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Peptide-loaded chimeric influenza virosomes for efficient *in vivo* induction of cytotoxic T cells

Mario Amacker[1]*, Olivier Engler[2]*, Andreas R. Kammer[1]*, Sonia Vadrucci[3,5], David Oberholzer[1], Andreas Cerny[4] and **Rinaldo Zurbriggen[1]**

[1]Pevion Biotech Ltd, Rehhagstrasse 79, CH-3018 Bern, Switzerland
[2]Clinic for Rheumatology and Clinical Immunology/Allergology, Inselspital, University of Bern, CH-3010 Bern, Switzerland
[3]Department of Biology Institute of Biochemistry, Federal Institute of Technology ETH, Hoenggerberg, CH-8093 Zurich, Switzerland
[4]Dipartimento di Medicina, Ospedale Regionale di Lugano, Ospedale Civico, CH-6900 Lugano, Switzerland

[5]Present address: Space Biology Group, ETH Technopark, Technoparkstrasse 1, CH-8005 Zurich, Switzerland

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Abstract

Virus-specific CD8$^+$ T cells are thought to play an important role in resolving acute hepatitis C virus (HCV) infection as viral clearance has been associated with a strong and sustained CD8$^+$ T cell response. During the chronic state of HCV infection virus-specific T cells have a low frequency and a reduced responsiveness. Based on this, a therapeutic vaccine increasing the frequency of specific T cells is a promising alternative for the treatment of chronic HCV infection. We improved an existing vaccine platform based on immunopotentiating reconstituted influenza virosomes (IRIVs) for efficient delivery of peptide epitopes to the MHC class I antigen presentation pathway. IRIVs are proteoliposomes composed of phospholipids and influenza surface glycoproteins. Due to their fusogenic activity, IRIVs are able to deliver encapsulated macromolecules, e.g. peptides to immunocompetent cells. We developed a novel method based on chimeric virosomes [chimeric immunopotentiating reconstituted influenza virosomes (CIRIVs)] combining the high peptide-encapsulation capacity of liposomes and the fusion activity of virosomes. This new approach resulted in a 30-fold increase of the amount of incorporated soluble peptide compared with current preparation methods. To study the immunogenicity of chimeric virosomes HLA-A2.1 transgenic mice were immunized with CIRIVs containing the HCV Core132 peptide. Core132-CIRIVs efficiently induced specific cytotoxic and IFNγ-producing T cells already with low peptide doses. Vaccine formulations, which include combinations of different HCV-derived CTL epitopes could be used to induce not only a strong but also a multi-specific CTL response, making them potential candidates for therapeutic and maybe prophylactic T cell vaccines in humans.

Introduction

Immunopotentiating reconstituted influenza virosomes (IRIVs) have been developed to obtain a potent and well-tolerated adjuvant for vaccines, already registered for use in humans (1–8). IRIVs are spherical, unilamellar vesicles with a mean diameter of ~150 nm. Their base is a liposome comprised of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and lipids derived from the influenza virus. Phospholipids are virtually non-immunogenic and have enjoyed a long history of use in human pharmaceutical preparations. The haemagglutinin (HA) and trace quantities of viral neuraminidase and phospholipids from influenza virus are intercalated within the phospholipid bilayer, whereby the presence of HA is necessary to enhance the immunopotentiating effect to antigens associated with IRIVs (9). The influenza HA plays a key role in the mode of action of the IRIVs. HA is a trimeric integral membrane protein (M_r 220 000) comprised of an ectodomain of identical subunits, each of which contain two polypeptides, HA_1 and HA_2, linked by a disulphide bond (10). The two polypeptides arise from a proteolytic cleavage event and are essential for fusion activity of the virus with the endosomal

*These authors contributed equally to this study.

Correspondence to: R. Zurbriggen; E-mail: rinaldo.zurbriggen@pevion.com

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membrane (11–13). HA_1 has a high affinity for sialic acid, which is present at relatively high concentrations on the surface of antigen-presenting cells (APCs; e.g. macrophages, dendritic cells). Like influenza virus IRIVs bind to sialic acid residues on the cell surface and the entry of influenza virosomes into cells occurs through receptor-mediated endocytosis (14). At the low pH of the host cell endosome (pH ~5), a conformational change occurs in the HA which is a prerequisite for fusion to occur. The HA_2 subunit of HA then mediates the fusion of virosomal and endosomal membranes. As a result of this fusion the contents of the virosome are released into the cytosol. Virosomes, therefore, are an elegant carrier system for the delivery of macromolecules like antigenic peptides, nucleic acids and others into the cell cytoplasm.

Synthetic peptide-based vaccines, which are designed to stimulate $CD8^+$ cytotoxic T cells, are an attractive approach to the treatment or prevention of infectious diseases and malignant disorders. One major drawback of such peptide vaccines is the rapid degradation of the peptide by proteases (15). However, macromolecules encapsulated into virosomes are protected from enzymatic degradation (16).

Upon endosomal fusion of the virosome containing cytotoxic T cell (CTL) epitopes within the APC, the T cell epitopes are released into the cytoplasm and directed into the MHC class I antigen presentation pathway thereby leading to the induction of $CD8^+$ T cells. It has been shown *in vitro* that virosomes containing a synthetic peptide are able to deliver the peptide antigen to the MHC class I presentation pathway, stimulating specific CTLs as well as rendering target cells susceptible to antigen-specific CTL-mediated lysis (17). In order to induce a CTL response, not only CTL epitopes have to be delivered but also T helper epitopes. These epitopes play a critical role in the induction of MHC class I-restricted CTL (18–21). The virosomal carrier is also able to trigger a T helper response since T helper epitopes are located on the influenza HA, the major component of IRIVs. It has been demonstrated that a synthetic peptide enclosed in virosomes is able to induce a CTL immune response in mice (22). In addition, the use of virosomes avoids the induction of tolerance, which has been observed under certain conditions after immunizing mice intra-peritoneally with soluble peptides (23).

Hepatitis C virus (HCV) is a major cause of chronic liver diseases, such as cirrhosis and liver cancer. About 3% of the world's population are infected with HCV and ~70–80% of newly infected patients progress to chronic infection (24). Anti-viral drugs such as IFNα/pegylated IFNα, alone or in combination with ribavirin, are effective in up to 80% of patients (25, 26) but many patients do not tolerate combination therapy (24). As for many other viral infections, vaccination might be an effective tool to control disease and therefore, it is desirable to develop a vaccine for the treatment of chronic infection as well as to prevent *de novo* infection. Recent evidence indicates that the cellular immune response is responsible for viral clearance in HCV-infected individuals. In particular, CTL may be a major defence mechanism in HCV infection (27–30). The ability to induce cytotoxic and IFNγ-producing T cells is considered to be an important feature of a candidate HCV vaccine. Since the core protein is the best-conserved structural protein in the HCV genome (31), we used the HCV Core132 epitope (DLMGYIPLV, aa 132–140) (32) as a model antigen.

One of the problems in current virosome technology is the lack of methods for the efficient entrapment of peptides or other molecules. For the induction of a good antibody immune response (B cell immune response), the antigens must be cross-linked to the virosome surface or integrated into the virosome bilayer via a lipid anchor. With the widely used detergent-removal procedure (4), efficient loading can be achieved for such antigens (33). In contrast, for CTL epitopes, which need to be encapsulated into virosomes, <1% of the aqueous phase can be entrapped in the virosome particle with the detergent-removal method and a given lipid concentration of 1 mM and an assumed virosome diameter of ~150 nm. Therefore, this is a rather inefficient and expensive method for the delivery of T cell antigens *in vivo*. On the other hand, high encapsulation efficiencies can be achieved by the freeze/thaw technique used to prepare pure lipid vesicles (34). However, liposomes lack the specific targeting to sialic acid residues on the surface of APCs and the adjuvant effect of the HA observed with virosomes. As a consequence, a method was developed that combines the positive properties of virosomes and liposomes with the result of a substantially increased entrapment of peptides or other molecules within functional virosomes. IRIVs prepared with this novel method induce a strong and dose-dependent CTL response against the peptide used.

Methods

Reagents and peptides

Octaethyleneglycol-mono-(*n*-dodecyl)ether (OEG, $C_{12}E_8$), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (PG), acetonitrile, triethylammonium phosphate (TEAP) solution, Streptomycin, HEPES, penicillin and RPMI medium were purchased from Fluka Chemie GmbH and Sigma (Buchs, Switzerland), respectively. FCS was purchased from GIBCO BRL (Basel, Switzerland). IL-2 was obtained from EuroCetus B.V. (Amsterdam, The Netherlands). Egg PC was obtained from Lipoid (Cham, Switzerland). PE was obtained from R. Berchtold (Biochemical Laboratory, University of Bern, Switzerland). SM2 Bio-Beads were from Bio-Rad Laboratories (Glattbrugg, Switzerland). *N*-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Bodipy 530/550-DHPE), Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (N-Rh-DHPE) and biotin-DHPE (*N*-(biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) were from Molecular Probes Europe (Leiden, The Netherlands). Sephadex G50 coarse was obtained from Amersham Biosciences (Otelfingen, Switzerland). Dynabeads MyOne™ Streptavidin (10 mg ml^{-1}) were purchased from Dynal Biotech (Hamburg, Germany).

The HLA-A2.1-binding HCV peptides Core132 (DLMGYIPLV, aa 132–140) (32) and Core35 (YLLPRRGPRL, aa 35–44) (32) were obtained from Bachem AG (Bubendorf, Switzerland).

Mice

Immunization experiments were performed in HHD mice transgenic for HLA-A2.1 (A0201) monochain histocompatibility class I molecule and deficient for both $H\text{-}2D^b$ and murine

β_2-microglobulin (35). Mice were housed in appropriate animal care facilities and handled according to international guidelines.

Viruses

Influenza viruses of the X-31 strain and the A/Sing (A/Singapore/6/86) strain, propagated in the allantoic cavity of embryonated eggs (36), were obtained from Berna Biotech AG (Bern, Switzerland) and purified as described previously (37). The HA : phospholipid ratio was determined by phospholipid determination following the method of Böttcher (38) and HA quantification after SDS-PAGE with the Coomassie-extraction method following the method of Ball (39).

Preparation of chimeric immunopotentiating reconstituted influenza virosomes

Chimeric virosomes with HA from the X-31 and the A/Sing Influenza strain, respectively, were prepared by the method described previously (3, 40). Briefly, 32 mg egg PC and 8 mg PE were dissolved in 2 ml of PBS, 100 mM OEG (OEG-PBS). Four micrograms HA of each influenza virus was centrifuged at 100 000 × *g* for 1 h at 4°C and the pellet was dissolved in 2 ml of OEG-PBS. The detergent-solubilized phospholipids and viruses were mixed and sonicated for 1 min. This mixture was centrifuged at 100 000 × *g* for 1 h at 20°C and the supernatant was sterile filtered (0.22 μm). Virosomes were then formed by detergent removal using 180 mg of wet SM2 Bio-Beads for 1 h at room temperature with shaking and three times for 30 min with 90 mg of SM2 Bio-Beads each.

Preparation of liposomes with encapsulated peptide

A total of 36.4 μmol (28 mg) PC and 15.6 μmol (11 mg) PG (molar ratio 70 : 30) were dissolved in methanol : chloroform (2 : 1). The solvent was removed by a rotary evaporator (Rotavapor R-205, Büchi Labortechnik, Switzerland) at 40°C at a gradual vacuum of 30–10 kPa. The dried lipid film was hydrated with 350 μl PBS containing 2–3.5 mg Core132 peptide to be encapsulated. Before extrusion, the volume was adjusted to 500 μl with PBS. The liposome dispersion was extruded 10 times through polycarbonate membranes (Nucleopore Track-Etch membrane, 0.2 μm; Whatman, Kent, UK) with a 1.5-ml Lipex Extruder (Northern Lipids, Vancouver, Canada). Size determination of extruded liposomes was done by light scattering using a Zetasizer 1000HS instrument (Malvern Instruments, Worcestershire, UK).

Preparation of Core132-chimeric immunopotentiating reconstituted influenza virosomes

Chimeric immunopotentiating reconstituted influenza virosomes (CIRIVs; 600 μl in PBS, ~6 mg phospholipid) were incubated with 200 μl (~15 mg phospholipid) of PC/PG-extruded liposomes (0.2 μm diameter) containing the Core132 peptide at 10°C in PBS under constant stirring. To trigger fusion the pH was adjusted to 4.5 with 15 μl of 1 M HCl. After incubation for 30 min, the mixture was neutralized with 15 μl of 1 M NaOH and fusion products were extruded 10 times through polycarbonate membranes (Nucleopore Track-Etch membrane, 0.2 μm) with a 1.5-ml Lipex Extruder (Northern Lipids).

Core132 peptide quantification

Peptide quantification was done by HPLC on a Äkta Explorer 10 (Amersham Biosciences) using a CC 125/4.6 Nucleosil 100-5 C8 reversed-phase column by HPLC (RP-HPLC; Macherey-Nagel, Oensingen, Switzerland). The following eluents were used: buffer A, 10 mM TEAP in H_2O; buffer B, 100% acetonitrile; HPLC program: flow rate 1.3 ml min^{-1}; buffer and column temperature 25°C; buffer starting concentration: 25% B; 0–7 min: increase of buffer B to 38%; 7–12.4 min: increase of buffer B to 100%; 12.4–17 min: 100% buffer B. For quantification of encapsulated peptide, a fraction (5–30 μl) of liposomes or virosomes, respectively, were loaded on freshly prepared, PBS-equilibrated 1 ml Sephadex G50 Coarse gel-filtration spin columns. Vesicles with encapsulated peptide only were obtained after centrifugation of the spin column at 300 × *g* for 2 min, as the non-encapsulated peptide was retarded in the column. For spiking experiments, 0.5 mg of peptide was added before purification to 30 μl of empty liposomes or to 30μl of inactivated A/sing Influenza virus.

Fluorescence resonance energy transfer assay

For *in vitro* fusion measurements by fluorescence resonance energy transfer (FRET) (41, 42), the following assay was developed: 0.75 mol% of Bodipy 530/550-DHPE and 0.25 mol% of N-Rh-DHPE were incorporated into liposomes consisting of PC : PG (70 : 30). Fluorescence measurements were carried out at discrete temperatures between 4 and 42°C in 5 mM sodium phosphate buffer pH 7.5, 100 mM NaCl, in a final volume of 0.8 ml in 2.5 ml poly(methyl methacrylate) (PMMA) micro-cuvettes (VWR, Dietikon, Switzerland) under continuous stirring. Typically, 1 μl of labelled liposomes (0.3 nmol phospholipid) was mixed with 5–20 μl of virosomes (0.1–0.4 nmol phospholipid) and fusion was triggered by the addition of 3.75–7 μl of 1 M HCl, resulting in a pH of 4.5. The increase in fluorescence was recorded every 5 s at excitation and emission wavelengths of 538 and 558 nm, respectively, with an excitation slit of 2.5 nm and an emission slit of 15.0 nm. Measurements were carried out with an LS 55 Luminescence spectrometer (Perkin Elmer Instruments, Schwerzenbach, Switzerland) equipped with a thermostated cuvette holder and a magnetic stirring device. The maximal fluorescence at infinite probe dilution was reached after addition of Triton X-100 (0.5% v/v final concentration). For calibration of the fluorescence scale the initial residual fluorescence of the liposomes was set to zero and the fluorescence at infinite probe dilution to 100% (maximal fluorescence).

Affinity precipitation of Core132-CIRIV$_{Bio}$

Chimeric virosomes with HA from the X-31 and the A/Sing Influenza strain were prepared as described in Preparation of Chimeric Immunopotentiating Reconstituted Influenza Virosomes with addition of 4 mg biotin-DHPE to the phospholipid mixture (CIRIV$_{Bio}$ with 1 mg ml^{-1} biotin-DHPE). Core132-CIRIV$_{Bio}$ were prepared as described in Preparation of Core132-Chimeric Immunopotentiating Reconstituted Influenza Virosomes with CIRIV$_{Bio}$ instead of regular CIRIVs. For affinity precipitation of Core132-CIRIV$_{Bio}$, 5 μl of Core132-CIRIV$_{Bio}$ was added to buffer A (50 mM sodium phosphate, 0.1 M NaCl, pH 7.5) to a final volume of 200 μl. As controls,

Core132-CIRIV$_{Bio}$ were supplemented with Triton X-100 to 0.5 % (v/v), or Core132-liposomes (equal amount of peptide as Core132-CIRIV$_{Bio}$) were mixed with 5 μl of empty CIRIV$_{Bio}$, in a volume of 200 μl. Hundred microlitres of MyOne Streptavidin paramagnetic beads was washed twice in buffer A and incubated with the sample mixtures at 4°C for 1.5 h with continuous shaking. The precipitate was washed twice in buffer A and re-suspended in 25 μl OEG-PBS. After incubation at 4°C for 5 min, the beads were removed and the supernatant was analysed by RP-HPLC as described in Core132 Peptide Quantification.

Immunization and cytotoxicity assay

Where indicated HLA-A2.1 transgenic mice were immunized intramuscularly with inactivated Influenza A/Sing (1 μg HA per dose) 3 weeks prior to immunization with vaccine formulations. Mice were immunized subcutaneously at the base of the tail with 100 μl of virosome formulation, liposomes with encapsulated peptide, peptide with empty virosomes, peptide in saline solution, empty virosomes or saline solution. Mice received two injections at a 3-week interval and the response was analysed 2 weeks after the last injection. Spleen cells (4×10^6 per well) from individual immunized mice were re-stimulated for 5 days in 24-well tissue culture plates with 2×10^6 irradiated (1500 rad) spleen cells that have been pulsed with 10 μg ml^{-1} Core132 peptide, in complete RPMI medium containing 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} Streptomycin, 5 mM HEPES, 10% FCS and 5×10^{-5} M 2-mercaptoethanol at 37°C and 5% CO_2. On day 2, 5 U ml^{-1} IL-2 was added. Specific cytolytic activity was tested in a standard ^{51}Cr-release assay against EL4S3^{-}Rob HHD target cells pulsed with 10 μg ml^{-1} of Core132 peptide or medium control. After a 4-h incubation, ^{51}Cr release was measured by using a γ-counter. Spontaneous and maximum release was determined from wells containing medium alone or after lysis with 1 N HCl, respectively. Lysis was calculated by the formula: (release in assay − spontaneous release)/(maximum release − spontaneous release) × 100. Peptide-specific lysis was determined as the percentage of lysis obtained in the presence or in the absence of peptide. Spontaneous release was always <15% of maximum release.

Enzyme-linked immunospot assay

To quantify the frequency of epitope-specific IFNγ-producing cells we used the IFNγ enzyme-linked immunospot (ELISPOT) kit from U-CyTech (Utrecht, The Netherlands). Spleen cells (6×10^6 per well) from immunized mice were re-stimulated in 24-well tissue culture plates with 10 μg ml^{-1} Core132 peptide or Core35 peptide (negative control) in complete RPMI medium containing 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} Streptomycin, 5 mM HEPES, 10% FCS and 5×10^{-5} M 2-mercaptoethanol at 37°C and 5% CO_2. After overnight stimulation 10^5 and 10^4 cells from the re-stimulation cultures were transferred in duplicates to a pre-coated and albumin-blocked IFNγ ELISPOT plate (U-CyTech) and incubated for 5 h at 37°C to allow cytokine secretion. Spots were developed following the manufacturer's instructions and were counted by using a microscope. The background level was assessed in wells where effector cells were stimulated with splenocytes pulsed with Core35 peptide. The number of peptide-specific spots was obtained by subtracting the background from the number of spots induced after Core132 peptide stimulation.

Intracellular IFNγ staining

Spleen cells (12×10^6) were incubated with 10 μg ml^{-1} Core132 peptide or Core35 peptide (negative control) in complete RPMI medium containing 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} Streptomycin, 5 mM HEPES, 5% FCS and 5×10^{-5} M 2-mercaptoethanol at 37°C and 5% CO_2 in the presence of 5 μg ml^{-1} Brefeldin A for 4 h. Cells were stained with FITC-conjugated anti-CD8 antibodies, permeabilized and stained with PE-conjugated anti- IFNγ antibodies using the Cytofix/Cytoperm kit following the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). Data were acquired on a FACSCalibur flow cytometer and analysed with WinMDI2.8 software. Frequency of IFNγ-producing cells was calculated as percentage of IFNγ-positive and CD8-positive cells among total CD8-positive cells. The percentage of peptide-specific cells was obtained by subtracting the percentage in samples stimulated with Core35 peptide from the percentage in samples stimulated with Core132 peptide.

Results

The concept of chimeric virosomes

A method was developed that combines the positive properties of virosomes and liposomes with the result of a substantially increased entrapment of peptides or other molecules within functional virosomes (Fig. 1). In a first step, a dried lipid film of PC and phosphatidylglycerol (PG) is prepared, and liposomes are formed by re-hydration of the film with a small volume of a concentrated peptide solution. The formed liposomes efficiently encapsulate the peptide, but are multi-lamellar and heterogeneous. Unilamellar and homogeneous liposomes are obtained after a defined number of cycles of extrusion through a 200-nm filter. In a second step, IRIVs with a binary mixture of HA molecules from different influenza virus strains are prepared (hereafter called CIRIVs). The HA molecules of the used strains display different temperature-dependent fusion characteristics: At pH 5 or lower, the X-31 HA triggers fusion efficiently already at low temperatures, whereas at the same pH, the HA from the influenza virus strain A/Sing requires elevated temperatures (>25°C) (13, 16). Thus, CIRIVs containing the HA molecules from both the X-31 and A/Sing virions are able to catalyse two distinct fusion reactions at a pH ~4.5, the first one at a low temperature (<20°C) and the second one at an elevated temperature (>25°C). Finally, peptide-containing CIRIVs result from fusion of liposomes with encapsulated peptides and CIRIVs at a pH ~4.5 and a temperature of 10°C. The fused vesicles are re-sized to a diameter of 200 nm by extrusion. These peptide-containing proteoliposomes are supposed, like intact influenza virus, to be taken up by cells through receptor-mediated endocytosis. In the acidic environment of the endosome, the A/Sing HA molecules are expected to trigger the second fusion reaction between the virosomal and endosomal

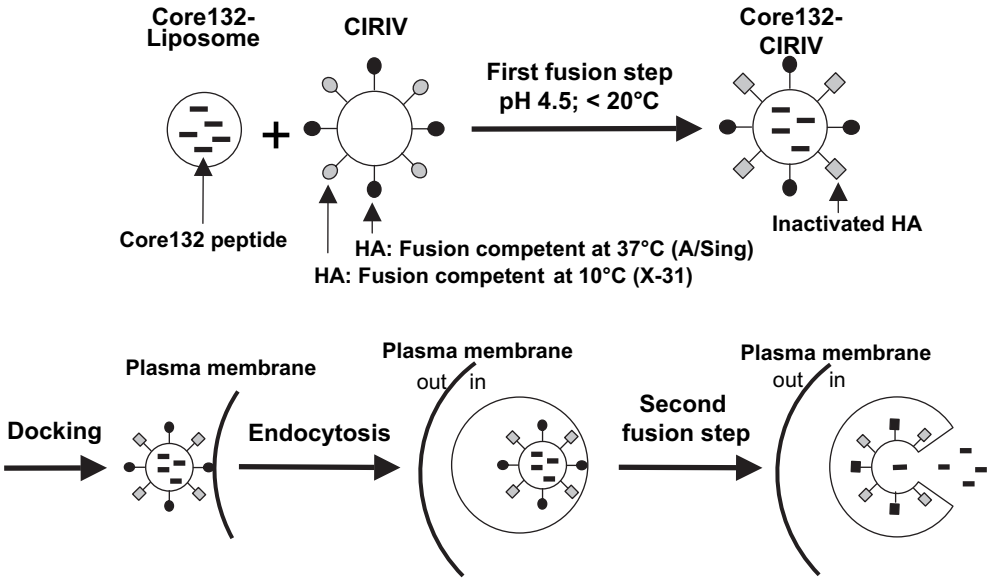

Fig. 1. Schematic representation of the preparation of chimeric virosomes containing the Core132 peptide with the two fusion steps. In a first fusion step, chimeric virosomes with HA from the A/Sing and the X-31 strains were fused with homogenized liposomes of a diameter of 200 nm containing the Core132 peptide inside the particle. Fusion takes place at a pH ~4.5 and at a temperature <20°C and is mediated by the HA derived from X-31. The resulting, neutralized fusion products were used for vaccination of mice. After receptor-mediated endocytosis, a second fusion step triggered by the low pH within endosomes and mediated by A/Sing HA takes place, releasing the Core132 peptide into the cytosol.

membranes, with the consequent release of the peptide into the cytosolic compartment of the cells.

Generation of Core132-CIRIVs

CIRIVs containing Core132 peptide were prepared with this novel procedure (Fig. 1). A concentrated peptide solution of 10 mg ml^{-1} in PBS was used to re-hydrate the lipid film. Determination of peptide concentrations was performed on a RP-HPLC. To remove free peptide, Core132-CIRIVs were purified over Sephadex G50 media with a fractionation range of M_r 1.5×10^3–3×10^4 for globular molecules. Therefore, virosomes were not fractionated, whereas peptide was retained by the media. As a control for the efficiency of this method, a defined amount of free Core132 peptide was added to empty liposomes or A/Sing virus and purified over Sephadex G50 (spiking control). No Core132 was found in the purified liposome and virus fractions, (data not shown). Fifty to seventy five percentage of the added Core132 peptide was encapsulated in the formed liposomes (Table 1). After extrusion, 30–40% of the starting material was still found inside the homogenized Core132-liposomes. To obtain the Core132-CIRIVs, CIRIVs and Core132-liposomes were incubated at a pH ~4.5 at 10°C for 20 min. After neutralization, the fused chimeric vesicles were homogenized by extrusion to a diameter of 200 nm. In contrast to the first extrusion step applied on Core132-liposomes, no encapsulated peptide was lost during the extrusion of the fused chimeric vesicles. Therefore, 30–40% of the starting amount of peptide was finally found entrapped inside the Core132-CIRIVs. Typical concentrations obtained for the encapsulated Core132 peptide were up to 300 μg ml^{-1}. With the conventional detergent-removal method an entrapment efficiency <1% was obtained for the Core132 peptide, as a maximal concentration of 10 μg ml^{-1} was determined (data not shown). The peptide concentration inside homogenic CIRIVs did not decrease when stored at 4°C for at least 4 weeks (data not shown). The peptide inside CIRIVs is protected against degradation by proteases *in vitro* (e.g. proteinase K; data not shown).

To demonstrate the efficiency of the fusion reaction between the Core132-liposomes and the CIRIVs, an affinity precipitation for Core132-CIRIVs was performed. For this purpose, biotin-DHPE was incorporated into CIRIVs ($CIRIV_{Bio}$) before the fusion with Core132-liposomes. Precipitation of different samples with and without fusion with magnetic streptavidin beads allowed the isolation of vesicles with biotin (CIRIV before fusion, Core132-CIRIV after fusion), whereas free peptide, and not fused Core132-liposomes, did not precipitate. The precipitate was dissolved in OEG-PBS and Core132 peptide in the precipitate was quantified by RP-HPLC, and compared with the starting amount of peptide (Fig. 2). After precipitation of fused Core132-$CIRIV_{Bio}$ ~90% of the peptide was found in the precipitate. The presence of free Core132 peptide did not influence the amount of affinity-precipitated Core132-$CIRIV_{Bio}$, indicating the irrelevance of the presence of free peptide on the fusion reaction (data not shown). When fused Core132-$CIRIV_{Bio}$ were incubated with streptavidin beads in the presence of detergent [0.5% (v/v) Triton X-100], no Core132 peptide was detected in the precipitate, excluding unspecific binding of Core132 peptide to the magnetic beads. When Core132-liposomes were mixed with empty $CIRIV_{Bio}$ without initiating a fusion reaction, no Core132 peptide was detected in the precipitate, excluding stable interactions between liposomes and empty CIRIVs. However, the presence of HA in the precipitate was demonstrated by immunoblotting with an anti-HA antibody (data not shown). These results show that

Table 1. Encapsulation efficiency for Core132

Particle type	Particle diameter (μm)	Encapsulated peptide[a](%)
Liposomes	up to 1.2	50–75
Homogenized liposomes	0.2	30–40
Fused chimeric virosomes	0.4–0.8	30–40
Homogenized virosomes	0.2	30–40

[a]In percentage of starting material. Data represent the range determined from at least five separate preparations.

under the specified conditions at least 90% of the Core132-liposomes fused with CIRIVs.

Core132-CIRIVs are fusogenic

To monitor the pH and temperature-dependent fusion activity of chimeric virosomes with liposomes *in vitro*, a FRET assay was used (41). This assay makes use of two fluorophores, an energy donor and an energy acceptor, both integrated in the membrane of the liposomes. The fluorescence signal of the energy donor is recorded continuously. Upon fusion of the labelled liposomes with unlabeled virosomes or influenza virus, the two fluorophores move apart and the fluorescence emitted by the donor increases. In this study, Bodipy 530/550-PE was used as the donor and rhodamine–PE as the acceptor. The fusion activity of Core132-CIRIVs was compared with that of CIRIVs and IRIVs (A/Sing) as well as with that of A/Sing and X-31 virus (Fig. 3). As target membranes, unilamellar liposomes prepared of PC : PG (70 : 30) and the two fluorophores (donor : acceptor, 0.75 : 0.25) were used (0.3 nmol of phospholipid) and mixed with virosomes or virus (~0.4 nmol of phospholipid), respectively, in a buffer at neutral pH and pre-incubated at the defined temperature (37 or 10°C). This fluorescence signal constituted the baseline and was set to 0% fluorescence. The fusion process was initiated by addition of HCl to reach a pH of ~4.5, and stopped by the addition of the detergent Triton X-100 to 0.5% (v/v). The latter fluorescence value corresponding to infinite probe dilution was set to 100% fluorescence. As expected, A/Sing virus could fuse to liposomes at 37°C but not at 10°C (Fig. 3A and G). IRIVs with A/Sing HA showed the same properties (Fig. 3B and H), whereas X-31 virus showed fusion activity at both temperatures (Fig. 3C and I). IRIVs with X-31 HA had the same properties as X-31 virus (data not shown). The CIRIVs with A/Sing HA and X-31 HA consequently could fuse both at 37 and at 10°C (Fig. 3D and J). In contrast, Core132-CIRIVs were fusogenic at 37°C, but could no longer fuse at 10°C, indicating that the A/Sing HA remained functional during the first fusion step at 10°C (Fig. 3E and K). As a control, no fusion was observed with Core132-CIRIVs when buffer instead of HCl was added (pH 7.5; Fig. 3F and L), or when unlabeled liposomes or inactivated virosomes were used (data not shown). Homogenic Core132-CIRIVs remained fusogenic for at least 4 weeks when stored at 4°C (data not shown).

$CD8^+$ T cell response induced by chimeric virosomes with encapsulated peptide

To study the immunogenicity of the CIRIVs HLA-A2 transgenic mice were immunized twice with CIRIV formulations containing various concentrations of the Core132 peptide (2, 5.5 and 10 μg per dose) or empty CIRIVs or PBS as negative controls. Two weeks after the last immunization spleen cells of individual mice were isolated and re-stimulated *in vitro* with peptide. After 5 days of *in vitro* stimulation, the CTL response for Core132 peptide was investigated by ^{51}Cr-release assay using HHD-transfected EL4 cells as target cells. In two independent experiments all the Core132 containing CIRIV formulations induced a strong CTL response in all of the immunized mice (Fig. 4A and B). The strength of the induced response was dose dependent with the highest dose giving the highest specific lysis of ~47% lysis at an E : T ratio of 33 : 1. Neither in naive mice nor in mice immunized with empty CIRIVs a substantial lysis of the target cells could be detected.

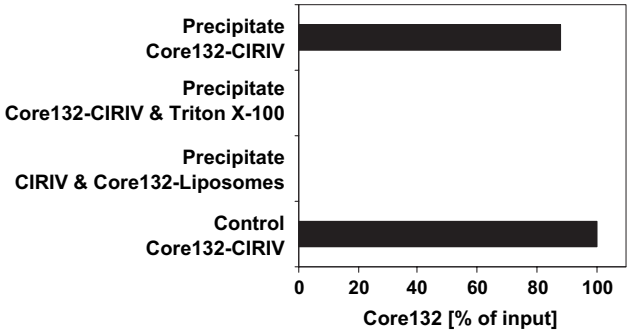

Fig. 2. Affinity precipitation of Core132-$CIRIV_{Bio}$ was carried out as described in Methods. Core132-$CIRIV_{Bio}$ were mixed with streptavidin magnetic beads and the precipitate was solubilized in OEG-PBS and analysed by RP-HPLC. In a first control experiment, Core132-$CIRIV_{Bio}$ were incubated with streptavidin magnetic beads in the presence of 0.5% (v/v) Triton X-100. In a second control experiment, Core132-liposomes were mixed with empty $CIRIV_{Bio}$ and incubated with streptavidin magnetic beads. An equal amount of Core132-$CIRIV_{Bio}$ starting material was purified over Sephadex G50 and analysed by RP-HPLC. A representative result out of three separate experiments is shown.

In addition, IFNγ release was chosen as another indicator of T cell response induced by Core132-CIRIV immunization. IFNγ release was quantified by ELISPOT assay (Fig. 5). The numbers of IFNγ-producing cells correlated well with the peptide-specific cytotoxicity with higher numbers induced by higher amounts of Core132 peptide in the CIRIV formulations.

In control experiments with three mice per group using the same immunization schedule and the same methods for analysis neither large unilamellar vesicles (LUVs) with encapsulated peptide (20 μg per dose), nor peptide with empty virosomes (20 μg per dose) nor peptide in saline solution (20 μg per dose) induced a detectable immune response in the ^{51}Cr-release assay and the ELISPOT assay (data not shown).

To get a better idea about the frequency of specific T cells in the $CD8^+$ population, another set of experiments were performed using staining for intracellular IFNγ. In addition, we wanted to test whether pre-existing immunity to influenza, which better reflects the situation in humans would affect the induction of HCV Core132-specific CTL. Therefore, naive and influenza immune mice were vaccinated and boosted 3 weeks later with the same Core132-CIRIV formulations or Core132-LUV and the frequency of specific IFNγ-producing cells was determined in the $CD8^+$ population with flow cytometry.

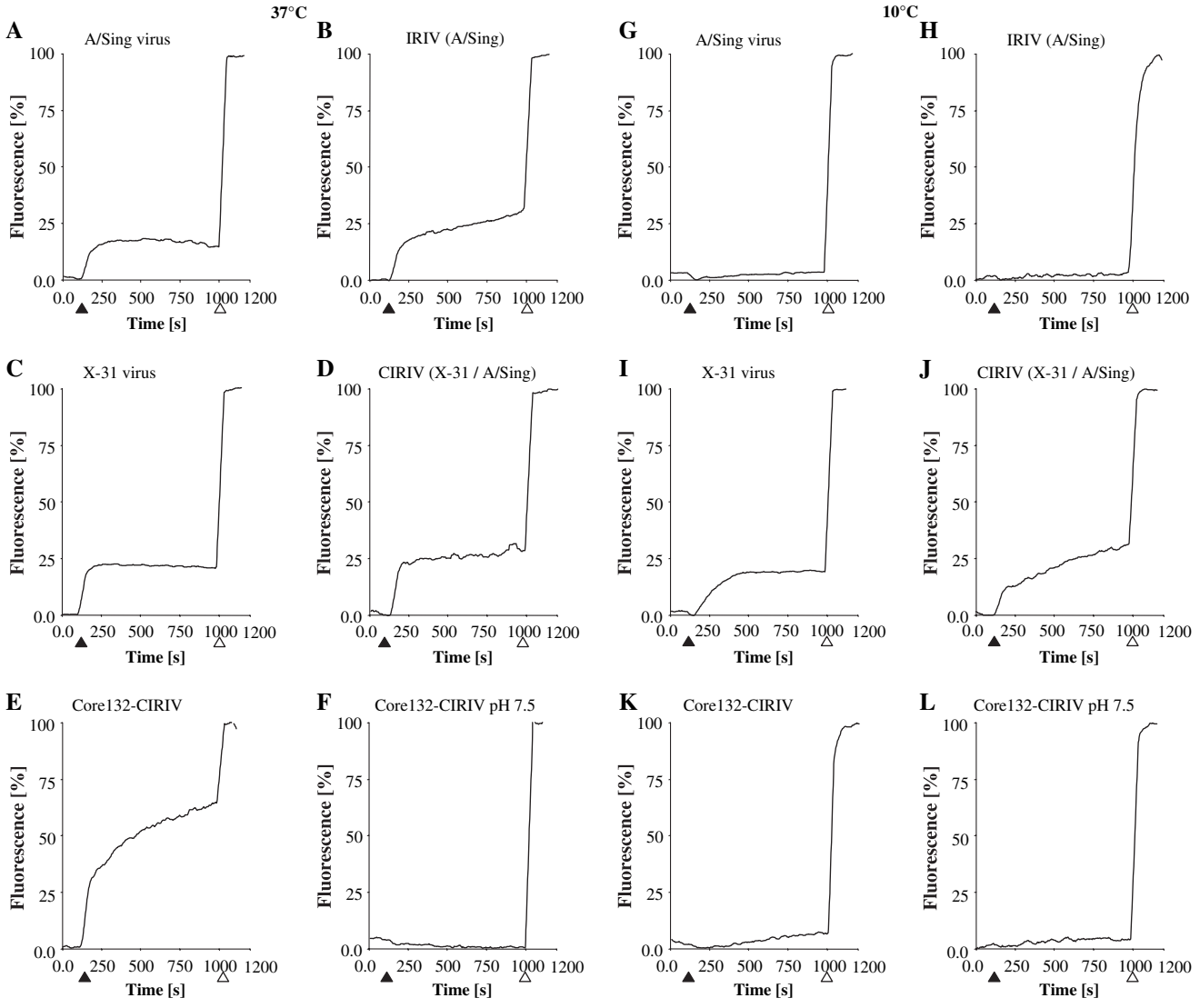

Fig. 3. Fusion activity of Core132-CIRIVs and its precursors. FRET measurements were carried out as outlined in Methods at 37°C (A–F) and 10°C (G–L), respectively, with the following samples: A/Sing Influenza virus (A and G), A/Sing virosomes (B and H), X-31 Influenza virus (C and I), chimeric A/Sing/X-31 virosomes before fusion with liposomes (D and J), Core132-CIRIVs (E and K) and Core132-CIRIVs without pH change (F and L). Labelled liposomes (0.3 nmol of phospholipid) were mixed with virosomes or virus (~0.4 nmol of phospholipid). Closed arrowheads indicate the addition of protons (pH change to ~4.5) and open arrowheads indicate the addition of Triton X-100 to 0.5% (v/v). For calibration of the fluorescence scale the initial residual fluorescence of the liposomes was set to zero and the fluorescence at infinite probe dilution to 100% (maximal fluorescence). Please note that for the measurements E, F, K and L, a higher amount (5×) of virosomes was used.

Compared with the Core132-LUV-vaccinated mice (negative control) in most of the Core132-CIRIV-vaccinated mice an increased frequency of peptide-specific $CD8^+$ T cells could be detected with a maximal frequency of 2.6% (Fig. 6). In addition pre-existing immunity to influenza had no effect on the frequency of peptide-specific $CD8^+$ T cells.

Discussion

The experiments described represent an effort to develop an improved virosome-based carrier system for the delivery of macromolecules (e.g. peptides) into cells, especially used as therapeutic vaccines. Since IRIVs are already registered for human use (4) and because of their safety profile and their rapid cellular uptake HA-virosomes represent a promising system. A further advantage of HA-virosomes is their potential targeting to specific cells or tissues by co-reconstituting receptors or antibodies (43) within the virosomal membrane. For this purpose a high concentration of the encapsulated macromolecules is of great importance. In addition, HA-virosomes have been shown to work as an excellent adjuvant in humans (44), a characteristic that is of interest for the use of HA-virosomes in vaccination. We have described a new methodology to improve the encapsulation efficiency by using CIRIVs. CIRIVs combine the advantages of liposomes and virosomes, namely the high encapsulation efficiency of liposomes, the receptor-mediated endocytosis and the membrane fusion properties of virosomes as well as the adjuvant

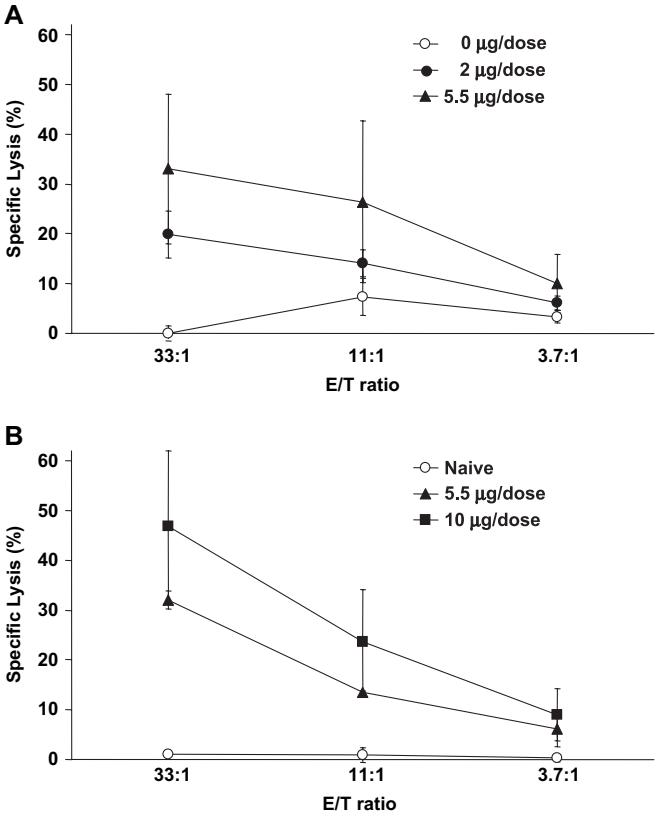

Fig. 4. Cytotoxic T cell responses in Core132-CIRIV-immunized HHD mice. In two independent experiments (A) and (B) HHD mice (2–3 mice per group) were immunized twice subcutaneously at a 3-week interval with empty IRIVs or Core132-CIRIVs. Spleen cells were isolated 2 weeks after the second immunization. After 5 days of *in vitro* re-stimulation with Core132 peptide, the stimulated spleen cells were used as effector cells against HHD-transfected EL4 as target cells in a Cr-release assay with an E : T ratio starting at 33 : 1. (A) Mice were immunized with empty IRIVs (open circles), or CIRIVs containing 2 µg (closed circles) or 5.5 µg (triangles) Core132 per dose. (B) Mice were naive (open circles) or immunized with CIRIVs containing 5.5 µg (triangles) or 10 µg (squares) Core132 per dose. Results show peptide mean specific lysis (lysis of target cells with peptide—lysis of target cells without peptide) of 2–3 individual mice ± standard deviation.

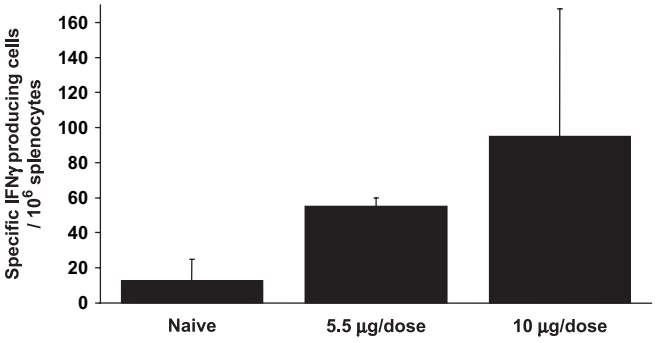

Fig. 5. IFNγ-producing cells in Core132-CIRIV-immunized HHD mice. HHD mice (2–3 mice per group) were immunized twice subcutaneously at a 3-week interval with Core132-CIRIVs. Spleen cells were isolated 2 weeks after the second immunization. The spleen cells were isolated and stimulated overnight with Core132 peptide or Core35 peptide (negative control). The number of IFNγ-producing T cells was determined in an ELISPOT assay as described in Methods. Specific IFNγ-producing cells were determined by counting the number of spots in the presence of Core132 peptide minus the number of spots in the presence of Core35 peptide. Shown are the mean values as derived from 2–3 individual mice ± standard deviation.

effect of virosomes. As a model antigen peptide, we showed that 30–40% of the supplied HCV Core132 nonapeptide could be incorporated into functional virosomes, whereas with the conventional detergent-removal method, entrapment efficiencies <1% were observed. The prepared proteoliposomes were found to undergo efficient fusion with model membranes at low pH and elevated temperatures. In contrast to previous reports with other virosomes (45, 46), no leakage was observed with Core132-CIRIVs during the fusion process, in agreement with the situation for alphaviruses (47).

The present method has also been used for the incorporation of other peptides with similar results. Lower entrapment efficiencies were generally obtained with hydrophobic peptides (M. Amacker, unpublished results). Furthermore, CIRIVs can also be used for different applications. Virosomes are, for example, a suitable carrier system for DNA and RNA since these fusogenic particles mimic a virus. The genetic material is enclosed in the virosome and is therefore protected from DNases and RNases. Virosomes could also serve as a drug delivery system to the cytosol. Using mAbs to tumour-associated antigens, virosomes could be targeted to cancer cells and therefore allow specific immunotherapy (48).

CTL epitope-based vaccine approaches offer a number of potential advantages (49). These include safety aspects, the possibility of inducing an immune response directed only against the relevant epitope and the ability to modify an epitope sequence to enhance the potency of the vaccine (50). In addition, they can be easily produced by peptide synthesis in large amounts at modest cost. Therefore, based on a suitable carrier, synthetic peptide epitopes are a very attractive approach for vaccine development.

As we have shown so far, we could efficiently encapsulate a peptide CTL epitope into CIRIVs. To study the efficiency of those CIRIVs in inducing a CTL response we used a model based on transgenic mice expressing human HLA-A2.1. The use of this model not only shows the immunogenicity of our newly developed CIRIVs but also may serve as an excellent tool for screening candidate epitopes for human vaccines in the future.

Our data indicate that CIRIVs containing Core132 peptide efficiently induce specific cytotoxic and IFNγ-producing T cells in HLA-A2.1 transgenic mice. Those responses were dose dependent and even the lowest dose of 2 µg peptide per injection induced a detectable CTL response.

As a very important point we were able to show that pre-existing immunity to influenza virus, reflecting the situation in humans, did not affect the induction of IFNγ-producing $CD8^+$ T cells by CIRIV. On the contrary it can be speculated that influenza-specific $CD4^+$ T cells could provide help to rise a CTL response directed against the peptide encapsulated in the virosome. Such a phenomenon has been observed *in vitro* with human PBMCs where virosomes activated $CD4^+$/$CD45RO^+$ T cells and induced a cytokine profile consistent with T_h1 stimulation (51). Alternatively, the lack of adjuvancy

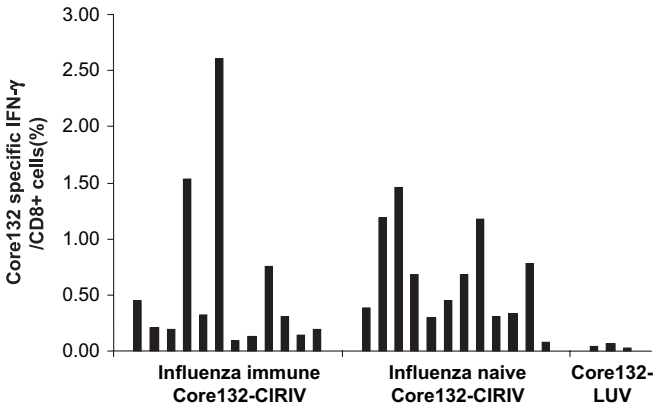

Fig. 6. IFNγ-producing $CD8^+$ T cells in influenza-immune and -naive Core132-CIRIV-immunized HHD mice. HHD mice (12 mice per group) either vaccinated with inactivated influenza A/Sing 3 weeks prior to immunization or naive mice were immunized twice subcutaneously at a 3-week interval with Core132-CIRIVs (20 μg per dose). The spleen cells were isolated and stimulated for 4 h with Core132 peptide or Core35 peptide (negative control) in the presence of Brefeldin A. The frequency of IFNγ-producing $CD8^+$ T cells was determined by flow cytometry after co-staining for CD8 and IFNγ as described in Methods. Frequency of specific IFNγ-producing cells was obtained by subtracting the percentage in samples stimulated with Core35 peptide from the percentage in samples stimulated with Core132 peptide. Shown are the values of 12 single mice per group immunized with Core132-CIRIV and 3 mice immunized with Core132-LUV as negative control.

found in these experiments may also reflect a limitation of the transgenic mouse model.

These data provide evidence that peptide bearing CIRIVs remain fusogenic and are effective at inducing specific, cytotoxic and IFNγ-producing T cells in HLA-A2.1 transgenic mice. Therefore, such vaccine formulations including combinations of different CTL epitopes could be used to induce not only a strong but also a multi-specific CTL response, making them potential candidates for prophylactic and/or therapeutic vaccines in humans against HCV and other targets, especially as virosome formulation are already accepted for use in humans.

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Abbreviations

APC	antigen-presenting cell
Bodipy 530/550-DHPE	*N*-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine
CIRIV	chimeric immunopotentiating reconstituted influenza virosome
ELISPOT	enzyme-linked immunospot
FRET	fluorescence resonance energy transfer
HA	haemagglutinin
HCV	hepatitis C virus
IRIV	immunopotentiating reconstituted influenza virosome
LUV	large unilamellar vesicles
N-Rh-DHPE	Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt
OEG	octaethyleneglycol-mono-(*n*-dodecyl)ether
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol)
RP-HPLC	reversed-phase column by HPLC
TEAP	triethylammonium phosphate

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