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Long-term generation of antiPCSK9 antibody using a nanoliposome-based vaccine delivery system

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HIGHLIGHTS

- PCSK9 inhibition is a pivotal approach for LDL cholesterol-lowering.
- PCSK9 inhibition using monoclonal antibodies is short-term and not cost-effective.
- We tested the efficacy of a nanoliposomal PCSK9-specific active vaccine.
- The tested nanoliposomal vaccine induced humoral immunity against PCSK9 in BALB/c mice.
- The tested vaccine was also safe and induced long-term generation of anti-PCSK9 antibodies.

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ABSTRACT

Background and aims: Proprotein convertase subtilisin kexin type 9 (PCSK9) is a liver secretory enzyme that controls plasma low-density lipoprotein cholesterol (LDL-C) levels through modulation of LDL receptor (LDLR). Inhibition of PCSK9 using monoclonal antibodies (mAbs) can efficiently lower plasma LDL-C. However, the relatively short half-life of mAbs necessitates frequent passive immunization, which is costly. These limitations can be circumvented by active immunization. Here, we evaluated the long-term antiPCSK9 antibody generation in BALB/c mice vaccinated with a nanoliposomal PCSK9-specific active vaccine.

Methods: Negatively charged nanoliposomes were used as a vaccine delivery system and prepared via lipid-film hydration method. We constructed a peptide vaccine termed Immunogenic Fused PCSK9-Tetanus (IFPT) by linking a short PCSK9 peptide (as B cell epitope) to a tetanus peptide (as T cell epitope). The IFPT peptide was conjugated to the surface of nanoliposome carriers using a DSPE-PEG- Maleimide (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(PEG)-2000]) linker. Nanoliposomal IFPT (L-IFPT) construct was formulated with alum vaccine adjuvant (L-IFPTA$^+$). To evaluate induction of antiPCSK9 antibody *in vivo,* BALB/c mice were subcutaneously inoculated four times in bi-weekly intervals with prepared vaccine formulations, including L-IFPT, L-IFPTA$^+$, IFPTA$^+$, IFPT, and empty liposomes as negative control. The long-term efficacy of antiPCSK9 antibodies was evaluated over 48 weeks after prime inoculation. Specificity of generated antiPCSK9 antibodies was assessed using ELISA method. To evaluate immunogenic safety, production of IL-4 and IFN-γ, and population of CD8$^+$ and CD4$^+$ T cells in splenic cells isolated from the vaccinated mice were analyzed.

Results: The L-IFPTA$^+$ vaccine was found to elicit the highest IgG antibody response against PCSK9 peptide in the vaccinated mice, when compared with the other vaccine formulations. Antibody titer analyses over 48 weeks post-prime vaccination revealed that the L-IFPTA$^+$ vaccine was able to stimulate a long-lasting humoral immune response against PCSK9 peptide, and thereby decrease plasma PCSK9. Generated antibodies could specifically target PCSK9 and thereby inhibit PCSK9-LDLR interaction. Analysis of splenic cells showed that the population of anti-inflammatory CD4$^+$ Th2 cells and production and secretion of IL-4 cytokine were increased in mice

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vaccinated with the L-IFPTA$^+$ vaccine, while population of inflammatory CD4$^+$ Th1 cell and cytotoxic CD8$^+$ T cells as well as production and secretion of IFN-γ were not altered.
Conclusions: The results indicate efficient activity of the tested nanoliposomal construct (L-IFPTA$^+$) to induce humoral immune response against PCSK9 in BALB/c mice. L-IFPTA$^+$ vaccine can induce immunogenic-safe and long-term generation of antiPCSK9 antibodies in BALB/c mice.

1. Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secretory protein that is primarily synthesized by hepatocytes. PCSK9 regulates hepatic low-density lipoprotein receptor (LDLR) and clearance of plasma LDL cholesterol (LDL-C) [1]. Mechanistically, secreted PCSK9 circulates in bloodstream and controls LDLR on the surface of hepatocytes through post-translational regulation *via* binding of its catalytic domain to the extracellular epidermal growth factor-like repeat A (EGF-A) domain of LDLR and targeting it to lysosomal degradation [2]. The EGF-A domain is responsible for recycling of LDLR to the cell surface [3–5], and binding of PCSK9 to this domain suppresses normal trafficking of the LDLR back to the cell surface and enhances lysosomal degradation of LDLR [6,7].

The central role of PCSK9 in the metabolism of LDL and LDLR, and also the approved safety of PCSK9 inhibition has encouraged the development of PCSK9 inhibitors [8]. Several novel approaches including the use of small molecule inhibitors, small interfering RNAs, antisense oligonucleotides, monoclonal antibodies (mAbs) and vaccines have recently been developed to inhibit PCSK9. The most widely studied method has been the disruption of interaction between PCSK9 and LDLR by mAbs [8–10]. The famous FDA-approved alirocumab [Praluent®] [11,12] and evolocumab [Repatha®] [13,14] are currently the most effective PCSK9-specific mAbs in the market.

Despite their efficacy, mAbs have major drawbacks that can limit their clinical utility. Due to their relatively short *in vivo* half-life, therapeutic use of mAbs for long-term requires frequent dosing that is associated with a high cost of > $14,000 per patient per year [15]. Furthermore, because of short *in vivo* half-life, antiPCSK9 mAbs need to be administrated frequently (once or twice a month) and at high doses (~140 mg for the 2 × month regimen), which may cause tolerability problems and poor compliance. These limitations emphasize the need for cheaper PCSK9 inhibitors that could serve either as alternatives or as adjuncts to PCSK9 mAbs to decrease the required dose and cost of treatment. The mentioned drawbacks can be circumvented by active vaccination that promotes therapeutic effects similar to those achieved with passive immunization using mAbs but with fewer injections and lower doses, and less risk of stimulating drug-neutralizing immune responses [16–18].

In this study, we developed a nanoliposome-based vaccine delivery system for an immunogenic PCSK9 epitope to elicit specific antiPCSK9 antibody. Using BALB/c mice, we showed that the active immunization with the developed construct can be a safe, durable and low-cost approach for PCSK9 antibody generation.

2. Materials and methods

2.1. Vaccine preparation

2.1.1. Nanoliposome preparation and characterization

Liposomal formulation containing 1,2-Dimyristoyl-sn-glycero-3-phosphorylcholine (DMPC), 1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG) and cholesterol (chol) (Lipoid GmbH, Germany) at the final concentration of 40 mM (total phospholipids and chol) was prepared using the lipid-film hydration method. Briefly, DMPC, DMPG and chol were separately dissolved in chloroform at the molar ratios of 75:10:15, respectively. Lipid solution was dried to a thin lipid film under reduced pressure using rotary evaporation (Heidolph, Germany). The film was then freeze-dried (VD-800F, Taitech, Japan) overnight to completely remove the solvent. Subsequently, the dried lipids were hydrated with 10 mM HEPES buffer (pH 7.2) containing 5% dextrose, and vortexed and bath-sonicated to disperse completely in the buffer. To obtain small unilamellar vesicles (SUVs) with a uniform size of 100 nm, the multilamellar vesicles (MLVs) were sequentially extruded using a mini extruder (Avestin, Canada) with polycarbonate membranes of 600, 400, 200, and 100 nm pore size, respectively. Vesicle size (diameter, nm), zeta potential (surface charge, mV) and poly dispersity index (PDI) of the prepared nanoliposomal formulation were evaluated using dynamic light scattering (DLS) technique on a Zetasizer (Nano-ZS, Malvern, UK) at the room temperature (RT). Liposomes were stored at 4°c under argon. The morphology and structure of the prepared nanoliposome particles was analyzed using a Philips CM10 transmission electron microscope (TEM).

2.1.2. Immunogenic peptide

Antibodies generated against PCSK9-derived linear peptides have been shown to effectively inhibit PCSK9 binding to LDLR [19]. On the basis of this data and according to the crystal structure and amino acid sequence of PCSK9 as a guide [20], we inspired the peptide sequence from the AFFiRiS group [16]. The selected sequence is a B cell epitope peptide, originally designed using AFFITOME® technology [16,21], that mimics an N-terminal sequence of human PCSK9 bound to LDLR (Table 1). The constructed mimotope peptide differs in its amino acid sequence from the original sequences (from human and murine), in which is identified as foreign by the immune system and therefore does not necessitate to break self-tolerance, and can provoke a PCSK9-specific antibody. The sequence of the mimotope peptide was also selected in a way that is closely identical between human and rodents. Therefore, the antibodies raised are supposed to block PCSK9 and LDLR interaction in both rodents and human. Furthermore, to increase CD4$^+$ T-cell response, a T-helper cell epitope belonging to tetanus toxin was used as a pharmaceutically acceptable carrier [22] and coupled to the C-terminal of the selected PCSK9 fragment using a 2-lysine-spacer sequence (Table 1). Two lysine residues serve as a sequence recognized by cathepsin B protease involved in the antigen processing [23].

Eventually, an immunogenic peptide construct termed IFPT was prepared, which includes an N-terminal cysteine bound by a 3-glycine-spacer sequence thereto (Table 1). This cysteine residue served as a reactive group in order to bind the peptide to DSPE-PEG-Mal (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(PEG)-2000]) linker (Lipoid GmbH, Germany) (Supplementary Fig. 1) that conjugates IFPT construct on the surface of nanoliposomes as adjuvant delivery system (Supplementary Fig. 2). Since many IFPT peptides can be

Table 1
Sequence of the immunogenic peptides used in the present study.

Peptide name	Sequence	Immunogenicity
PCSK9	S-I-P-W-N-L-E-R-I-T-P-V-R	B cell epitope
Tetanus	A-Q-Y-I-K-A-N-S-K-F-I-G-I-T-E-L	T cell epitope
IFPT	*CGGGSIPWNLERITPVRkkAQYIKANSKFIGITEL	

The bold amino acid codes are as a linker sequence for conjugation with DSPE-PEG-Mal. The bold lower cases show a 2-lysine-spacer which serves as a sequence recognized by cathepsin B protease involved in the antigen processing. IFPT; Immunogenic Fused PCSK9-TetanSus.

conjugated to the surface of liposome nanoparticles, we propose that IFPT-linked nanoliposomes can elicit a high-titer antibody against self-antigen PCSK9, maybe through elevating peptide valency.

The Immunogenic Fused PCSK9-Tetanus (IFPT) peptide with a purity grade of > 95% were synthesized and high performance liquid chromatography (HPLC)-purified by ChinaPeptides Co., Ltd. (Shanghai, China).

2.1.3. Construction of DSPE-PEG-IFPT micelles

DSPE-PEG-Mal linker was used to conjugate the immunogenic peptides and nanoliposomes.

N-terminal cysteine residue of the IFPT peptide provides a thiol group that reacts with pyrrole group of maleimide and produces thioether bond, causing the peptide to covalently bind to DSPE-PEG-Mal linker (Supplementary Fig. 1). Hereof, the IFPT peptide and DSPE-PEG-Mal at the molar ratios of 1.2:1, respectively, were dissolved in DMSO/chloroform solution at the volume ratio of 1:1, and then gently stirred at RT for 48 h. The linkage was confirmed using TLC (thin layer chromatography) method with the mobile phase containing chloroform, methanol and water at the volume ratio of 90:18:2. Thereafter, the DMSO/chloroform solution was dried by rotary evaporator and freeze-drying followed by hydration with sterile deionized water (pH 7.2) at 30 °C to construct DSPE-PEG-IFPT micelles. The efficiency of the linkage in the prepared micelles was measuresd by HPLC analysis. The true value of the linked micelles were measured using efficiency of linkage and content of total lipid determined by the Bartlett phosphateassay method [24].

2.1.4. HPLC analysis of the linkage efficiency

The efficinecy of linkage between the IFPT peptide and DSPE-PEG-Mal linker was indirectly measured by HPLC quantification of the free peptide content of the prepared DSPE-PEG-IFPT micelles. The HPLC apparatus was equipped with a Smart line HPLC Pump 1000, a PDA Detector 2800 (set at 220 nm), and a Degasser5000, all from Knauer (Berlin, Germany). Each sample (20 µl) was injected through a Smart line auto sampler and data were obtained and processed with ChromGate software (version 3.3.1) from Knauer (Berlin, Germany). Chromatographic separation was performed on a C18 reverse-phase column, 4.6 mm × 25 cm (Shimadzu, Japan), using an isocratic mobile phase of (0.1% TFA in water)/(0.1% TFA in acetonitrile) at gradient ratios of 55/45 to 45/55 in 10 min, at a flow rate of 1 mL min^{-1}. The IFPT peptide with HPLC purity > 95% was used as a standard solution. The free peptide peak within the chromatogram of the micelle sample was identified and assigned based on the retention time (2.2 min) of the standard solution, followed by sample spiking.

To quantify the free peptide content of the micelle sample, a calibration curve was constructed by injecting standard solution at five concentrations (50–500 µg/mL) in which was linear with a correlation coefficient (r [2]) of 0.9954. Using linear regression analysis of the calibration cure appearing in the standard chromatogram, the free peptide content of the micelle sample was estimated. Linkage efficiency in the constructed DSPE-PEG-IFPT micelles was calculated by subtracting the free peptide amount within the micelles quantified by HPLC from the amount of the IFPT peptide initially added.

2.1.5. Construction of nanoliposomal IFPT vaccine

Nanoliposomes were used as a delivery adjuvant to enhance immunogenicity of the peptide. To conjugate the IFPT peptide to the nanoliposome surface, the post insertion approach was performed, in which the prepared DSPE-PEG-IFPT micelles (100 µg, based on the linked peptide) and liposome nanoparticles (1 mL) were mixed and then gentely shaked at 45 °C for 3 h. The micelles were inserted in the nanoliposome bilayer *via* DSPE phospholipid moiety, and expose IFPT peptides on the nanoliposome surface through the PEG chains (Supplementary Fig. 2). Particle size, surface charge and PDI of the prepared nanoliposomal IFPT (L-IFPT) particles were evaluated using DLS technique on a Zetasizer (Nano-ZS, Malvern, UK) at the RT. The IFPT-conjugated nanoliposomes were adsorbed to 0.4% alum vaccine adjuvant (Alhydrogel®, Sigma-Aldrich) at a 1:1 (v:v) ratio in a total volume of 200 µl and stored at 4 °C under argon. Prior to injection, the vaccine (Nanoliposomal IFPT plus alum vaccine adjuvant) was brought to RT and carefully mixed.

2.2. Animal

A total of fifty 6–8 week old male BALB/c mice (15 ± 3 g) were purchased from the Laboratory animal research center of Pasteur Institute of Tehran, Iran. All animal handling procedures were carried out in strict accordance with the Animal Welfare guidelines approved by Institutional Ethics Committee and Research Advisory Committee of the Mashhad University of Medical Sciences, Mashhad, Iran (project code: 940492). The animals were housed in an air-conditioned room at a constant temperature of 22 ± 2 °C with 12:12 h light/dark cycle and fed a standard rodent diet and water *ad libitum*. At the end of study all animals were euthanized by intravenous injection (30 mg/kg) of thiopental sodium.

2.3. Vaccination scheme

Following one week of taming prior to the experimental procedures, fifty male BALB/c mice were randomly and equally divided into five groups (10 mice per group), including 1) IFPT vaccine group, 2) IFPT plus alum vaccine adjuvant ($IFPTA^+$) vaccine group, 3) nanoliposomal IFPT (L-IFPT) vaccine group, 4) L-$IFPTA^+$ vaccine group, 5) empty nanoliposome (negative control) group. All groups were inoculated 4 times subcutaneously with the mentioned formulations (200 µl in both the right and left flanks), in bi-weekly intervals. The time point at which the first inoculation was performed in order to induce antiPCSK9 antibody refers to week 0 (W0). Three more boosters were then carried out at W2, W4, and W6. To evaluate antiPCSK9 antibody titers, blood was withdrawn two weeks after each inoculation and at the pre-defined time points during 48 weeks after the first inoculation (Supplementary Fig. 3).

2.4. Quantification of plasma antiPCSK9 peptide antibody

To determine the titer of antiPCSK9 antibodies, plasma samples were collected and analyzed by ELISA method. Briefly, PCSK9 peptide (injected peptide) at the concentration of 5 µg/mL in 0.1 M $NaHCO_3$ (pH 9.2–9.4) was coated overnight in a 96-well Nunc-MaxiSorp plate [16]. Free binding sites were then blocked by the incubation with blocking buffer (1 × PBS, 1% BSA) for 1 h at 37 °C. Diluted plasma (1:400 in dilution buffer [1 × PBS/0.1% BSA/0.05% Tween-20]) were added, serially diluted 1:4, and incubated for 1 h at 37 °C. Each ELISA plate contained a standard antibody as internal control. Detection was done by HRP-conjugated anti-mouse IgG (H + L) (Sigma Aldrich; dilution 1:1000), incubated for 1 h at 37°Cfollowed by the addition of the substrate TMB (3,3′,5,5′-tetramethylbenzidine) (Sigma-Aldrich; 10 min at RT). The optical density (OD) at 450 nm was measured with a microwell plate reader (BioTek, Synergy 2 plate reader, VT, USA) and the titers were defined as the dilution factor referring to 50% of the maximal optical density ($OD_{max}/2$). The mean titers ± SD of all animals per group are presented.

2.5. Plasma PCSK9 quantification

Plasma PCSK9 concentration of vaccinated BALB/c mice was measured by CircuLex mouse PCSK9 ELISA (CircuLexTM, Cy-8078, MBL, Woburn, MA) according to the manufacturer's instructions. Briefly, 100 µl of the diluted 1:100 plasma samples was added on a 96-well microplate and incubated for 1 h at RT. A HRP-conjugated antiPCSK9 antibody was added for 1 h, followed by the substrate reagent and stop

solution at RT. The OD was detected at 450 nm with the Microwell plate reader. A standard curve provided by the supplier was defined to measure PCSK9 concentration.

2.6. PCSK9 inhibition assessment

To evaluate inhibition of mouse PCSK9 by vaccine-induced antibodies, interaction of generated-antibodies with mouse PCSK9 was assayed. For this purpose the same kit CircuLex mouse PCSK9 ELISA was employed, but instead of HRP-conjugated antiPCSK9 antibody, detection was performed with HRP-conjugated anti-mouse IgG (H + L) (Sigma Aldrich; dilution 1:5000) incubated for 1 h at RT, followed by the substrate TMB and stop solution provided by the supplier [16]. The OD was detected at 450 nm with the Microwell plate reader.

2.7. Inhibition of PCSK9-LDLR interaction in vitro

CircuLex PCSK9-LDLR *in vitro* binding assay kit (CircuLex™, Cy-8150, MBL, Woburn, MA) was employed to analyze the ability of vaccine-generated antibodies for inhibition of the PCSK9-LDLR interaction *in vitro*. Briefly, 100 µl of vehicle control or the plasma samples of vaccinated mice was added to a 96-well microplate pre-coated with a recombinant LDLR-AB domain, which contains binding site for PCSK9. Immediately after that, reaction was initiated by adding a "His-tagged PCSK9 wiled type" solution incubated for 2 h followed by adding a biotinylated anti-His-tag monoclonal antibody for 1 h at RT. Then, HRP-conjugated streptavidin was coated for 1 h at RT followed by the substrate reagent and stop solution. In this method, the higher amount of PCSK9-LDLR interaction is associated with higher ELISA OD, in which at the presence of antiPCSK9 antibody this interaction is inhibited and consequently ELISA OD is decreased. A dose-response curve with appropriate serial dilutions of "His-tagged PCSK9 wild type" solution was drawn to measure accurate inhibition percentage of test samples.

2.8. ELISpot assay

One week after the last vaccination, the mice inoculated with PCSK9 peptide or Tetanus peptide, and non-inoculated naïve mice as negative control (5 animals per group) were sacrificed and their splenocytes were aseptically isolated. Subsequently, the isolated splenocytes form each group were homogenized, and then passed through a 70 µm cell strainer (BD Falcon). Red blood cells (RBCs) were lysed using ACK buffer (0.15 M NH4Cl, 1.0 M KHCO3, 0.1 mM Na2EDTA), and the RBC-depleted splenocytes were counted using trypan blue (Gibco). The mouse INF-γ cytokine assay (Mouse INF-γ ELISpot Kit, Mabtech) was performed according to the manufacture protocol. Briefly, 96-well PVDF-plates (Millipore Corp.) were coated with 100 µl/well of 15 µg/mL anti-INF-γ antibody solution (diluted in sterile PBS pH 7.4) and incubated overnight at 4 °C. Afterward, the coated plates were washed 5 times with PBS in sterile condition prior to incubation with 200 µl RPMI medium (Gibco) supplemented with 10% Fetal Calf Serum (FCS) (Gibco) and 1% Penicillin (1U/mL)/Streptomycin (100 µg/mL) (P/S) (Gibco) for at least 30 min at RT to block non-specific binding. After blocking, splenocytes were seeded at 5×10^5 cells per well and re-stimulated either with 15 µg/mL PCSK9 peptide, 20 µg/mL PHA (phytohemagglutinin) (Sigma-Aldrich), or supplemented RPMI medium and incubated for 24 h at 37 °C. Eventually, spot-forming cells (SFC) were detected according to the manufacturer's guidelines, and the amount of INF-γ producing cells were determined by counting the number of spots per well using Kodak 1 D image analysis software (Version3.5, Eastman Kodak, Rochester, New York). Values were expressed as the number (Median-interquartile range) of anti-INF-γ SFC per 10^6 cells.

2.9. Flow cytometry analysis

Flow cytometry analysis was conducted to evaluate CD4 and CD8 T cells and their intracellular IFN-γ and IL-4 cytokines. Therefore, 1×10^6 RBC-depleted splenocytes were re-stimulated with medium containing PCSK9 peptide (15 µg/mL), PMA/ionomycin cocktail (2 µL/ml), or supplemented RPMI medium for 4 h at 37 °C. Then, 10^5 splenocytes were transferred into flow cytometry tubes and washing was done two times with stain buffer (2% FCS in phosphate buffered saline (PBS). Splenocytes were stained with 1 µl anti CD4-PE-cy5 antibody and 1 µl anti-CD8 APC antibody for 30 min at 4 °C. The cells were washed with stain buffer and fixed by Cytofix/Cytoperm™ solution. Washing was done two times with Perm/Wash™ buffer and then stained with 1 µl anti-IFN-γ- FITC antibody for 30 min at 4 °C. CD4 cells were stained with 1 µl anti-IL-4-PE antibody. Then washing was done with Perm/Wash™ buffer and cells were suspended in 300 µl stain buffer for flow cytometric analysis (BD FACSCalibur™, BD Biosciences, and San Jose, USA).

2.10. Statistical analysis

Unpaired two-tailed Student's *t*-test and one-way ANOVA followed by Tukey's *post-hoc* test were used to assess the significance of the difference among various groups (GraphPad Prism Software, version 7, San Diego, CA). $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Characterization of nanoliposomal formulations

Physical properties of the free nanoliposomal formulations including size, polydispersity index (PDI) and zeta potential are shown in Table 2. Free nanoliposomes and the IFPT-linked nanoliposomes exhibited a size range from 140 nm to 180 nm in diameter, in which PDI was less than 0.2, showing homogeneity of particles (Supplementary Fig. 4). Zeta potential analysis also revealed negative charge on the surface of nanoparticles (Supplementary Fig. 5). The nanoliposomal vesicles were visualized by transmission electron microscopy indicating unilamellar vesicles with the lamellae of vesicles evenly spaced to the core as shown in the Supplementary Fig. 6.

3.2. TLC and HPLC analysis of DSPE-PEG-IFPT micelles

Based on the disappearance of DSPE-PEG-Mal spot (lipid spot) on the TLC plate, conjugation of DSPE-PEG-Mal linker to IFPT peptide was verified after a 48-h reaction (Supplementary Fig. 7). Afterward, DSPE-PEG-IFPT micelles were made and the efficiency of conjugation was indirectly measured using HPLC analysis, by quantification of the free peptide content of the micelle sample. HPLC analysis indicated that 96% of the initially added IFPT peptides were conjugated to DSPE-PEG-Mal linker (Supplementary Fig. 8).

Table 2
Physical properties of nanoliposomal formulations. https://www.atherosclerosis-journal.com/article/S0021-9150(18)31474-6/fulltext?dgcid=raven_jbs_etoc_email

Formulation	Z-average (nm) [Mean ± SD, n = 3]	Zeta potential (mV) [Mean ± SD, n = 3]	PDI [Mean ± SD, n = 3]
The empty nanoliposome	150 ± 9.5	−41 ± 4.8	0.03 ± 0.01
The IFPT linked-nanoliposome	181 ± 11.5	−25 ± 4.2	0.136 ± 0.08

PDI, polydispersity index.

3.3. Antibody titres against PCSK9 peptide in the vaccinated mice

Nanoliposomal vaccine exposing IFPT peptide adsorbed to alum vaccine adjuvant (L-IFPTA$^+$ vaccine) was found to provoke the highest IgG antibody response against PCSK9 peptide in the vaccinated mice, when compared with the other vaccine formulations (Fig. 1A). The ELISA analysis revealed that, two weeks after the last vaccination, mice inoculated with L-IFPTA$^+$ vaccine showed 0.4, 0.6, and 4.2 fold higher serum antibody titers than mice inoculated with the nanoliposomal IFPT, IFPTA$^+$, IFPT vaccine, respectively. Of note, mice inoculated with vaccine formulation containing nanoliposome particles and IFPT peptide exhibited higher antibody titers when compared with vaccine formulations containing IFPT peptide without nanoliposome particles, including IFPT and IFPTA$^+$ vaccine formulations. The results indicate efficient adjuvant activity of nanoliposomes to induce humoral immuno response against peptide antigen.

3.4. Vaccine-generated antiPCSK9 antibodies target plasma PCSK9

To evaluate specific targeting of vaccine-induced antiPCSK9 antibodies *in vivo*, plasma levels of PCSK9 in vaccinated mice were evaluated, two weeks after the last immunization. Vaccine-induced antiPCSK9 antibody was found to specifically target plasma PCSK9 in BALB/c mice. Hence, plasma levels of PCSK9 in the L-IFPTA$^+$ vaccine and control group were 27.85 ± 4.2 ng/mL and 58.26 ± 5.7 ng/mL, respectively (Fig. 1B). Compared with control mice, plasma levels of PCSK9 was significantly decreased by 52.2% (−30.41 ± 7.125, $p = 0.013$) in vaccinated mice. For further evidence of specific targeting, PCSK9 inhibition was evaluated using ELISA-based assay to detect vaccine-generated antibodies directly bound to plasma PCSK9. For this aim, plasma PCSK9 isolated from vaccinated and control mice was captured onto murine antiPCSK9 antibodies-coated ELISA plates, and vaccine-generated PCSK9-bound murine antibodies were detected using an anti-mouse IgG antibody. As shown in Fig. 1C, the significantly higher OD450 signal in plasma of mice vaccinated with L-IFPTA$^+$ vaccine confirmed a direct binding of vaccine-generated antiPCSK9 antibodies to the target protein (PCSK9). To sum up, L-IFPTA$^+$ vaccine induces specific antibodies that directly target and bind mouse PCSK9 in the bloodstream.

3.5. Vaccine-generated antiPCSK9 antibodies inhibit in vitro PCSK9-LDLR interaction

CircuLex PCSK9-LDLR *in vitro* binding assay kit was used to evaluate the ability of vaccine-generated antibodies for inhibition of the PCSK9-LDLR interaction *in vitro*. Based on this kit, in the presence of plasma antiPCSK9 antibodies, *in vitro* binding of murine PCSK9 and LDLR (supplied in kit) is inhibited. In accordance with this approach, we showed that vaccine-induced antiPCSK9 antibodies isolated from BALB/c mice immunized with L-IFPTA$^+$ could efficiently inhibit

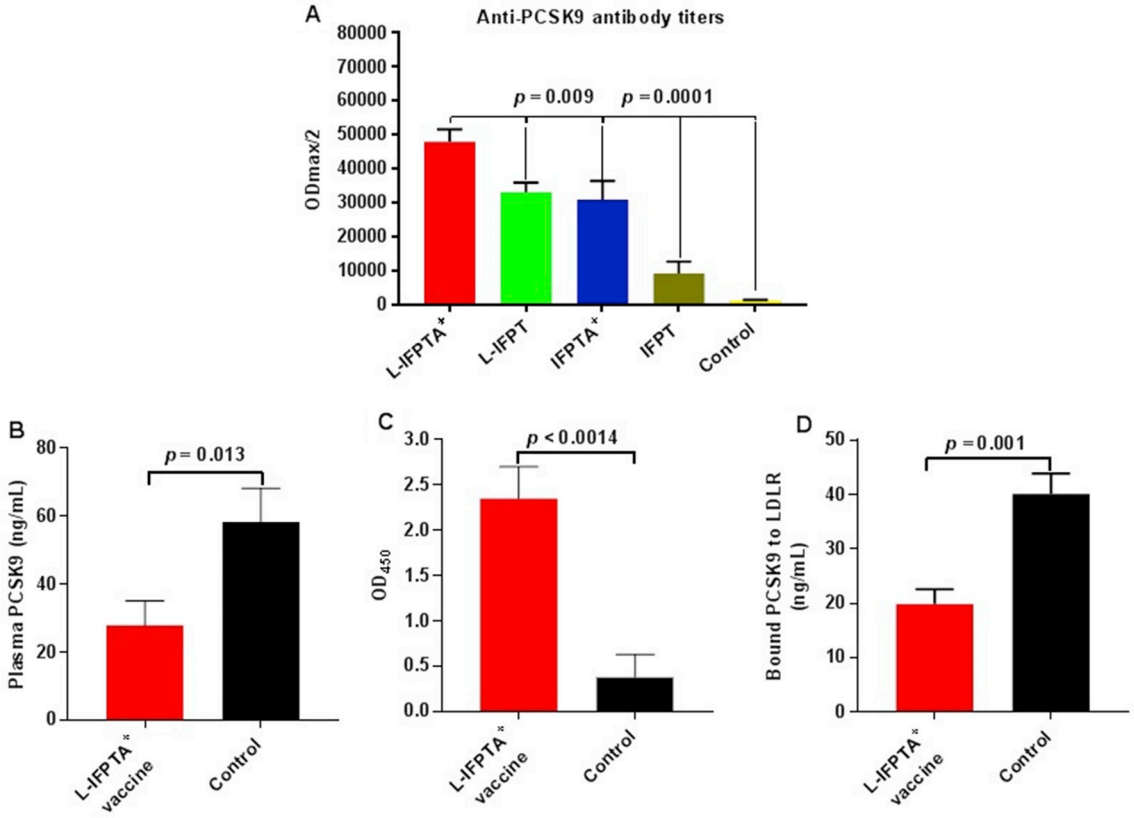

Fig. 1. Efficacy of antiPCSK9 vaccines in BALB/c mice, two weeks after the last immunization.
Antibody titers (ODmax/2) against PCSK9 peptide were determined in the plasma of mice vaccinated with different vaccine formulations or empty nanoliposomes as control. Values are means ± SEM, n = 10 mice/group. (A) One-way ANOVA followed by Tukey's *post-hoc* test was used to assess the significance of the difference among various groups. (B) Plasma levels of PCSK9 in vaccinated and control groups were 27.85 ± 4.192 ng/mL and 58.26 ± 5.762 ng/mL, respectively. Bars show mean concentrations of PCSK9 in plasma samples. (C) Plasma PCSK9 is targeted by vaccine-generated antiPCSK9 antibodies. Direct detection of antibodies bound to plasma PCSK9 in plasma samples from vaccinated and control mice. Increased OD450 is indicative for vaccine generated antiPCSK9 antibodies directly binding to PCSK9. (D) *In vitro* PCSK9/LDLR binding assay. Plasma sample of the vaccine group could decrease PCSK9 binding to LDLR by 50.2%, when compared with plasma sample of the control group. Bars show mean values, error bars show ± SD, n = 3 replicates of the pooled samples of 10 mice per group. Significance compared to control values was analyzed by unpaired 2-tailed Student's t-test. IFPT, immunogenic Fused PCSK9-Tetanus; IFPTA$^+$, IFPT plus Alhydrogel; L-IFPT; nanoliposomal IFPT.

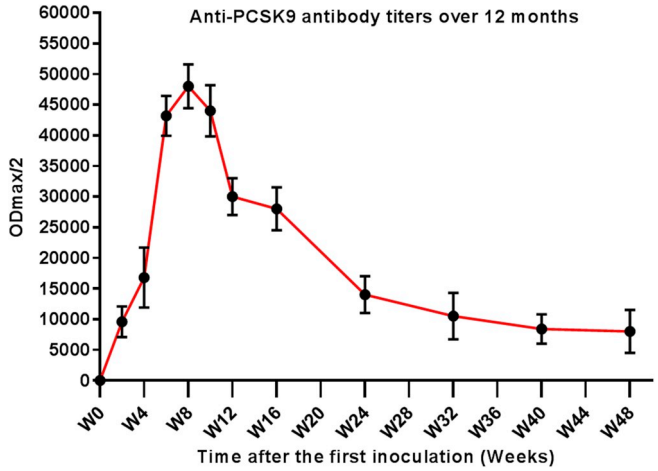

Fig. 2. Long-term boostability of nanoliposomal IFPTA$^+$ vaccine.
Long-term evaluation over 48 weeks post prime inoculation of titers against PCSK9 peptide (ODmax/2), generated upon 4 inoculations in a biweekly interval. Values are means ± SD, n = 5 mice/group.

PCSK9-LDLR interaction *in vitro*. Plasma sample of the L-IFPTA$^+$ vaccine group significantly decreased PCSK9 binding to LDLR by 50.2%, when compared with plasma sample of the control group (Fig. 1D).

3.6. Long-term efficacy of nanoliposomal IFPTA$^+$ vaccine in mice

To understand the persistence of the induced antiPCSK9 antibody, longer-term studies were undertaken on BALB/c mice inoculated with L-IFPTA$^+$ vaccine. Antibody titer analyses over 48 weeks post prime vaccination revealed that L-IFPTA$^+$ vaccine was able to stimulate a long-lasting humoral immune response against PCSK9 peptide, which acquired a maximum mean titer (ODmax/2 = 48000) eight weeks after prime vaccination. The level of plasma antiPCSK9 antibodies was found to drop with an *in vivo* half-life of about 5 months (Fig. 2).

3.7. Evaluation of PCSK9-specific T cell activation

Absence of target-specific T cell response is one of the most important safety prerequisites for vaccinations against self-proteins. Therefore, we examined whether the antiPCSK9 vaccine is capable to induce cytotoxic T cells *in vivo*. BALB/c mice were either immunized with L-IFPTA$^+$ vaccine, tetanus peptide alone or remained untreated, and T cell activation was evaluated using IFN-γ ELISpot assay and flow cytometry analysis. As demonstrated by ELISpot assay results (Fig. 3), *ex vivo* re-stimulation of splenocytes isolated from immunized mice with the peptide used for immunization could not induce IFNγ-releasing T cells. However, tetanus re-stimulation or stimulation with the mitogen phytohemagglutinin (PHA) as positive control induced IFN-γ release. The results are further supported by flow cytometric analysis that revealed percentages of IFNγ-producing CD8$^+$ T cells were significantly and much lower in the population of splenocytes re-stimulated with PCSK9 peptide used for immunization than in those simulated with PMA as positive control (Fig. 4). Hence, PCSK9 peptide used for immunization does not act as a T cell epitope leading to the induction of PCSK9-specific cytotoxic T cells.

3.8. L- IFPTA$^+$ vaccine modulate Th1/Th2-associated cytokines

Flow cytometry analysis of splenic cells showed that nanoliposomal IFPTA$^+$ vaccine significantly increased the population of anti-inflammatory CD4$^+$ IL-4$^+$ Th2 cells in the immunized mice when compared with the naïve mice ($p = 0.001$). However, there was no significant effect on the numbers of pro-inflammatory CD4$^+$ IFN-γ$^+$ Th1 cells and cytotoxic CD8$^+$ IFN-γ$^+$ T cells. The results showed that the

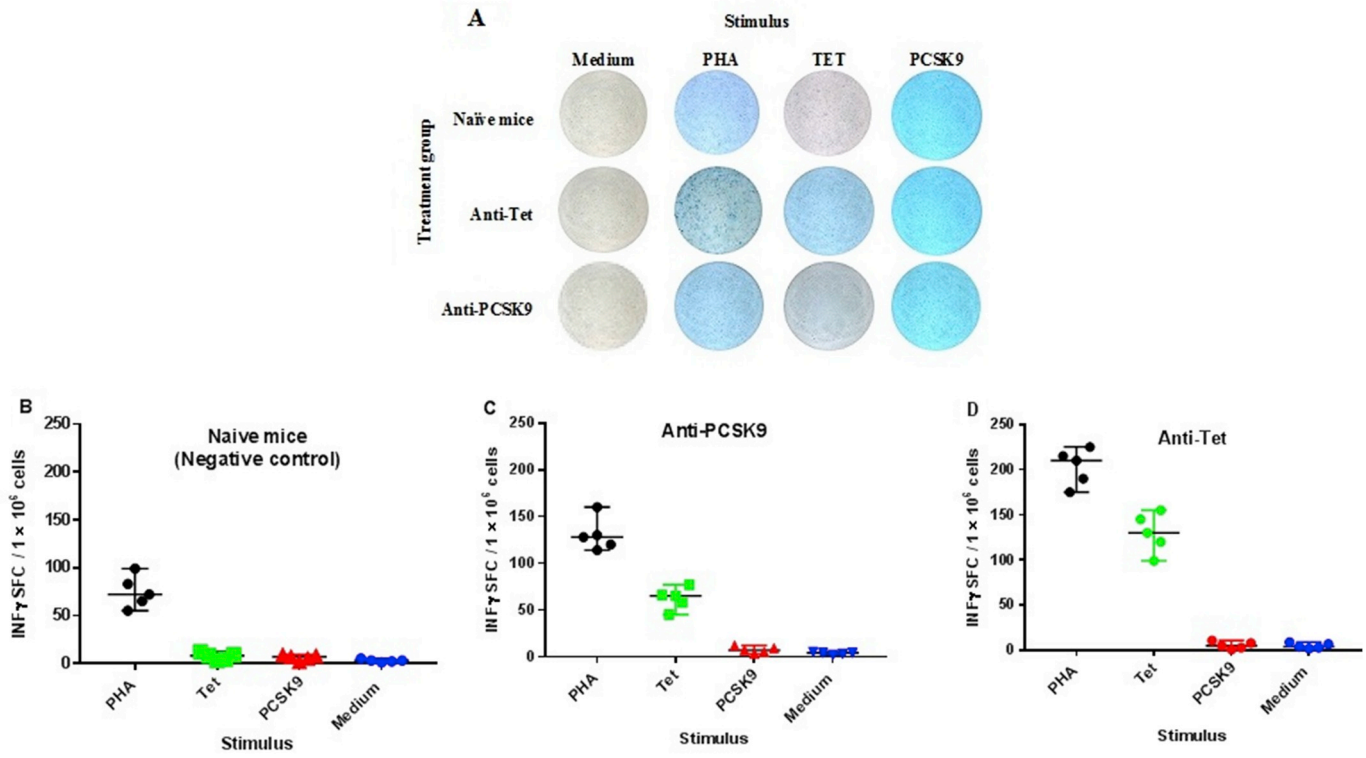

Fig. 3. PCSK9-specific T cells were not activated by PCSK9 vaccination.
(A) Representative IFNγ ELISPOT wells for splenocytes isolated from various treatment groups. ELISpot analysis for IFN-γ releasing splenocytes isolated from naïve mice as negative control (B), and mice immunized with either PCSK9 peptide (C) or tetanus peptide (D).The isolated splenocytes were stimulated with PHA (as positive control), medium, PCSK9 and tetanus (Tet) peptides. Data are presented as mean ± SD of five mice in each group.

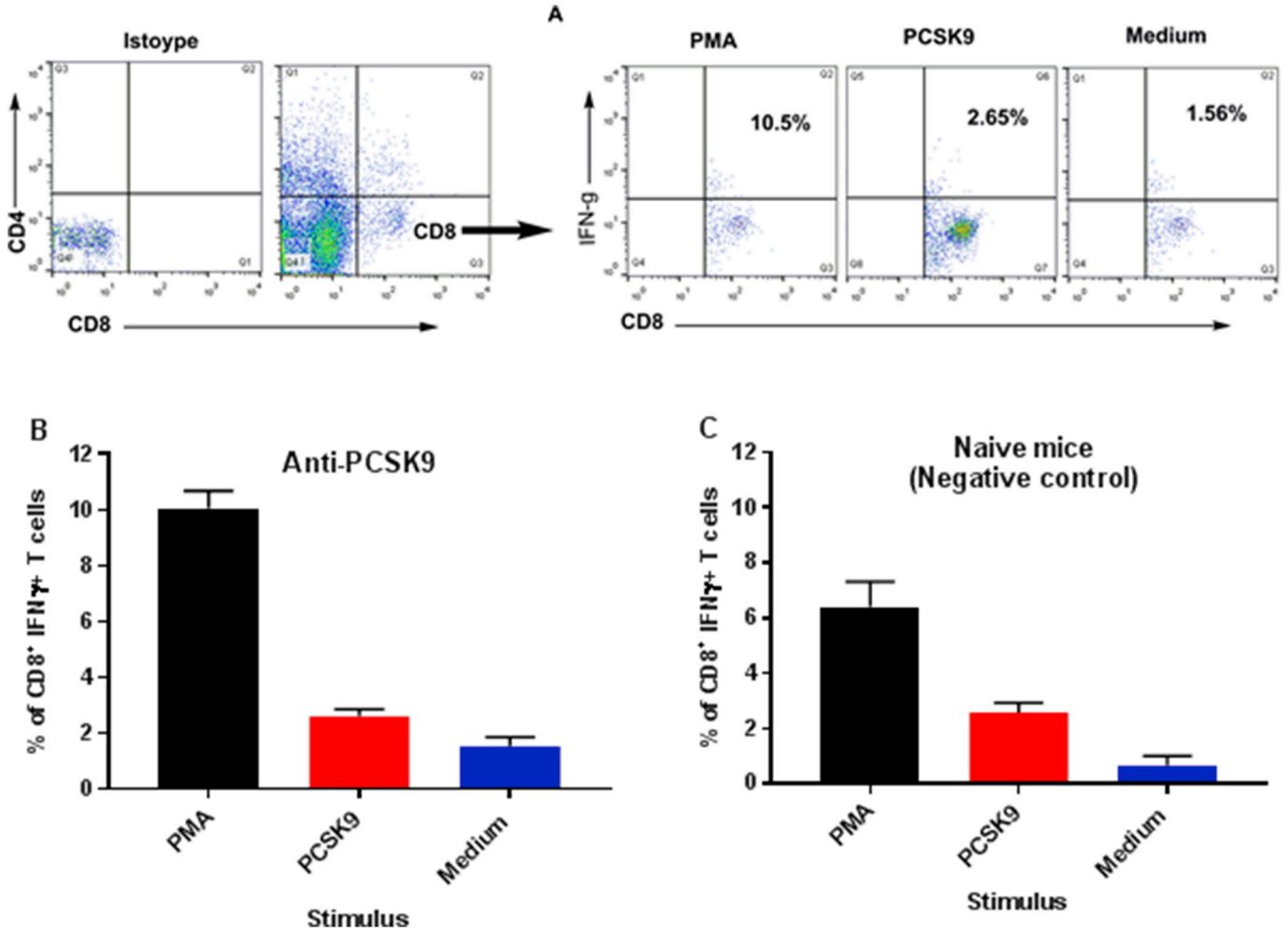

Fig. 4. PCSK9-specific CD8$^+$ T cells were not activated by antiPCSK9 vaccination.
(A) Representative dot plots show the gating strategy for flow cytometric analysis of IFNγ-producing CD8$^+$ cells on the basis of isotype control. IFNγ-producing CD8$^+$ cells were analyzed after stimulation of splenocytes with PMA (as positives control), medium (as negative control), or PCSK9 peptide. Percentage of CD8$^+$ cells producing IFN-γ within the gated cell population isolated from splenocytes of the antiPCSK9 vaccine group (B) and negative control group (C) are presented. Results are indicated as mean ± SD of five mice in each group.

percentage of splenic CD4$^+$ IL-4$^+$ T cell population in the immunized mice was 6.2 folds higher than in naïve mice (Fig. 5).

4. Discussion

PCSK9 inhibition is known to be one of the most promising LDL-lowering approaches for the treatment of hypercholesterolemia and cardiovascular disease. Currently, PCSK9 mAbs, including alirocumab and evolocumab, are the only approved PCSK9 inhibitors which markedly reduce LDL-C level and decrease the incidence of cardiovascular events in clinical trials [12,14,25]. Nevertheless, widespread implementation of PCSK9 mAbs has been hindered due to limitations such as high cost and the need for frequent administration [26]. To overcome these shortcomings, active vaccination could provide an efficient and cost-effective therapeutic approach for hypercholesterolemia [27–29]. Hence, it is desirable to develop a vaccine that actively and durably immunizes patients against PCSK9.

PCSK9 regulates plasma levels of LDL-C through a direct interaction between the EGFA domain of LDLR and the catalytic domain in the N-terminal sequence of PCSK9 protein [7,20]. An effective antiPCSK9 vaccine should elicit antibodies targeting catalytic domain of PCSK9, thereby preventing the binding of PCSK9 to LDLR. The present study revealed that active immunization against PCSK9 using L-IFPTA$^+$, a complex containing nanoliposome carrier and IFPT peptide formulated with alum vaccine adjuvant (IFPTA$^+$), could efficiently and safely induce long-lasting PCSK9-specific antibodies in the vaccinated BALB/c mice. L-IFPTA$^+$ vaccine induced antibodies that bind PCSK9 and efficiently inhibit PCSK9 –LDLR interaction (Fig. 1).

An important concern is that inducing antibodies against self-antigens activates a target-specific T cell response and enhances accumulation of auto-reactive T cells that can provoke life-threatening and irreversible tissue damage [30]. The IFPT peptide used in this study contained two different epitopes related to PCSK9 and tetanus proteins. To circumvent T cell tolerance, and based on the designed PCSK9 epitopes by the AFFiRiS group [16], we used a short peptide sequence (13 amino acids) posing a B cell epitope mimicking an N-terminal sequence of human PCSK9 protein responsible for binding to LDLR. To prevent the activation of PCSK9-specific T cells while preserving the capacity to promote an effective B cell response, the selected peptide sequence was fused to the foreign carrier peptide tetanus that poses T helper cell epitope (Table 1). Analysis of T cell activation using ELISpot (Fig. 3) and flow cytometry (Fig. 4) demonstrated that the selected PCSK9 peptide could not stimulate PCSK9-specific T cells in the vaccinated mice, confirming the peptide as an exclusive B cell epitope, while tetanus peptide could significantly promote T cell activation, showing safety of the peptide vaccine.

Adjuvants have also impressive effects on the intensity of immune response. Adjuvant activity of nanoliposome particles has been clearly

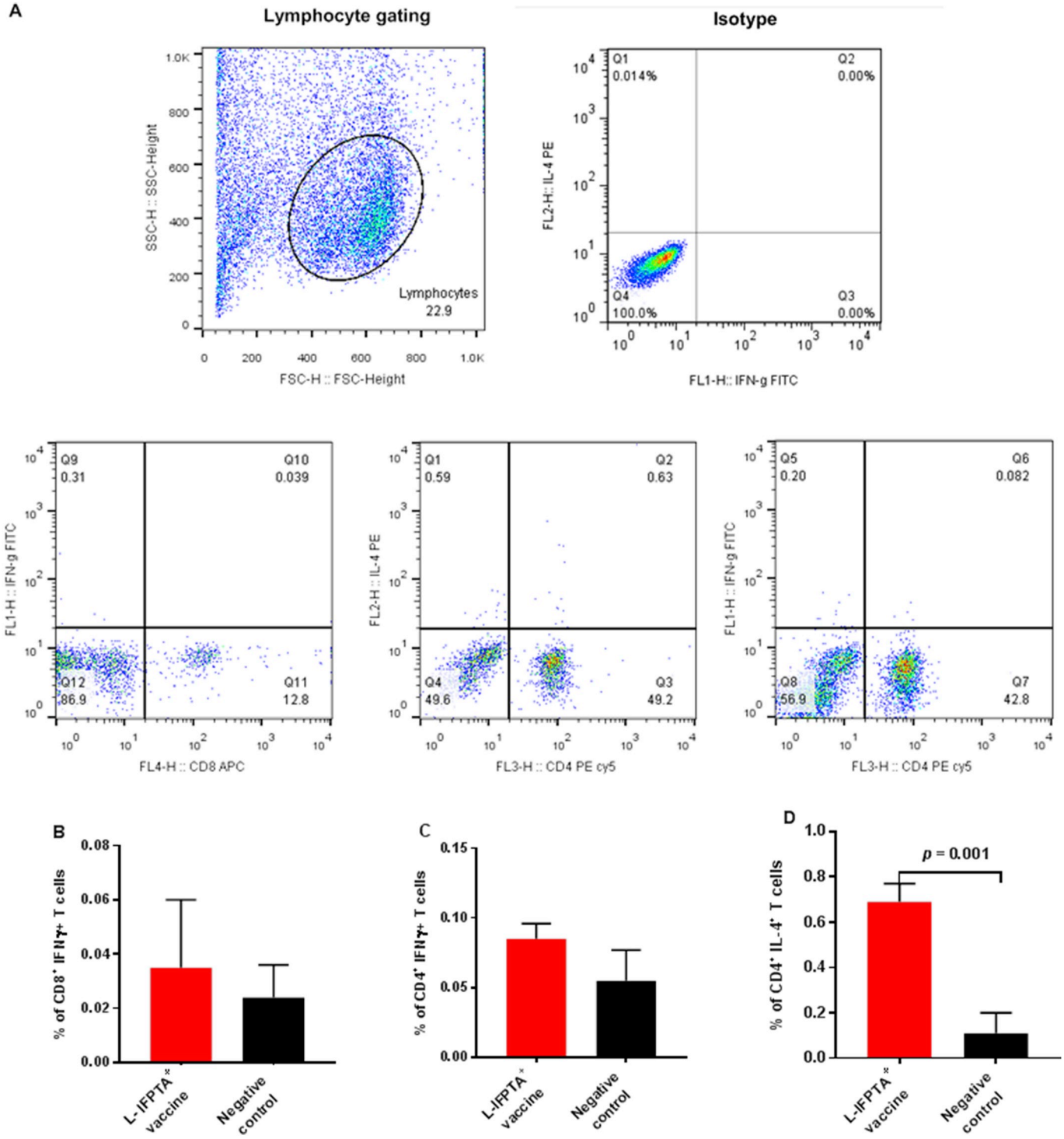

Fig. 5. Representative dot plot of gating strategy for $CD8^+$ IFN- γ^+, $CD4^+$ IL-4^+ and $CD4^+$ IFN- γ^+ T cells.
(A) Gating and quadrant analysis was performed on the basis of isotype control. Nanoliposomal $IFPTA^+$ vaccine exerted no significant effect on splenic $CD8^+$ IFN-γ^+ (B) and $CD4^+$ IFN-γ^+ (C) T cells, when compared with naïve mice (negative control). (D) $CD4^+$ IL-4^+ T cell population was found to be significantly increased in mice vaccinated with nanoliposomal $IFPTA^+$ vaccine. Data are presented as mean ± SD of five mice in each group.

documented [31–35]. The present study showed that nanoliposomes with negative surface charge can profoundly enhance the humoral immune response to surface-exposed peptide antigen. Negatively charged nanoliposomes (DMPC/DMPG/Chol) were used as an adjuvant delivery system to enhance PCSK9 antibody response in the vaccinated mice. Negatively charged liposomes have shown strong adjuvant activity by facilitating antigen uptake and, thereby, activation of dendritic cells [36]. It has been reported that nanoliposomes containing negatively charged phospholipids, like DSPG, could increase the migration of dendritic cells to the lymph nodes [37]. Our results showed that immunogenicity of the IFPT peptide was significantly increased when it was displayed on the surface of nanoliposome carriers. It was found that surface-exposed IFPT nanoliposomes (L-IFPT or L-$IFPTA^+$) elicited significantly higher PCSK9 antibody than IFPT alone. This finding is consistent with other studies that have shown strong antibody induction by surface conjugation of antigens [38–42]. This is not

surprising because peptide antigen on the liposome surface can be better recognized by B cell receptors [41].

Interestingly, the prepared nanoliposomes revealed, at least partially, more adjuvanticity than alum vaccine adjuvant, as L-IFPT vaccine could elicit slightly, but not significantly, higher antibody response against PCSK9 than IFPTA$^+$ vaccine. Of note, formulation of nanoliposome carriers with Alhydrogel adjuvant showed cumulative effect on humoral immune response, in which L-IFPTA$^+$ vaccine elicited significantly highest antiPCSK9 antibody titers when compared with the other vaccine formulations. It was found that L-IFPTA$^+$ vaccine specifically target mouse PCSK9, as it could decrease plasma levels of PCSK9 and also engaged mouse PCSK9 *in vitro*. High titers following prime vaccination extend the time duration in which levels of the antibody are high enough to adequately block plasma PCSK9. The long-term analysis showed that the half-life time of antiPCSK9 antibodies elicited by L-IFPTA$^+$ vaccine was about five months (Fig. 2), surpassing the half-life time of mAbs.

To our knowledge, there are other active attempts to develop an efficient PCSK9 vaccine. AFFiRiS AG has reported a peptide-based vaccine in which short PCSK9 peptide that is the same as the peptide used in our study, is conjugated to a KLH-carrier, and gives fascinating data in mice [16]. Crossey et al. [43] and Pan et al. [18] generated "virus-like particle" peptide vaccines targeting PCSK9, and the developed vaccine induced high titers of IgGs against PCSK9 in mice and macaques. Nonetheless, it is unknown whether VLP or KLH-peptide conjugates will be sufficiently immunogenic in humans.

More importantly, we evaluated inflammatory response in mice vaccinated with L-IFPTA$^+$ vaccine. Study of splenic cells revealed that IL-4 secretion and IL4-producing CD4$^+$ cell population, representing anti-inflammatory Th2 cells, were elevated in the vaccinated mice, while IFN-γ secretion and IFNγ-producing CD4$^+$ cell population, representing inflammatory Th1 cells, were unchanged (Fig. 5), showing immunological safety of L-IFPTA$^+$ vaccine.

In conclusion, L-IFPTA$^+$ vaccine can effectively, safely, and durably provoke PCSK9 antibody in BALB/c mice. Thus, L-IFPTA$^+$ vaccine might serve as a potential candidate for further proof-of-concept studies regarding its LDL-lowering effects.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

AS and MRJ conceived the study. MRJ, AS and AB designed the study. AAM conducted the experimental work, collected the data and drafted the manuscript. AS, MRJ and AB evaluated the data and critically revised the manuscript. All authors approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.02.001.

References

[1] G. Lambert, B. Sjouke, B. Choque, et al., The PCSK9 decade thematic review series: new lipid and lipoprotein targets for the treatment of cardiometabolic diseases, J. Lipid Res. 53 (2012) 2515–2524.
[2] Y.-W. Qian, R.J. Schmidt, Y. Zhang, et al., Secreted PCSK9 downregulates low density lipoprotein receptor through receptor-mediated endocytosis, J. Lipid Res. 48 (2007) 1488–1498.
[3] C.G. Davis, J.L. Goldstein, T.C. Südhof, et al., Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region, Nature 326 (1986) 760–765.
[4] G. Rudenko, L. Henry, K. Henderson, et al., Structure of the LDL receptor extracellular domain at endosomal pH, Science 298 (2002) 2353–2358.
[5] D. Van der Westhuyzen, M. Stein, H. Henderson, et al., Deletion of two growth-factor repeats from the low-density-lipoprotein receptor accelerates its degradation, Biochem. J. 277 (1991) 677–682.
[6] D.-W. Zhang, T.A. Lagace, R. Garuti, et al., Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation, J. Biol. Chem. 282 (2007) 18602–18612.
[7] H.J. Kwon, T.A. Lagace, M.C. McNutt, et al., Molecular basis for LDL receptor recognition by PCSK9, Proc. Natl. Acad. Sci. Unit. States Am. 105 (2008) 1820–1825.
[8] A. Catapano, N. Papadopoulos, The safety of therapeutic monoclonal antibodies: implications for cardiovascular disease and targeting the PCSK9 pathway, Atherosclerosis 228 (2013) 18–28.
[9] R.Q. Do, R.A. Vogel, G.G. Schwartz, PCSK9 Inhibitors: potential in cardiovascular therapeutics, Curr. Cardiol. Rep. 15 (2013) 1–12.
[10] A. Sahebkar, G.F. Watts, New therapies targeting apoB metabolism for high-risk patients with inherited dyslipidaemias: what can the clinician expect? Cardiovasc. Drugs Ther 27 (2013) 559–567.
[11] U. Food, D. Administration, FDA Approves Praluent to Treat Certain Patients with High Cholesterol, 2015.
[12] J.G. Robinson, M. Farnier, M. Krempf, et al., Efficacy and safety of alirocumab in reducing lipids and cardiovascular events, N. Engl. J. Med. 372 (2015) 1489–1499.
[13] U. Food, D. Administration, FDA Approves Repatha to Treat Certain Patients with High Cholestero L, 2015.
[14] M.S. Sabatine, R.P. Giugliano, S.D. Wiviott, et al., Efficacy and safety of evolocumab in reducing lipids and cardiovascular events, N. Engl. J. Med. 372 (2015) 1500–1509.
[15] D.S. Kazi, A.E. Moran, P.G. Coxson, et al., Cost-effectiveness of PCSK9 inhibitor therapy in patients with heterozygous familial hypercholesterolemia or atherosclerotic cardiovascular disease, J. Am. Med. Assoc. 316 (2016) 743–753.
[16] G. Galabova, S. Brunner, G. Winsauer, et al., Peptide-based anti-PCSK9 vaccines-an approach for long-term LDLc management, PLoS One 9 (2014) e114469.
[17] E. Fattori, M. Cappelletti, P.L. Surdo, et al., Immunization against proprotein convertase subtilisin-like/kexin type 9 lowers plasma LDL-cholesterol levels in mice, J. Lipid Res. 53 (2012) 1654–1661.
[18] E. Crossey, M.J. Amar, M. Sampson, et al., A cholesterol-lowering VLP vaccine that targets PCSK9, Vaccine 33 (2015) 5747–5755.
[19] C.J. Duff, M.J. Scott, I.T. Kirby, et al., Antibody-mediated disruption of the interaction between PCSK9 and the low-density lipoprotein receptor, Biochem. J. 419 (2009) 577–584.
[20] D.E. Piper, S. Jackson, Q. Liu, et al., The crystal structure of PCSK9: a regulator of plasma LDL-cholesterol, Structure 15 (2007) 545–552.
[21] A. Schneeberger, M. Mandler, O. Otava, et al., Development of AFFITOPE vaccines for Alzheimer's disease (AD)—from concept to clinical testing, JNHA-The Journal of Nutrition, Health and Aging 13 (2009) 264–267.
[22] C.L. Slingluff, G. Yamshchikov, P. Neese, et al., Phase I trial of a melanoma vaccine with gp100280–288 peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes, Clin. Cancer Res. 7 (2001) 3012–3024.
[23] L.J. Cruz, F. Rueda, L. Simón, et al., Liposomes containing NY-ESO-1/tetanus toxoid and adjuvant peptides targeted to human dendritic cells via the Fc receptor for cancer vaccines, Nanomedicine 9 (2014) 435–449.
[24] G.R. Bartlett, Phosphorus assay in column chromatography, J. Biol. Chem. 234 (1959) 466–468.
[25] S.J. Nicholls, R. Puri, T. Anderson, et al., Effect of evolocumab on progression of coronary disease in statin-treated patients: the GLAGOV randomized clinical trial, Jama 316 (2016) 2373–2384.
[26] M.J. Korman, K. Retterstøl, I.S. Kristiansen, T. Wisløff, Are PCSK9 inhibitors cost effective? Pharmacoeconomics 36 (2018) 1031–1041.
[27] M.F. Bachmann, M.R. Dyer, Therapeutic vaccination for chronic diseases: a new class of drugs in sight, Nat. Rev. Drug Discov. 3 (2004) 81.
[28] B. Chackerian, A. Remaley, Vaccine strategies for lowering LDL by immunization against proprotein convertase subtilisin/kexin type 9, Curr. Opin. Lipidol. 27 (2016) 345–350.
[29] F. Zhu, Z. Zhou, Y. Liao, The renin-angiotensin system and therapeutic vaccines for hypertension, Curr. Opin. Investig. Drugs 9 (2008) 286–294 (London, England: 2000).
[30] I. Ferrer, M.B. Rovira, M.L.S. Guerra, et al., Neuropathology and pathogenesis of

encephalitis following amyloid β immunization in Alzheimer's disease, Brain Pathol. 14 (2004) 11–20.
[31] M.M. Abhyankar, M.T. Orr, S. Lin, et al., Adjuvant composition and delivery route shape immune response quality and protective efficacy of a recombinant vaccine for Entamoeba histolytica, npj Vaccines 3 (2018) 22.
[32] A. Razazan, J. Behravan, A. Arab, et al., Conjugated nanoliposome with the HER2/neu-derived peptide GP2 as an effective vaccine against breast cancer in mice xenograft model, PLoS One 12 (2017) e0185099.
[33] P. Zamani, A.A. Momtazi-Borojeni, M.E. Nik, et al., Nanoliposomes as the adjuvant delivery systems in cancer immunotherapy, J. Cell. Physiol. 233 (2018) 5189–5199.
[34] F. Khademi, R.A. Taheri, A.A. Momtazi-Borojeni, et al., Potential of Cationic Liposomes as Adjuvants/Delivery Systems for Tuberculosis Subunit Vaccines, Reviews of Physiology, Biochemistry and Pharmacology, (2018).
[35] A. Arab, J. Nicastro, R. Slavcev, et al., Lambda phage nanoparticles displaying HER2-derived E75 peptide induce effective E75-CD8+ T response, Immunol. Res. 66 (2018) 200–206.
[36] N. Yanasarn, B.R. Sloat, Z. Cui, Negatively charged liposomes show potent adjuvant activity when simply admixed with protein antigens, Mol. Pharm. 8 (2011) 1174–1185.
[37] S.R. Gameiro, R.M. Straubinger, Adjuvancy of Negatively-Charged Liposomes for Pulmonary Delivery of Vaccines, Pulmonary Delivery of Liposome-Based Vaccines: Application to a Murine Model of Schistosoma Mansoni Infection, (2008), p. 32.
[38] L. Tan, V. Weisslg, G. Gregorladls, Comparison of the immune response against polio peptides covalently-surface-linked to and internally-entrapped in liposomes, Asian Pac. J. Allergy Immunol. 9 (1991) 25.
[39] H.-M. Thérien, D. Lair, E. Shahum, Liposomal vaccine: influence of antigen association on the kinetics of the humoral response, Vaccine 8 (1990) 558–562.
[40] E. Shahum, H.-M. Thérien, Correlation between in vitro and in vivo behaviour of liposomal antigens, Vaccine 12 (1994) 1125–1131.
[41] W. Vannier, S. Snyder, Antibody Responses to Liposome-Associated Antigen, NAVAL MEDICAL RESEARCH INST, BETHESDA MD, 1988.
[42] H.H. Guan, W. Budzynski, R.R. Koganty, et al., Liposomal formulations of synthetic MUC1 peptides: effects of encapsulation versus surface display of peptides on immune responses, Bioconjug. Chem. 9 (1998) 451–458.
[43] Y. Pan, Y. Zhou, H. Wu, et al., A therapeutic peptide vaccine against PCSK9, Sci. Rep. 7 (2017) 12534.