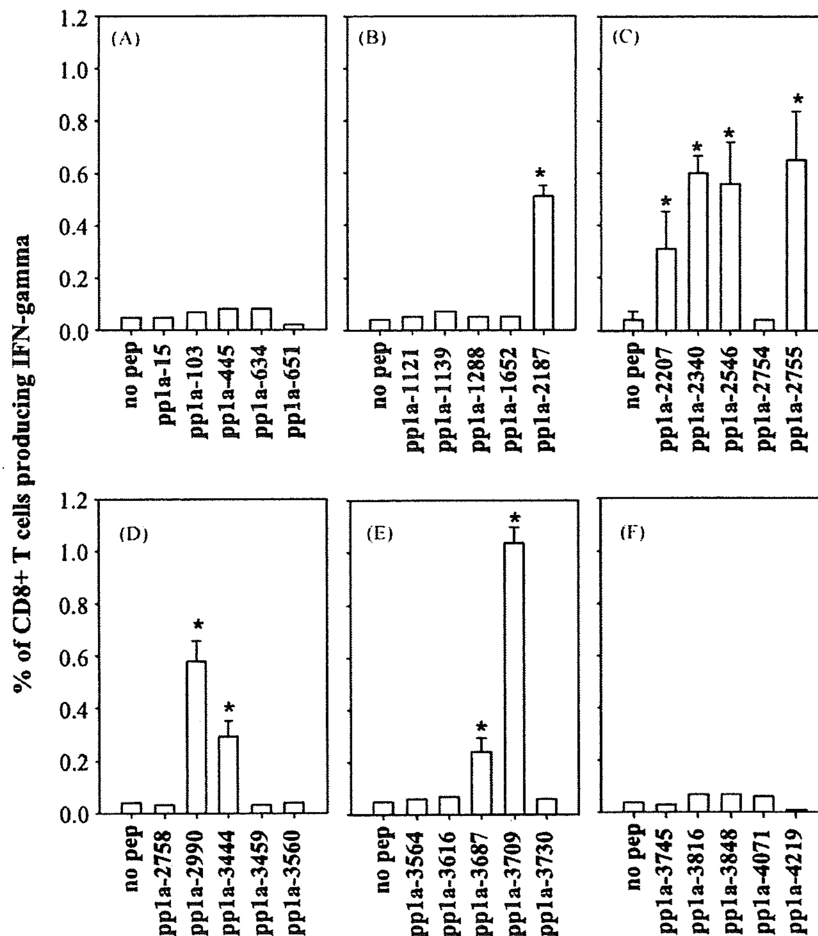

Fig. 2. Intracellular IFN-γ staining of $CD8^+$ T cells specific for pp1a-derived peptides in spleen cells of mice immunized i.v. with peptide-pulsed spleen cells. HHD mice were immunized i.v. once with syngeneic spleen cells pre-pulsed with each of pp1a-derived peptides. One week after immunization, spleen cells were prepared and stimulated with or without (no pep) a relevant peptide for 5 h. Cells were then stained for their surface expression of CD8 and their intracellular expression of IFN-γ (y axis). The data are shown as percentages of intracellular IFN-γ^+ cells within $CD8^+$ T cells. Three mice were used in each group and the data are shown as the mean ± SD of three mice. One-way ANOVA was used for comparison of data between groups in each of the panels (A–F). $^*p < 0.01$ compared to data of negative controls (no pep).

evant peptide. However, numbers of IFN-γ^+ $CD8^+$ T cells varied among these liposomal peptides (Fig. 3), suggesting a variety of immunogenicity. In particular, Lip-pp1a-3709 was most effective for the induction of peptide-specific IFN-γ^+ $CD8^+$ T cells (Fig. 3). In contrast, Lip-pp1a-2207 and Lip-pp1a-3444 marginally elicited IFN-γ-producing $CD8^+$ T cells, and Lip-pp1a-3687 failed to induce IFN-γ^+ $CD8^+$ T cells in mice. To further assess immunogenicity of the liposomal pp1a peptides, three known CTL epitopes derived from SARS-CoV spike protein, Spike-978, -1167 and -1203 (Wang et al., 2004a; Wang et al., 2004b) were conjugated on the surface of liposomes (Lip-Spike-978, -1167, and -1203), and were compared to the pp1a-derived peptides for their induction of IFN-γ-secreting $CD8^+$ T cells. As shown in Fig. 3, high percentages of IFN-γ^+ cells within $CD8^+$ T cells were detected in mice immunized with Lip-Spike-1203. However, this liposomal peptide was likely to be slightly less effective than Lip-pp1a-3709 in the induction of IFN-γ^+ $CD8^+$ T cells (Fig. 3). On the contrary, either Lip-Spike-978 or Lip-Spike-1167 did not elicit IFN-γ-producing $CD8^+$ T cells in mice (Fig. 3).

We next examined *in vivo* killing activities of peptide-specific CTLs in mice immunized with liposomal peptides. Two weeks after immunization, both peptide-pulsed $CFSE^{high}$ and unpulsed $CFSE^{low}$ target cells were delivered into the mice via i.v. injection, and then peptide-specific lysis was analyzed by flow cytometry (Fig. 4). In agreement with the ICS data in Fig. 3, high killing activities were observed in mice immunized with Lip-pp1a-3709 as well as Lip-Spike-1203 (Fig. 4). Lip-pp1a-2990 also induced a high level of killing activity in mice (Fig. 4). On the other hand, modest killing responses were elicited in mice immunized with each of five liposomal peptides involving Lip-pp1a-2187, -2340, -2546, -2755, -3687, whereas any significant cell lysis was not observed in mice injected with either Lip-pp1a-2207, -pp1a-3444, -Spike-978, or -Spike-1167 (Fig. 4). These data also suggest that each of the liposomal peptides exhibits different immunogenicity.

3.4. Recognition of naturally processed peptides by liposomal peptide-induced CTLs

To address whether the pp1a-derived peptides identified are naturally processed and presented, liposomal peptide-induced CTLs were tested for their capacity to lyse C1R-A2-pp1a cells. C1R-A2-pp1a cells carry the multiepitope minigene that encodes multiple predicted epitopes with several natural flanking amino acid residues at the N and C termini of each epitope (Fig. 1A), and thereby express naturally processed epitopes. Expression of the multiepitope minigene was confirmed by detection of GFP in C1R-A2-pp1a cells (Fig. 1B). HHD mice were immunized twice

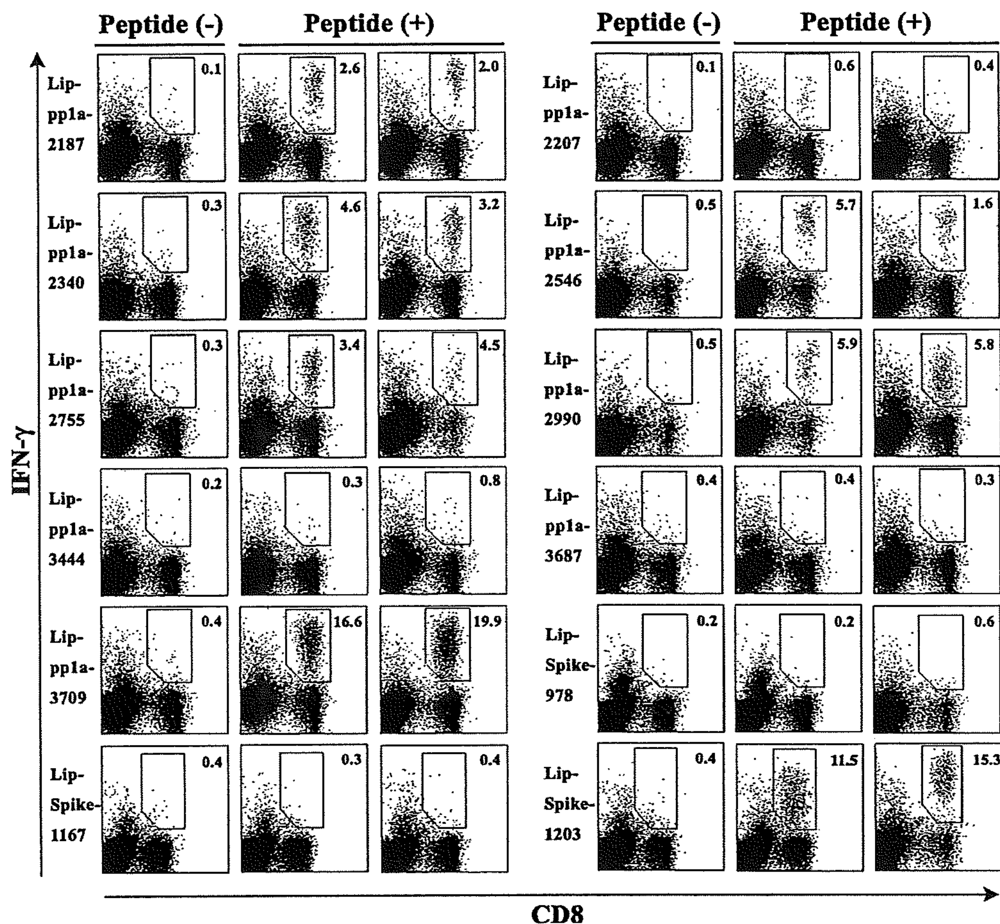

Fig. 3. Intracellular IFN-γ staining of CD8⁺ T cells specific for peptides derived from pp1a and spike of SARS-CoV in spleen cells of mice immunized with surface-linked liposomal peptides. HHD mice were immunized once with either Lip-pp1a-2187, Lip-pp1a-2207, Lip-pp1a-2340, Lip-pp1a-2546, Lip-pp1a-2755, Lip-pp1a-2990, Lip-pp1a-3444, Lip-pp1a-3687, Lip-pp1a-3709, Lip-Spike-978, Lip-Spike-1167, or Lip-Spike-1203 together with CpG.. After one week, spleen cells were prepared and stimulated with (+) or without (−) a relevant peptide for 5 h. Cells were then stained for their surface expression of CD8 (*x* axis) and their intracellular expression of IFN-γ (*y* axis). Two representatives per group are shown in the data of spleen cells stimulated with a peptide. The numbers shown indicate the percentages of intracellular IFN-γ⁺ cells within CD8⁺ T cells. The data shown are representative of three independent experiments.

with each of seven liposomal pp1a-derived peptides including Lip-pp1a-2187, -2340, -2546, -2755, -2990, -3687, and -3709 that significantly induced IFN-γ⁺ CTLs (Fig. 3) and/or *in vivo* killing activities (Fig. 4). CTL activities in spleen cells of the mice were then determined by ^{51}Cr-release assays using C1R-A2-pp1a cells as a target. As shown in Fig. 5, C1R-A2-pp1a cells were significantly ($p < 0.01$ or $p < 0.05$) lysed by liposomal peptide-induced CTLs, whereas C1R-A2 cells were not recognized by them. These data indicate that the seven pp1a-derived peptides are naturally processed and presented to CTLs.

3.5. Induction of long-lasting memory CTLs

We next examined whether long-lasting peptide-specific CTLs could be elicited by immunization with liposomal peptides. HHD mice were immunized three times at one-week intervals with either Lip-pp1a-2990 or Lip-pp1a-3709. Spleen cells were then prepared at various days after the final immunization, and stimulated *in vitro* once with a relevant peptide for 5 h at 37 °C. CD8⁺ T cells were then analyzed for their peptide-induced expression of intracellular IFN-γ. In both of the cases, the total numbers of IFN-γ-producing CD8⁺ T cells per mouse spleen were slightly increased at day 3 after immunization, rose to a peak at day 7, and gradually decreased as days went by (Fig. 6A and B). As shown in Fig. 7, IFN-γ-producing CD8⁺ T cells were still detected in mice 75 days after immunization with any of the two liposomal peptides. These results demonstrate that immunization with these liposomal peptides generated long-lasting memory CTLs. Especially, the frequency of IFN-γ⁺ CD8⁺ T cells in mice injected with Lip-pp1a-3709 was quite high (Fig. 7), indicating that Lip-pp1a-3709 may be an excellent vaccine candidate.

4. Discussion

In the current study, high-performing computational algorithms have extensively been utilized for the prediction of CTL epitopes derived from pp1a protein of SARS-CoV. As shown in Table 1, 30 peptides were selected as potential CTL epitopes using SYFPEITHI (Rammensee et al., 1999) and BIMAS (Parker et al., 1994). Out of them, only nine peptides could significantly induce IFN-γ-producing CD8⁺ T cells in mice (Fig. 2). Furthermore, there was not always a good correlation between peptides identified by the algorithms and their activity in the biological assays. For instance, pp1a-2340 and pp1a-3709 showed relatively inferior scores in BIMAS (Table 1), whereas both peptides stimulated good CTL responses (Figs. 2-4). Thus, currently available algorithms have limited accuracy to find actual epitopes (Chentoufi et al., 2008),

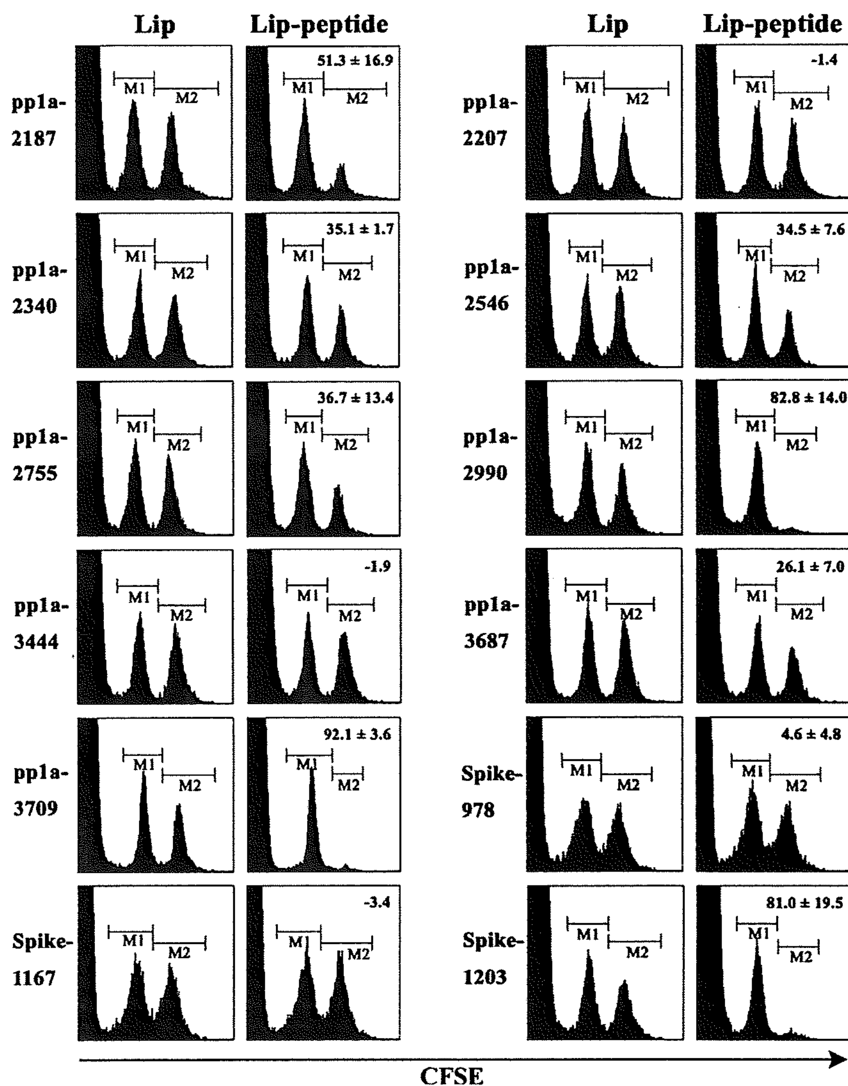

Fig. 4. *In vivo* killing activities specific for peptides derived from pp1a and spike of SARS-CoV in mice immunized with surface-linked liposomal peptides. HHD mice were immunized once with either each liposomal peptide (Lip-peptide) or liposomes alone (Lip) together with CpG. One week later, an equal number of a relevant peptide (pp1a-2187, pp1a-2207, pp1a-2340, pp1a-2546, pp1a-2755, pp1a-2990, pp1a-3444, pp1a-3687, pp1a-3709, Spike-978, Spike-1167, or Spike-1203)-pulsed CFSE^{high} targets (M2) and unpulsed CFSE^{low} targets (M1) were transferred into the immunized mice by i.v. injection. After 12 h, CFSE-labeled cells were recovered from spleens of recipient mice and analyzed by flow cytometry. The numbers are the percentages of specific lysis shown as mean values ± SD of three independent experiments.

However, they are still quite useful because we can easily choose a cluster of promising peptides within a huge protein such as pp1a on the programs. Multiple immunological screenings have been advanced to validate predicted CTL epitopes. When used individually, each screen is not sufficient for identifying actual epitopes. However, various combinations of these screens are usually successful (Chentoufi et al., 2008; Ohno et al., 2009). Therefore, we performed multiple screenings, including cell surface stabilization of HLA-A*0201 molecules on T2 cells, detection of antigen-driven IFN-γ-producing CD8$^+$ T cells, and functional *in vivo* and *in vitro* CTL assays.

In these experiments, we took advantage of highly reactive HLA-A*0201 transgenic mice, termed HHD mice (Pascolo et al., 1997). In HHD mice, the innate H-2D^b and mouse β2m genes have been disrupted by homologous recombination, and therefore, the only MHC class I molecule on the cell surface, HLA-A*0201, is efficiently utilized by HLA-A*0201-restricted CTLs. As a consequence, six peptides conjugated on the surface of liposomes significantly induced IFN-γ-producing CD8$^+$ T cells (Fig. 3), and seven liposomal peptides including the six peptides primed CTLs showing peptide-specific killing activities in mice (Fig. 4). However, it has to be taken account that there may be differences between the immunogenic variation observed in HLA class I transgenic mice and that in humans primarily because the antigen processing, presentation and ultimately, immunodominance may differ between them. In fact, it was shown that several HLA-A*0201-restricted CTL epitopes derived from human papillomavirus were not processed in HDD mice although these epitopes were naturally processed in HLA-A*0201$^+$ humans (Street et al., 2002), indicating that cross-species incompatibility in antigen-processing and presentation machinery skews the presentation of some CTL epitopes.

Because pp1a-specific CTLs induced were generated by stimulation with synthetic peptides, it was necessary to test whether they would recognize naturally processed peptides. To this end, we generated an HLA-A*0201 positive C1R-A2-pp1a cell line in substitution for SARS-CoV-infected target cells because it is quite

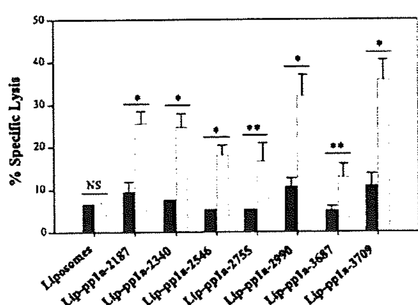

Fig. 5. Recognition of naturally processed epitopes derived from pp1a. HHD mice were immunized twice with either Lip-pp1a-2187, Lip-pp1a-2340, Lip-pp1a-2546, Lip-pp1a-2755, Lip-pp1a-2990, Lip-pp1a-3687, Lip-pp1a-3709, or liposomes alone. Two weeks after immunization, spleen cells were prepared and stimulated *in vitro* with a relevant peptide. After one week, ^{51}Cr release assays were performed at an E:T ratio of 150 with C1R-A2-pp1a cells (gray bars) or C1R-A2 (black bars) as targets. Data are shown as the means ± SD of triplicate wells. The experiment was repeated twice with similar results. At least three mice per group were used in each experiment. *$p < 0.01$; **$p < 0.05$; NS, not significant.

difficult to obtain live SARS-CoV in Japan. C1R-A2-pp1a cells carry the multiepitope minigene that encodes nine predicted epitopes with several natural flanking amino acid residues at the N and C termini of each epitope (Fig. 1). The basic idea to utilize flanking amino acids (Fig. 1) comes from the observation that flanking amino acid sequences modulate antigen processing of CTL epitopes (Le Gall et al., 2007). In fact, it was shown that several mutations at N-terminal (Draenert et al., 2004; Milicic et al., 2005) and C-terminal (Allen et al., 2004; Milicic et al., 2005) flanking residues of CTL epitopes disrupt proteasomal processing of HIV Gag and Nef proteins. Therefore, addition of flanking amino acid sequences allows natural antigen processing of CTL epitopes in C1R-A2-pp1a cells. As shown in Fig. 5, CTLs induced by seven liposomal peptides recognized HLA-A*0201 positive C1R-A2-pp1a cells. These data indicate that the seven peptides are naturally processed epitopes. As a matter of course, it will be necessary to examine whether pp1a-derived peptides can induce protective CTLs using SARS-CoV at the final screening.

So far, several CTL epitopes have been identified from spike and nucleocapsid proteins of SARS-CoV (Wang et al., 2004a; Wang et al., 2004b; Chen et al., 2005; Tsao et al., 2006; Zhou et al., 2006; Ohno et al., 2009). To our knowledge, however, the current study is the first report to demonstrate CTL epitopes derived from a non-structural protein of SARS-CoV such as pp1a protein. In fact, several liposomal peptides derived from pp1a induced high frequencies of IFN-γ-producing CD8$^+$ T cells (Fig. 3) in comparison with liposomal peptides derived from nucleocapsid which we have recently published (Ohno et al., 2009). In particular, Lip-pp1a-3709 turned out to be most effective in the induction of antigen-driven IFN-γ-producing CD8$^+$ T cells (Fig. 3), indicating that pp1a-3709 is a highly immunogenic, dominant CTL epitope.

It was demonstrated that the surface-linked liposomal peptide was effective for peptide-specific CTL induction in the current study as well as in the previous study (Ohno et al., 2009). It is noteworthy that long-lasting memory CTLs were detected in mice 75 days after immunization with liposomal peptides (Fig. 7). These data suggest that the surface-linked liposomal peptide may be an effective tool for CTL-based immunotherapy against infectious diseases such as SARS. The surface-linked liposomal peptide might be similar to the lipopeptide, a form of palmitoyl-lipidated peptide that is currently under intense investigation as human vaccines (Zhu et al., 2004; Zhang et al., 2009). Although both effectively induce peptide-specific CTLs, there are several differences between them. The lipopeptide is self-adjuvanting to stimulate peptide-specific CTLs via Toll-like receptor (TRL)-2 (Zhu et al., 2004; Zhang et al., 2009), but the surface-linked liposomal peptide requires external TLR ligands such as CpG (Nagata et al., 2007). However, CpG causes toxicity in humans (Davila et al., 2003), and hence, it is essential to find out a safe adjuvant for clinical use of liposomal peptides. On the other hand, the lipopeptide alone without a CD4$^+$ T-cell epitope

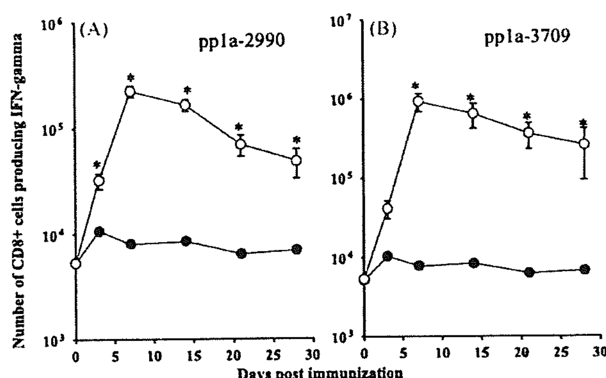

Fig. 6. Kinetics of pp1a-specific CD8$^+$ T cell responses after immunization with surface-linked liposomal peptides. Spleen cells were prepared from mice at various days after immunization with either Lip-pp1a-2990 (open circles in A), Lip-pp1a-3709 (open circles in B) or liposomes alone (closed circles in A and B). After stimulation with a relevant peptide for 5 h, cells were stained for their surface expression of CD8 and their intracellular expression of IFN-γ. The data indicate the total numbers of intracellular IFN-γ$^+$ CD8$^+$ T cells per mouse spleen, and are shown as the means ± SD of three mice per group. Significant (*$p < 0.05$) difference compared to mice immunized with liposomes alone on the same day post immunization.

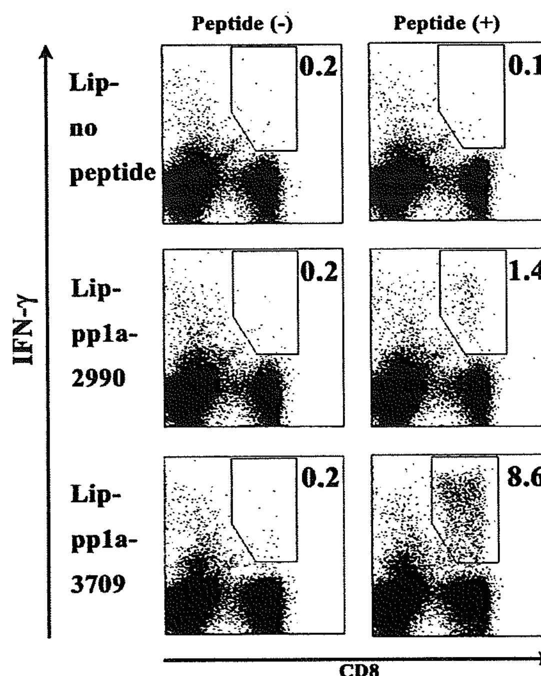

Fig. 7. Induction of long-lasting IFN-γ-producing CD8$^+$ T cells in mice immunized with surface-linked liposomal peptides. HHD mice were immunized three times at one-week intervals with either Lip-pp1a-2990, Lip-pp1a-3709 or liposomes alone (Lip-no peptide). Spleen cells were then prepared 75 days after the final immunization, and stimulated *in vitro* once with (+) or without (−) a relevant peptide for 5 hours at 37 °C. Cells were then stained for their surface expression of CD8 (*x* axis) and their intracellular expression of IFN-γ (*y* axis). The numbers shown indicate the percentages of intracellular IFN-γ$^+$ cells within CD8$^+$ T cells. The experiment was repeated twice with similar results, and the data shown are representative of the two independent experiments. At least three mice per group were used in each experiment, and spleen cells of mice per group were pooled.

failed to induce CTL-based protective immunity, whereas a helper peptide is not necessary for the surface-linked liposomal peptide to induce peptide-specific CTLs.

Although we focused this study on CTL epitopes restricted by HLA-A*0201 which is the most common HLA class I allele in the world, this is just a model system that could be applied to any haplotypes. The HLA polymorphism should hinder the development of our system, but the supertypes of HLA class I may solve this issue. Sette and Sidney, 1999 defined only nine HLA class I supertypes that almost cover the entire repertoire of HLA class I molecules. Epitopes related to all of the nine supertypes should be identified and incorporated into the liposomal vaccine.

In summary, we have identified seven HLA-A*0201-restricted CTL epitopes derived from pp1a protein of SARS-CoV using computational algorithms, HLA-A*0201 transgenic mice and the surface-linked liposomal peptide. It was shown that one of the liposomal pp1a peptides was effective for peptide-specific CTL induction in mice, and efficiently elicited long-lasting memory CTLs. These data suggest that surface-linked liposomal peptides derived from pp1a protein may offer an effective and safe CTL-based vaccine against SARS.

Acknowledgments

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Highly Efficient Antiviral CD8$^+$ T-Cell Induction by Peptides Coupled to the Surfaces of Liposomes[▽]

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In previous studies, we have demonstrated that liposomes with differential lipid components display differential adjuvant effects when antigens (Ags) are chemically coupled to their surfaces. When ovalbumin was coupled to liposomes made by using unsaturated fatty acids, it was found to be presented not only to CD4$^+$ T cells but also to CD8$^+$ T cells and induced cytotoxic T lymphocytes (CTLs) which effectively eradicated the tumor from mice. In this study, we coupled liposomes to immunodominant CTL epitope peptides derived from lymphocytic choriomeningitis virus (LCMV) and evaluated its potency as an antiviral vaccine. The intramuscular immunization of mice with the peptide-liposome conjugates along with CpG resulted in the efficient induction of antiviral CD8$^+$ T-cell responses which conferred complete protection against not only LCMV Armstrong but also a highly virulent mutant strain, clone 13, that establishes persistent infections in immunocompetent mice. The intranasal vaccination induced mucosal immunity effective enough to protect mice from the virus challenge via the same route. Complete protection was achieved in mice even when the Ag dose was reduced to as low as 280 ng of liposomal peptide. This form of vaccination with a single CTL epitope induced Ag-specific memory CD8$^+$ T cells in the absence of CD4$^+$ T-cell help, which could be shown by the complete protection of CD4-knockout mice in 10 weeks as well as by the analysis of recall responses. Thus, surface-linked liposomal peptide might have a potential advantage for the induction of antiviral immunity.

The development of practical vaccines has been greatly potentiated by the availability of synthetic antigens (Ags), but progress has been hampered by the poor immunogenicity of Ags. Liposomes have successfully been used as drug carriers (35) and have also been proposed to be carriers of Ags and adjuvants to induce immune responses (33). Most of the liposomal vaccines proposed have been prepared by Ag encapsulation within the aqueous lumen of liposomes. However, it is known that the immune responses induced by encapsulated liposomal Ags are different from those induced by surface-linked liposomal Ags.

We have demonstrated that Ags chemically coupled to the surfaces of liposomes induce Ag-specific immunoglobulin G (IgG) but not IgE antibody production (28). The inducibility of Ag-specific IgG production was found to vary among liposome preparations: the greater that the membrane mobility in the liposomes is, the greater that the antibody production induced by Ag-liposome conjugates is (29). In our previous study, we have reported that ovalbumin (OVA) coupled to liposomes made with unsaturated fatty acids was presented to both CD4$^+$ and CD8$^+$ T cells, whereas OVA coupled to liposomes made with saturated fatty acids was presented only to CD4$^+$ T cells. Furthermore, the cross-presentation of OVA coupled to liposomes consisting of unsaturated fatty acids was further confirmed by the in vivo induction of cytotoxic T lymphocytes (CTLs) which conferred tumor eradication (42) and protection against influenza virus (27) in mice. However, the advantages of the surface-linked liposomal peptides for other forms of vaccines, especially regarding the efficiency of effector CD8$^+$ T cells and the inducibility of long-term memory CD8$^+$ T cells, have not been demonstrated.

In the present study, we evaluated the potency of surface-coupled liposomal peptides as an antiviral vaccine using the infection of mice with lymphocytic choriomeningitis virus (LCMV) as a model. Use of that model enabled us to compare the effectiveness of this system with that of various vaccine formulas prepared with the same epitope peptide as the Ag reported elsewhere. We also elucidate the induction and maintenance of memory CD8$^+$ T cells by a minimum CTL epitope peptide which does not seem to stimulate the help of CD4 cells.

MATERIALS AND METHODS

Mice and viruses. Female C57BL/6 mice (age, 6 weeks) were purchased from Clea Japan, Inc. (Tokyo, Japan), and Tokyo Laboratory Animal Science Co. Ltd. (Tokyo, Japan). CD4-knockout (KO) mice of a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). After they were bred, 6-week-old male CD4-KO mice were used for the experiments. The mice were housed in appropriate animal care facilities at Saitama Medical University (Saitama, Japan) and were handled according to international guidelines. The experimental protocols were approved by the Animal Research Committee of Saitama Medical School (approval number 634). The mice received 2 × 10^5 PFU of LCMV Armstrong (Arm) intraperitoneally (i.p.) or intranasally (i.n.) to ini-

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