CD8+ T-cells in Atherosclerosis: mechanistic studies revealing a protective role in the plaque microenvironment

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7

Liposome-based vaccination against an ApoB100-derived $CD8^+$ T-cell epitope does not affect atherosclerosis development in $LDLr^{-/-}$ mice

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ABSTRACT

Aims Atherosclerosis is a chronic disease of the vessel wall, characterized by the buildup of plaques consisting of lipids and inflammatory cells. We have previously shown that CD8$^+$ T-cells, which make up an important part of the inflammatory component of the lesions, confer a protective effect on atherosclerosis. The objective of this study was to boost the protective function of CD8$^+$ T-cells in atherosclerosis via vaccination against ApoB100, the main protein component of the cholesterol-carrier LDL.

Methods and Results Prediction of ApoB100-derived MHC-I binding peptides revealed several peptides that could induce strong CD8$^+$ T-cell responses *in vivo*. The QSFDLSVK peptide was effective at inducing immune responses in a dendritic cell vaccination study and therefore selected to be encapsulated in liposomes containing the lipids DOPC and DOTAP. LDLr$^{-/-}$ mice were fed a Western-type diet for 10 weeks to induce atherosclerosis, and vaccinated with the liposomal formulation containing the peptide followed by two boost injections with the peptide adjuvanted with poly(I:C) and an anti-CD40 antibody. Unexpectedly, the treatment affected neither plaque burden nor stability. Plaque macrophage content and serum cholesterol levels were unaffected as well. Ten weeks following vaccination, we were unable to measure any antigen-specific immunity towards our peptide. Since OVA-specific CD8$^+$ T-cell responses were readily detected using the SIINFEKL peptide in the same prime-boost strategy in a subsequent experiment, we conclude that the QSFDLSVK peptide could not be efficiently cross-presented, and thus failed to induce CD8$^+$ T-cell responses. Interestingly, we did observe an increase in weight upon vaccination against the QSFDLSVK peptide.

Conclusion In short, a pro-inflammatory vaccination against the ApoB100-derived peptide QSFDLSVK formulated in DOPC/DOTAP liposomes does not affect atherosclerosis development in LDLr$^{-/-}$ mice, suggesting that using different antigens to boost CD8$^+$ T-cell responses may be more relevant for the treatment of atherosclerosis.

1. Introduction

Cardiovascular diseases are among the leading causes of morbidity and mortality worldwide [1]. Atherosclerosis, the main underlying pathological process that drives cardiovascular disease, is characterized by lipid accumulation in the form of low-density lipoprotein (LDL) as well as chronic inflammation within the vessel wall of medium- and large-sized arteries [2]. Currently, patients are mainly treated with statins, drugs that lower the LDL cholesterol levels in blood. However, statins reduce the risk of a cardiovascular event by only 20-25% [3], emphasizing the need for additional therapeutic strategies. Targeting the immune component of atherosclerosis might be an interesting option to pursue, as a recent large clinical trial has demonstrated that modulating inflammatory responses via blockade of IL-1β improves cardiovascular outcome [4]. Among the inflammatory cell-buildup within the atherosclerotic plaque, $CD8^+$ T-cells are abundant and comprise up to 50% of all lymphocytes in advanced human plaques [5, 6]. $CD8^+$ T-cells have been reported to show both pro- and anti-atherogenic properties, depending on their inflammatory status and interactions with other cell types within the atherosclerotic lesion [7].

We have previously shown a protective role for $CD8^+$ T-cells in the advanced stages of atherosclerotic lesion development, by limiting lesional macrophage content and $CD4^+$ T-cell responses via FasL-induced cell death [8]. Moreover, we reported that activation of $CD8^+$ T-cells specifically in the lesion microenvironment decreases inflammatory cytokine production through upregulation of the ectonucleotidase CD39 [9]. Therefore, increasing the number of $CD8^+$ T-cells in the atherosclerotic lesion, for instance through vaccination, may be a potential strategy to stabilize atherosclerotic plaques. Although the antigens that drive the adaptive immune response in atherosclerosis are still a matter of debate [10], there is a strong body of evidence suggesting a role for LDL- and ApoB100-derived peptides. Several studies have shown that antibodies direct against ApoB100 are generated in both atherosclerotic patients and mouse models [11–14]. Moreover, T-cells from human atherosclerotic lesions can recognize oxidized LDL [15], whereas blocking T-cells from recognizing ApoB100 (the primary protein in LDL) reduces atherosclerosis formation in mice [16].

Previous work has demonstrated an atheroprotective effect of multiple immunizations with the ApoB100-derived p210 peptide conjugated to cationic bovine serum albumin and using Alum as an adjuvant [17], which was mediated by $CD8^+$ T-cells [18]. Interestingly, Alum preferentially primes a type 2 immune response [19] and has not been described as a potent inducer of cytotoxic $CD8^+$ T-cells. Here, we aimed to raise the number of atheroprotective $CD8^+$ T-cells using a cationic liposomal formulation specifically designed to induce protective $CD8^+$ T-cell responses in a murine model of atherosclerosis. Liposomes are a promising antigen-delivery system to use *in vivo*, as they enhance the uptake of the encapsulated antigen in dendritic cells (DCs) [20]. Furthermore, by altering the physicochemical properties of the liposomes, the ensuing immune response can be skewed in the desired direction [21]. For instance, cationic liposomes are known to induce strong $CD8^+$ T-cell responses [22], possibly due to their ability to facilitate endosomal escape [23], resulting in increased presentation of their peptide load on ma-

jor histocompatibility complex class I (MHC-I) molecules. Encouraging results using cationic liposomes for inducing CD8$^+$ T-cells in cancer treatment have been reported previously [24–27], which is why we opted to use DOPC:DOTAP liposomes in our study. We show that vaccination with an ApoB100-derived peptide encapsulated in these liposomes, unexpectedly, does not affect the atherosclerotic burden in LDLr$^{-/-}$ mice.

2. Materials & Methods

2.1. Materials

The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Göttingen, Germany). The ApoB100-derived peptides (LSIQNYHVF, VMWLMDSFI, VTYLMALI, STNVYSNL, VPYAFKSL, SAINNEHNI, ANILNSEEL, TNLKYSPL, QSFDLSVK and TTKQSFDL) were synthesized by GenScript (Piscataway, New Jersey, USA). SIINFEKL was purchased from Tebu-bio (Heerhugowaard, the Netherlands) Poly(I:C) was purchased from Invivogen (Toulouse, France) and monoclonal anti-CD40 antibody (rat IgG2a clone FGK4.5) was obtained from BioXcell (Hanover, New Hampshire, USA).

2.2. Liposome preparation

Empty or peptide-containing cationic liposomes (DOTAP:DOPC, 1:1 molar ratio) were produced by making use of the thin film dehydration-rehydration method as previously described [28]. Briefly, DOTAP and DOPC were mixed in a 1:1 molar ratio to reach a final concentration of 10 mg lipids in 1 mL chloroform. A lipid film was formed by rotary evaporation. The lipid film was hydrated in the presence of glass beads with a 2 mL solution of 0.04% NH_4OH for non-loaded (empty) liposomes or with a 2 mL of a solution of 0.04% NH_4OH containing 500 µg QSFDLSVK for the loaded liposomes. The liposomal dispersion was snap-frozen and freeze-dried in a Christ alpha 1–2 freeze-dryer (Osterode, Germany) overnight. After freeze-drying overnight, the lipid cake was rehydrated with 10 mM phosphate buffer, pH 7.4 (PB), in three consecutive steps: twice 250 µL was added followed by vortexing and 30 min equilibration after each addition, and as a third step the remaining 500 µL was added, followed by 1 h equilibration. The liposomes were next sized using high-pressure extrusion at room temperature with a LIPEX Extruder (Northern Lipids Inc., Canada). To obtain monodisperse liposomes, the liposome mixture was passed four times through a 400 nm-pore and a 200 nm-pore sized membrane (Nuclepore, Millipore, Kent, UK). The liposomes were purified and concentrated using Vivaspin 2 centrifugation concentrators (PES membrane, molecular weight cut-off 300 kDa) which were centrifuged at 1500 RPM and 4 °C for 5–6 hours until the suspension was concentrated five-fold. The filtrate containing the free peptide was removed and the concentrated liposomes were washed with 1 mL PB and again centrifuged until the volume was concentrated five-fold.

2.3. Liposome characterization

The Z-average diameter and polydispersity index (PDI) of the liposomes were measured by dynamic light scattering (DLS). Zeta-potential was determined by using laser Doppler electrophoresis, both using a NanoZS Zetasizer (Malvern Ltd., Malvern, UK). Samples were diluted 100-fold in PB to a total volume of 1 mL. Peptide encapsulation efficiency was measured using reversed-phase ultra-pressure liquid chromatography (UPLC) on a Waters ACQUITY UPLC (Waters, MA, USA). 5 µL of the sample was injected into a 1.7 µm BEH C18 column (2.1 × 50 mm, Waters). The column temperature was set to 40 °C and the sample temperature set to 4 °C. The mobile phases were Milli-Q water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). For detection, the mobile phases were applied in a linear gradient from 5% to 95% solvent B over 6 min at a flow rate of 0.370 mL min^{-1}. Peptides were detected by absorbance at 214 nm using an ACQUITY UPLC TUV detector (Waters).

2.4. Animals

LDLr$^{-/-}$ mice and C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and bred in-house. Animals were kept under standard laboratory conditions; food and water were provided *ad libitum*. All animal work was performed in compliance with the Directive 2010/63/EU of the European Parliament and the Dutch government guidelines. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

2.5. Bone marrow-derived dendritic cells (BMDCs)

Bone marrow was isolated from the tibias and femurs of C57BL/6 mice. A single-cell suspension of bone marrow cells was obtained by using a 70 µm cell strainer (Greiner Bio-One B.V., Alphen aan den Rijn, NL). The cells were cultured in IMDM (Lonza, Breda, NL) supplemented with 2 mM L-glutamine, 8% (v/v) FCS, 100 U mL^{-1} penicillin/streptomycin (Lonza), and 50 µM β-mercaptoethanol (Sigma, Zwijndrecht, NL) at 37 °C and 5% CO_2 in 95 mm Petri dishes with 20 ng mL^{-1} GM-CSF (Immunotools, Friesoythe, Germany) for 10 days.

2.6. Cell preparation and flow cytometry

Mice were sacrificed by injection of a lethal dose of ketamine (40 mg mL^{-1}), sedazine (8 mg ml^{-1}) and atropine (0.1 mg ml^{-1}) and blood, spleens, heart lymph nodes (hLN), inguinal lymph nodes (iLN), and aortas were harvested after in situ perfusion with phosphate-buffered saline (PBS, pH 7.4, Lonza). White blood cells were obtained by lysing the blood twice for 2 min with lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na2EDTA; pH 7.3). Single-cell suspensions of spleens and LNs were obtained by using a 70 µm cell strainer (Greiner Bio-One). Splenocytes were lysed for 1 min with lysis buffer to obtain white blood cells. For peptide restimulations, approximately

1×10^6 cells were plated out in 96-well U-bottom plates (Greiner Bio-One) in RPMI 1640 Medium (Lonza, Basel, Switzerland) supplemented with fetal bovine serum (10%, Greiner Bio-One), L-glutamine (2%, Lonza), penicillin/streptomycin (1%, Lonza), sodium pyruvate (1%, Sigma-Aldrich) and β-mercaptoethanol (60 µM, Sigma-Aldrich) at 37 °C and 5% CO_2. Cells were incubated for 5 hours in the presence of 3 µg mL^{-1} Brefeldin A (ThermoFisher Scientific, MA, USA) and 10 µm, 5 µM, 1 µM or 10 nM of the ApoB100-derived peptides. Cells were stained with the appropriate antibodies for flow cytometric analysis. For intracellular staining, cells were fixed and permeabilized by using an intracellular staining kit (BD bioscience) according to the manufacturer's protocol. Flow cytometry analyses were performed on a Beckman Coulter Cytoflex S and FlowJo software (Treestar).

2.7. Dendritic cell vaccination studies

BMDCs were cultured as described above and stimulated overnight with 100 ng mL^{-1} of LPS and 50 µM of the ApoB100-derived peptides LSIQNYHVF, VMWLMDSFI, VTYLMALI, STNVYSNL, VPYAFKSL, SAINNEHNI, ANILNSEEL, TNLKYSPL, QSFDLSVK and TTKQSFDL. The next day, 18 LDLr$^{-/-}$ mice were injected i.v. with 1E6 DCs loaded with a combination of the different peptides (n = 12), LPS-treated DCs (n = 3) or PBS (n = 3) or the ovalbumin-derived SIINFEKL peptide as controls. After one week, the immune response was boosted by intraperitoneal (i.p.) injection with 200 µg of the peptides, 50 µg anti-CD40 antibody, and 50 µg poly(I:C) in PBS. One week after the boost injections, mice were sacrificed by cervical dislocation and spleens were removed for flow cytometric analysis.

2.8. Atherosclerosis studies

LDLr$^{-/-}$ mice of between 10 and 13 weeks of age were randomized into 4 groups of 10 mice each, based on age, weight, and plasma cholesterol levels. At the start of the experiment, the mice were immunized intravenously (i.v.) with either 100 µL PBS, 3.5 nanomole of free QSFDLSVK peptide in PBS, 0.9 mg DOPC:DOTAP liposomes, or 3.5 nanomole QSFDLSVK peptide encapsulated in 0.9 mg DOPC:DOTAP liposomes. From the moment of immunization, mice were fed a Western-type diet (WTD) containing 15% cocoa butter, 10% maize starch, 20% casein, 40.5% sucrose, 0.25% cholesterol and 5.95% cellulose (Special Diet Services, Witham, Essex, UK) for 10 weeks to induce atherosclerotic lesion formation. One week and four weeks after the start of the experiment, the PBS and empty liposome-treated mice received a boost injection with 50 µg poly(I:C) and 50 µg anti-CD40 antibody, while the free peptide and peptide liposome-treated mice were boosted with 200 µg QSFDLSVK peptide, 50 µg poly(I:C) and 50 µg anti-CD40 antibody in 200 µL PBS via i.p. injection. In weeks 3 and 6, 100 µL blood was obtained via venipuncture of the tail vein for the determination of plasma cholesterol levels. After 10 weeks, mice were sacrificed and organs were harvested as described above. Total cholesterol levels were assessed using an enzymatic colorimetric assay (Roche Diagnostics, Almere, The Netherlands).

2.9. Liposomal vaccination studies

$LDLr^{-/-}$ mice that were 10 weeks of age were divided into two groups of three mice each. At the start of the experiment, the mice were immunized i.v. with either 3.5 nanomole QSFDLSVK peptide or SIINFEKL peptide encapsulated in DOPC:DOTAP liposomes. One week after the initial vaccination, the mice received a boost injection with 50 µg poly(I:C), 50 µg anti-CD40 antibody, and 200 µg QSFDLSVK or SIINFEKL via i.p. injection. One week after the boost vaccination, mice were sacrificed via cervical dislocation and spleens were harvested.

2.10. Histological analysis

All hearts were embedded in optimal cutting temperature (O.C.T.) compound (Sakura, Alphen aan den Rijn, The Netherlands) and sectioned horizontally to the aortic axis and towards the aortic arch. Upon identification of the aortic root, defined by the trivalve leaflets, consecutive 10 µm sections were collected. Analysis of lesion size was performed on aortic root lesions stained with Oil-Red O and hematoxylin (Sigma-Aldrich). Corresponding sections were stained with Sirius Red (Sigma-Aldrich) to determine collagen content. Plaque macrophages were stained immunohistochemically by using a rat-anti-mouse monocytes/macrophages antibody (MOMA, 1:1000, AbD Serotec) as a primary antibody, biotinylated rabbit anti-rat IgG (1:100; Vector) as a secondary antibody, and Vectastain ABC horseradish peroxidase in combination with ImmPACT Nova Red for visualization (Vector). The average plaque size (in μm^2) was calculated from 5 sequential sections. For all other stainings, three subsequent sections displaying the highest plaque content per mouse were analyzed. All microscopic analyses were performed on a Leica DM-RE microscope using Leica QWin software and were blinded for independent analysis. The percentages of collagen and macrophages in the atherosclerotic lesions were determined by dividing the area in μm^2 stained positive for collagen or MOMA-2 by the total lesion surface area and calculated as a percentage.

2.11. Statistical analysis

Data are presented as individual values or as mean ± SEM, the number of animals in each group is stated in the text. Data were tested for normal distribution and analyzed using a one-way ANOVA or two-way ANOVA, as appropriate. Statistical analysis was performed using Prism (GraphPad). Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. Identification of peptide candidates to induce ApoB100-specific CD8$^+$ T-cells

We set out to investigate which peptides derived from the ApoB100 protein would be likely bind strongly to the murine MHC-I molecules and thus show potential for CD8$^+$ T-cell activation, using the Immune Epitope Database Analysis Resource (IEDB). We restricted our predictions to the H2-K^b and H2-D^b alloantigens, as these are expressed in mice on a C57Bl/6 background. The IEDB T Cell Epitope Prediction Tool [29] uses a combination of the artificial neuronal network (ANN) [30], stabilized matrix method (SSM) and Scoring Matrices derived from Combinatorial Peptide Libraries (Comblib_Sidney2008) [31] to predict which peptides in a given sequence can bind strongly to MHC-I molecules. Peptide length was set to octamers for H2-K^b and to nanomers for H2-D^b to ensure optimal binding [32]. We selected 8 peptide sequences for synthesis that showed high predicted binding scores in which we included both H2-K^b- and H2-D^b-binding peptides throughout all regions of the ApoB100 protein (Table 1). Furthermore, the P210-derived peptides QSFDLSVK and TTKQSFDL, which were described to be recognized by CD8$^+$ T-cells in atherosclerotic mice [18], were synthesized as well (Table 1).

To evaluate whether these peptides are able to induce antigen-specific CD8$^+$ T-cell responses in an atherosclerotic mouse model, we performed a DC vaccination study. Bone-marrow-derived DCs were activated with LPS and pulsed with the peptides and subsequently injected into LDLr$^{-/-}$ mice. One week later, the mice were boosted with 200 µg of each of the peptides, 50 µg anti-CD40 antibody and 50 µg poly(I:C). One week after the boost injections, splenocytes were obtained and restimulated with the peptides in the presence of Brefeldin A. We observed significant dose-dependent increases in IFN-γ production by CD8$^+$ T-cells from vaccinated mice stimulated with the peptides QSFDLSVK, TNLKYSPL, VTYLMALI, LSIQNYHVF, and STNVYSNL compared to the effect of these peptides on CD8$^+$ T-cells derived from mice treated with LPS-stimulated DCs (Fig. 1). Based on these results, we identified TNLKYSPL and QSFDLSVK as the best peptide candidates for further studies, as they are able to induce the highest antigen-specific CD8$^+$ T-cell responses in our mouse model. When considering the physicochemical properties of the peptides, QSFDLSVK was the best candidate for encapsulation in liposomes. We were unable to dissolve TNLKYSPL in a range of solvents, including water, methanol, chloroform and acetonitrile, which hampered the possibility to further study this peptide in a liposomal vaccination setting. QSFDLSVK, however, is water-soluble and therefore easier to work with.

3.2. Preparation of liposomes

Cationic DOPC:DOTAP liposomes, empty or loaded with the QSFDLSVK (Abbreviated to QSF) peptide, were prepared using the film dehydration-rehydration method. The resulting empty and loaded liposomes had a very similar size (146 vs. 147 nm, respec-

Table 1: ApoB100-derived peptides selected from the epitope binding prediction.

Allele	Sequence		Length	Peptide	Method	Percentile rank
	Start	End				
H-2-D^b	3070	3078	9	SAINNEHNI	ann/smm/ comblib_ sidney2008	0.1
H-2-K^b	1631	1638	8	TNLKYSPL	ann/smm	0.15
H-2-K^b	384	391	8	VTYLMALI	ann/smm	0.15
H-2-K^b	1332	1339	8	STNVYSNL	ann/smm	0.15
H-2-K^b	4254	4261	8	VPYAFKSL	ann/smm/ comblib_ sidney2008	0.2
H-2-D^b	571	579	9	ANILNSEEL	ann/smm/ comblib_ sidney2008	0.3
H-2-D^b	4436	4444	9	LSIQNYHVF	ann/smm/ comblib_ sidney2008	0.3
H-2-D^b	4103	4111	9	VMWLMDSFI	ann/smm/ comblib_ sidney2008	0.6
H-2-K^b	3131	3138	8	TTKQSFDL	ann/smm	11.5
H-2-K^b	3134	3141	8	QSFDLSVK	ann/smm	19.5

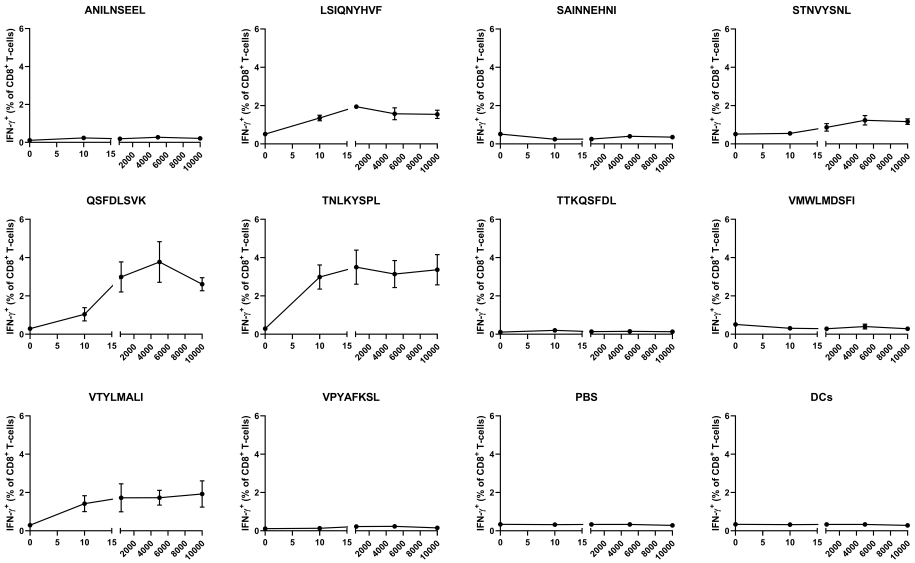

Figure 1: Dendritic cell vaccination with ApoB100-derived peptides in LDLr$^{-/-}$ mice identifies two peptide candidates that induce cytokine production in CD8$^+$ T-cells upon restimulation. Splenocytes were isolated from the vaccinated mice and 1×10^6 cells were stimulated with the indicated concentrations of peptides in the presence of Brefeldin A for 5 hours. Subsequently, cells were stained and analyzed by flow cytometry. Percentages of CD8$^+$ T-cells that are positive for IFN-γ are plotted for each tested peptide. Two-way ANOVA shows a significant difference between the DC control group and QSF***, TNL***, VTY***, LSI***, and STN*. Mean ± SEM, n = 3 per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 3: Physicochemical properties of liposomal formulations. Data are averages ± SD of four independent batches. Z_{ave} is the Z-average particle diameter, PDI is the polydispersity index.

	Z_{ave} (nm)	PDI	Zeta potential (mV)	QSF loading efficiency (%)
Empty	147 ± 6	0.15 ± 0.01	30.7 ± 0.7	-
QSF-loaded	146 ± 6	0.16 ± 0.01	31.2 ± 3.6	13.2 ± 5.1

tively), PDI (0.15 vs. 0.16, respectively) and zeta potential (30.7 vs. 31.2 mV, respectively). The peptide loading efficiency was on average 13% (Table 3).

3.3. Vaccination using DOPC:DOTAP liposomes with encapsulated ApoB100-derived peptides does not affect atherosclerosis development

To investigate the effect of our liposomal formulation containing the QSF peptide on atherosclerosis development, $LDLr^{-/-}$ mice were fed a WTD and immunized i.v. with either 100 µL PBS, 3.5 nanomole of free QSF peptide in 100 µLl PBS, 0.9 mg DOPC:DOTAP liposomes in 100 µL PBS, or 3.5 nanomole QSFDLSVK peptide encapsulated in 0.9 mg DOPC:DOTAP liposomes in 100 µL PBS. The mice were boosted twice with 50 µg anti-CD40 antibody, 50 µg poly(I:C) and 200 µg of the peptide. Ten weeks after the start of the treatment, the mice were sacrificed and immunological responses as well as atherosclerotic lesion formation were assessed. The atherosclerotic lesion size was assessed in the aortic root lesions using Oil-Red-O staining. No significant differences were observed in lesion size between the different treatment arms (Fig. 2A). Moreover, analysis of collagen content in the lesions, a measure for the stability of the plaques, revealed no significant differences (Fig. 2B). Finally, we stained the aortic root lesions for MOMA2 using immunohistochemistry, which visualizes the monocyte and macrophage content in the plaques, and observed no differences between the groups (Fig. 2C). As expected, all mice gained weight during the experiment due to their consumption of a WTD. However, the QSF liposome treated mice showed a significantly stronger gain in weight compared to the control-treated mice, resulting in an average weight of 34 vs. 31 grams at the end of the experiment, respectively (Fig. 3A). These differences were not associated with increases in cholesterol levels, as these were found not to differ in the plasma throughout the experiment (Fig. 3B) or in the serum at the end of the experiment (Fig. 3C).

As we did not observe changes in plaque size or composition as a result of vaccination, we next evaluated the immunological effects of our vaccination. We only observed limited, non-significant increased percentages of $CD8^+$ T-cells in blood (64% vs 61%, p = 0.42), iLN (47% vs 45%. p = 0.55), hLN (46% vs. 41%, p = 0.38) and spleens (38% vs. 36% p = 0.54) in the QSF liposome-treated group compared to PBS controls (Fig. 4A).

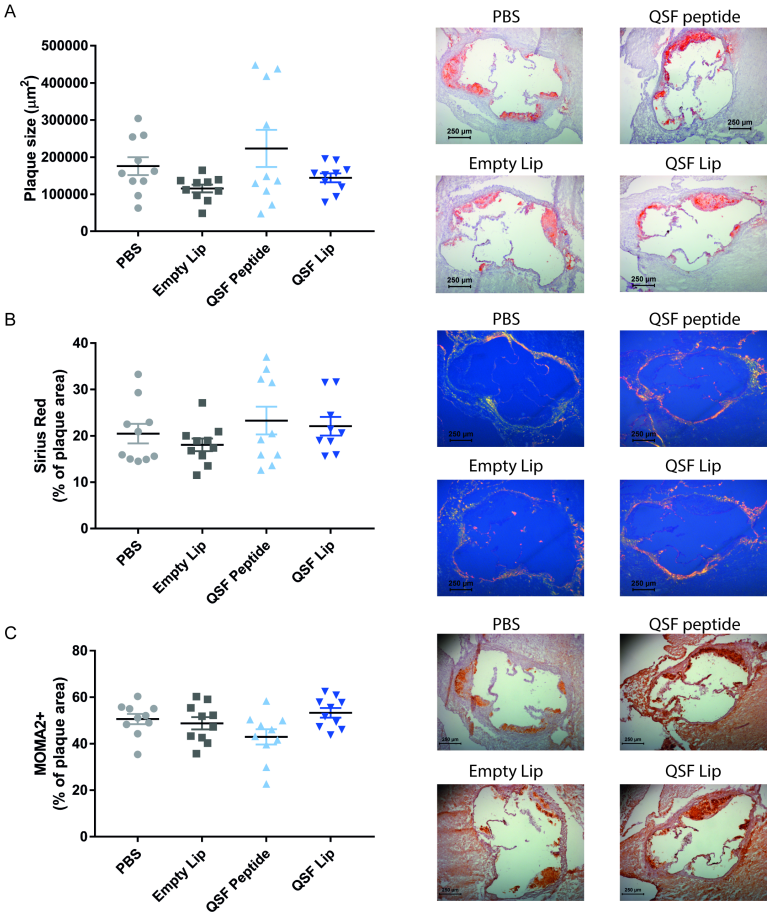

Figure 2: No differences in plaque size or composition in the aortic root lesions of LDLr$^{-/-}$ mice on a WTD vaccinated with PBS, empty liposomes, QSF peptide or QSF liposomes. (A) Quantification of lesion size in the aortic root by Oil-Red-O staining and representative pictures from each group. (B) Quantification of collagen content by Sirius Red staining in the aortic roots of LDLr$^{-/-}$ mice and representative pictures from each group. (C) Macrophage quantification in the aortic roots of the LDLr$^{-/-}$ mice by MOMA-2 staining and representative images of MOMA-2 staining. Data points represent individual values, lines denote mean ± SEM, n = 10 per group, significance was determined by one-way ANOVA and Bonferroni's multiple comparisons tests.

We analyzed the expression of CD62L and CD44 on these CD8$^+$ T-cells, to determine if these cells were naïve (CD62L$^+$CD44$^-$), effector memory (TEM, CD62L$^-$CD44$^+$), or central memory (TCM, CD62L$^+$CD44$^+$) cells. We observed no significant differences, but in the blood there appeared to be a small decrease in the percentage of TCM cells (40% vs. 48%, p = 0.29) and a small increase in the percentage of TEM cells (35% vs. 29% p = 0.48) in the QSF liposome treated group compared to PBS controls (Fig. 4B). Similarly, no differences were observed in the expression of the proliferation-associated transcription factor Ki-67 in these CD8$^+$ T-cells. Analysis of the transcription factors

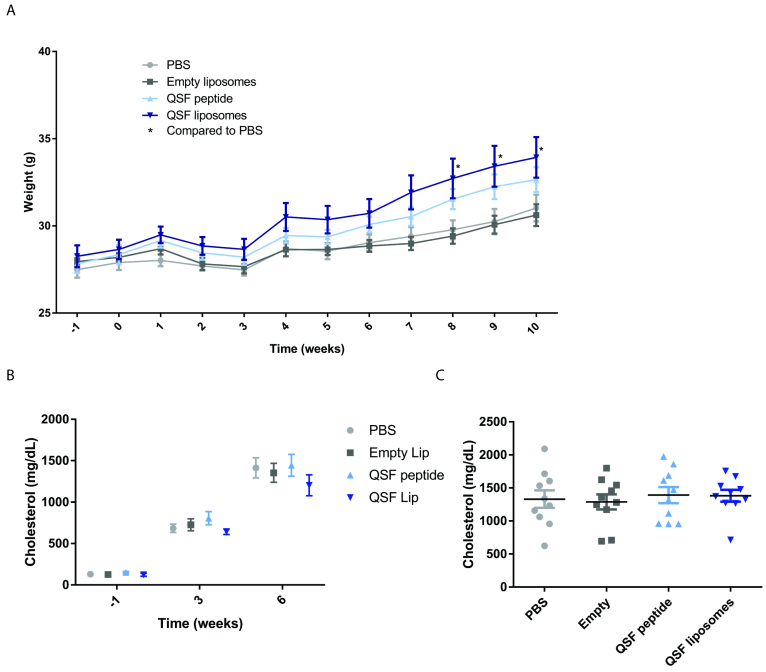

Figure 3: QSF liposome vaccinated mice show a significant increase in weight at the end of the experiment, but no differences in cholesterol levels. (A) Body weights of the LDLr$^{-/-}$ mice over time during the vaccination experiment, WTD feeding and vaccination were initiated at time point 0. (B) Plasma cholesterol levels in the LDLr$^{-/-}$ mice at baseline, week 3 and week 6. (C) Serum cholesterol levels in the LDLr$^{-/-}$ mice at the time of sacrifice. Mean ± SEM, n = 10 per group, significance was determined by either a one-way or two-way ANOVA and Bonferroni's multiple comparisons tests, as appropriate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

associated with different CD8^{+} T-cell subsets (T-bet, GATA3, RORγt, and FoxP3) were also not found to differ between the groups (data not shown). Apart from the effects of the treatment on the CD8^{+} T-cells, we also analyzed other immune cell subsets. We observed no differences in the number of inflammatory (Ly6Chi) and patrolling (Ly6Clo) monocytes in the blood (Fig. 4C).

We previously reported that CD8^{+} T-cells may affect the number of macrophages and CD4^{+} T-cells in the lesion, whereas such differences were not observed systemically. However, when analyzing the aortic lesions in this study, we observed no difference in the presence of CD11b^{+} APCs (Fig. 4D), nor in the expression levels of T-bet and GATA3 (data not shown), or in the ratio between Th1 and Th2 cells within the CD4^{+} T-cell compartment (Fig. 4E).

Although we found no significant difference in the bulk CD8^{+} T-cell population, vaccination might have induced changes in the number of antigen-specific CD8^{+} T-cells. This was analyzed by stimulating splenocytes with the QSF peptide, after which cytokine production was measured using flow cytometry. However, we observed no differences in IFN-γ production (Fig. 4F), TNF-α production, nor IL-10 production (data

not shown) between the different groups or between the stimulated samples and non-peptide treated controls.

3.4. DOPC:DOTAP liposomes are able to induce antigen-specific CD8$^+$ T-cell responses towards the SIINFEKL peptide, but not the QSFDLSVK peptide in LDLr$^{-/-}$ mice

As we observed no differences in both atherosclerotic lesion formation and immunological responses upon vaccination with the QSF peptide, we set out to determine whether or not there was any immune response induced using our vaccination strategy. We vaccinated LDLr$^{-/-}$ - mice with 3.5 nanomole of the QSF peptide in the liposomes described above or with 3.5 nanomole of the model antigen SIINFEKL, derived from ovalbumin (abbreviated as OVA peptide), in the same liposomes as a positive control. The injected liposomes were similar in size (146 vs. 143 nm, respectively), PDI (0.14 vs. 0.14, respectively) and zeta-potential (29.4 vs. 30.2 mV, respectively). The peptide loading efficiencies were 7.8% for QSF and 9.6% for the OVA peptide (Table 5). One week after the boost vaccination with 50 µg anti-CD40 antibody, 50 µg poly(I:C) and 200 µg of the respective peptides, the mice were sacrificed and the splenocytes were restimulated with a range of different concentrations of the peptides. Interestingly, we observed antigen-specific CD8$^+$ T-cell responses towards the OVA peptide in the OVA-vaccinated group upon restimulation. Flow cytometry revealed an increase from 0.2% to 10.9% in IFN-γ^+CD8$^+$ T-cells (Fig. 5A) and from 0.1% to 1.5% in TNF-α^+CD8$^+$ T-cells (Fig. 5B) upon restimulation with the lowest concentration (10 nM) of the peptide. However, no significant increases in either IFN-γ or TNF-α production were observed upon restimulation with the QSF peptide in the QSF-vaccinated group (Fig. 5A, B). So, whereas the liposomal formulation containing OVA peptide is effective in inducing CD8$^+$ T-cell-mediated antigen-specific responses in LDLr$^{-/-}$ mice, the QSF peptide in the same liposomes lacks immunogenicity in our model.

Table 5: Physicochemical properties of liposomal formulations containing the QSF peptide or OVA peptide. Data are averages ± SD of n = 3 measurements. Z_{ave} is the Z-average particle diameter, PDI is the polydispersity index.

	Z_{ave} (nm)	**PDI**	**Zeta potential** (mV)	**QSF loading efficiency (%)**
OVA-loaded	143.3 ± 4.6	0.137 ± 0.02	30.2 ± 1.2	9.6
QSF-loaded	145.9 ± 7.7	0.138 ± 0.003	29.4 ± 0.15	7.8

4. Discussion

Previous studies have identified T-cell responses and autoantibodies against (oxidized forms of) LDL and its main protein constituent, ApoB100, in both humans and animal

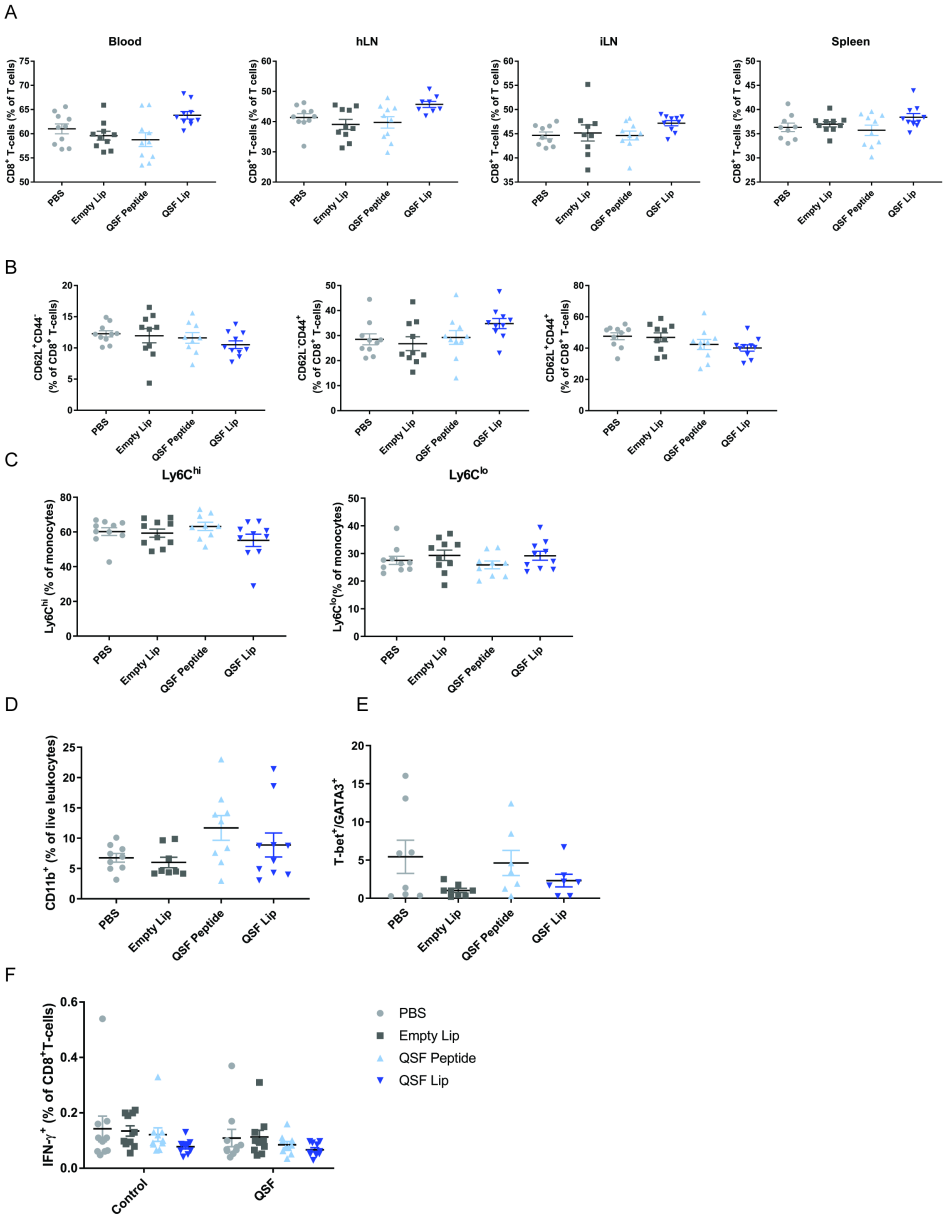

Figure 4: Flow cytometric analysis of CD8$^+$ T-cell responses in LDLr$^{-/-}$ mice on a WTD vaccinated with PBS, empty liposomes, QSF peptide or QSF liposomes. (A) Percentage of CD8$^+$ T-cells in the blood, hLN, iLN, and spleens of the LDLr$^{-/-}$ mice at the time of sacrifice. (B) Flow cytometric analysis of percentages of naïve CD44$^-$CD62L$^+$, TEM CD62L$^-$CD44$^+$, and TCM CD62L$^+$CD44$^+$ CD8$^+$ T-cells in the blood of the LDLr$^{-/-}$ mice at the time of sacrifice. (C) Percentages of Ly6C^{hi} and Ly6C^{lo} monocytes in the blood at the time of sacrifice. Monocytes were gated as viable, NK1.1$^-$Ly6G$^-$CD11b$^+$ cells. (D-E) Flow cytometry analysis of the percentage of CD11b$^+$ cells in the aorta (D) and the ratio between the percentages of aortic CD4$^+$ T-cells expressing T-bet and GATA3 at sacrifice (E). (F) CD8$^+$IFN-γ^+ T-cells in the splenocytes derived from the LDLr$^{-/-}$ mice at sacrifice, stimulated for 5 hours in the presence or absence of 10 µm QSFDLSVK peptide and Brefeldin A. Data points represent individual values, lines denote mean ± SEM, n = 10 per group, significance was determined by one-way or two-way ANOVA and Bonferroni's multiple comparisons tests, as appropriate. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

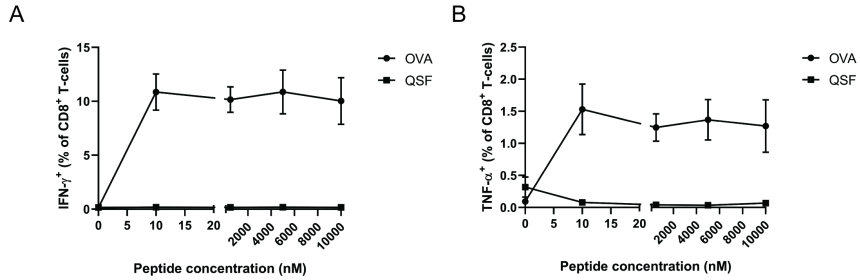

Figure 5: Liposomal vaccination against the QSFDLSVK peptide does not induce antigen-specific CD8$^+$ T-cell responses in LDLr$^{-/-}$ mice. Splenocytes were isolated from the mice that were vaccinated with either QSF or OVA peptide and 1×10^6 cells were restimulated with the indicated concentrations of the corresponding peptide in the presence of Brefeldin A for 5 hours. Subsequently, cells were stained and analyzed by flow cytometry. Percentages of CD8$^+$ T-cells that are positive for IFN-γ (A) and TNF-α (B) are plotted for both groups of mice. Mean ± SEM, n = 3 per group.

models of atherosclerosis [11–13, 15]. As protective roles for CD8$^+$ T-cells in atherosclerosis have been demonstrated previously [8, 17], we aimed to specifically boost antigen-specific CD8$^+$ T-cells, to induce an atheroprotective immune response that will result in cytolytic activity towards plaque-resident cells that present parts of this protein on their MHC-I molecules. However, we were unable to detect any antigen-specific CD8$^+$ T-cell responses using our liposomal vaccination strategy targeted towards the ApoB100-derived QSFDLSVK peptide. Of note, this peptide could be recognized by CD8$^+$ T-cells using a different vaccination strategy. As no differences were observed in immune responses in the peptide-liposome treated groups compared to the controls, it is not surprising that no differences were observed in atherosclerotic lesion size, plaque stability or plaque macrophage content at the end of the experiment.

There are several possible explanations as to why our vaccination was unsuccessful. Firstly, the choice of the antigen is obviously crucial for the efficacy of a vaccine. From our peptide predictions and DC vaccination study, we identified QSFDLSVK as a likely peptide candidate against which CD8$^+$ T-cells can mount a response. This is in agreement with previous publications, as vaccination with the ApoB100-derived peptide p210, which contains the QSF sequence, was previously shown to induce CD8$^+$ T-cell responses and reduce atherosclerosis development in apoE$^{-/-}$ mice [17]. Indeed, immunization of apoE$^{-/-}$ mice with p210 increased the number of CD8$^+$ T-cells that recognize the peptide sequence QSFDLSVK, which exerted increased cytolytic activity towards APCs [18]. Moreover, vaccination of mice against the ApoB100-derived peptides P2 and P45 induced CD8$^+$FoxP3$^+$ regulatory CD8$^+$ T-cells, which protect against atherosclerosis development via increased IL-10 production [33], confirming that CD8$^+$ T-cells are able to mount an immune response against ApoB100-derived peptides. We have successfully encapsulated the QSF peptide in liposomal particles consisting of DOPC and DOTAP, which were approximately 150 nm in size. Positively charged carriers of this size have been shown to be preferentially taken up by DCs and to elicit a significant CD8$^+$ T-cell response [25, 34–36]. In our LDLr$^{-/-}$ model, we observed no antigen-specific immunity towards the QSF peptide at the end of the

atherosclerosis experiment. However, we did observe antigen-specific $CD8^+$ T-cell responses in the DC vaccination experiment. This shows that there are $CD8^+$ T-cells that are able to react towards the QSF peptide in our mouse model. This discrepancy in immunological responses between the DC vaccination study and the liposomal vaccination study suggests that the failing of our vaccination strategy is likely not caused by the choice of a non-functional peptide epitope.

Secondly, the efficacy of a vaccine depends on the choice of the antigen delivery system. We observed that our liposomal delivery system is capable of inducing $CD8^+$ T-cell responses towards the model peptide SIINFEKL after two weeks, but vaccination with the QSF peptide in these liposomes did not result in any detectable antigen-specific $CD8^+$ T-cell responses even one-week after the boost injection. Thus, this indicates a problem with the liposomal delivery of the QSF peptide specifically, rather than a general incapability of peptide-loaded DOPC:DOTAP liposomes to induce a $CD8^+$ T-cell response.

In this study, we used a peptide consisting of 8 amino acids, the minimal length required to induce a $CD8^+$ T-cell response. For a DC vaccination strategy, the use of minimal peptides is favorable, as they can be loaded directly onto the MHC-I molecules without the need for processing. However, minimal peptides induce lower immune responses *in vivo* compared to longer peptides [37]. In this study, we opted for the shorter peptide in combination with an anti-CD40 antibody to bypass $CD4^+$ T-cell help [38]. However, this may have negatively affected the processing of the peptide and the ensuing immune responses. Indeed, previous work has shown that synthetic long peptides (SLPs), peptides which include flanking residues around the epitope of interest, offer superiority over the use of minimal peptides containing only the epitope recognized by the $CD8^+$ T-cell receptor [39, 40]. It has been suggested that a minimal peptide in the absence of a continuous danger signal, may induce T-cell tolerance towards this antigen [41]. However, as we delivered the peptide in a liposomal formulation, a danger signal should have been present, as the liposomes themselves act as immunostimulators [42]. APCs loaded with minimal peptides are less efficient in sustaining antigen presentation over longer periods of time compared to SLPs [43], which may have contributed to the negative results observed here. Alternatively, there could have been a problem in one of the steps of the cross-presentation process, by which the antigen gains access to the cytosol. Possibly, the acidity of the endosomes in which the liposomes are taken up may affect the stability of the QSF peptide more than that of the OVA peptide. Alternatively, the QSF peptide may have been more susceptible to degradation by enzymes present within the endosomes. For instance, cathepsins are known to preferentially cleave after small hydrophobic or basic amino acid residues [44], and could, therefore, cleave after the L and V residues. Cathepsin S may be able to cleave after the D and S residues [45]. Moreover, cathepsin B would be able to cleave off the terminal lysine residue by its exopeptidase activity [46]. Thus, the QSF peptide may have been degraded in the endosomes, preventing presentation upon MHC-I molecules. An alternative explanation could lie in the affinity of the peptides for binding to MHC-I. According to the IEDB database, SIINFEKL binds to MHC-I with an IC50 in the nanomolar range, whereas this is in the micromolar range for the QSF peptide. Thus, perhaps there is limited endosomal escape by both peptides, but as SIINFEKL binds stronger to the

MHC-I molecules, this peptide is still able to induce immunity, whereas the QSF peptide cannot. In the DC vaccination study, the peptide was freshly dissolved and loaded onto the DCs on the day of injection, thus endosomal processing played no role in this study.

Alternatively, the peptide stability may already have been negatively affected before the administration *in vivo*. The liposomal formulation process can take up to eight days between the dissolution of the peptide and the injection of the liposomes, during which the peptide may have become less stable. After the liposomal formulation process, the QSF peptide was successfully detected using UPLC, albeit at a low encapsulation efficiency. From this, it can be concluded that at least some of the QSF peptide is within the liposomes in the original form, although it cannot be excluded that some of the original peptide had undergone chemical modifications that were not detected by the UPLC method.

Interestingly, we observed a significant increase in weight in the QSF liposome treated mice compared to the PBS controls. The induction of immunity towards ApoB100 could be expected to reduce plasma cholesterol levels [47] and therefore reduce the weight of the mice. However, we found no differences in plasma cholesterol levels between the different treatment groups and as we did not observe any immunological effects demonstrating the effectiveness of our vaccine, it is unlikely that there would be effects on ApoB100 levels. Blast analysis revealed that the QSFDLSVK sequence is aligned for 87% to sequences encoding LPS binding protein (LBP) and dual oxidase maturation factor 2 (DUOXA2). LBP is expressed mainly in the liver and aids in the binding of bacterial lipopolysaccharide (LPS) to the CD14 receptor [48]. Increased levels of LBP and the resulting increased inflammatory responses have been linked to increased weight loss in cancer patients [49]. Therefore, cytotoxic activities against LBP-expressing cells could be a factor that affects weight gain. However, other studies report a role for LBP in adipogenesis and weight gain [50, 51]. Finally, a mutation in the DUOXA2 gene, expressed mainly in endocrine tissue and the liver, results in hypothyroidism [52], which is known to affect body weight. Thus, the differences in weight observed in this work could possibly be explained by off-target immunity of our vaccination directed towards the cells that present epitopes of the aforementioned proteins via MHC-I that share sequence homology with the peptide against which we vaccinated. Alternatively, our therapy may have exerted off-target pharmacological effects on other systems that we did not investigate, which could have caused the observed weight differences.

5. Conclusion

Collectively, our data show that vaccination against the ApoB100-derived peptide QSFDLSVK formulated in DOPC:DOTAP liposomes did not result in the induction of CD8$^+$ T-cell responses and did not affect atherosclerosis development in LDLr$^{-/-}$ mice. However, DC vaccination using the same peptide did induce antigen-specific CD8$^+$ T-cells, and vaccination with the same liposomes encapsulating SIINFEKL could induce OVA-specific CD8$^+$ T-cells. We suggest that our choice of peptide in combination with

this liposomal formulation is responsible for the lack of immune responses, probably due to problems occurring during one of the steps of the cross-presentation process. Further exploration into activating $CD8^+$ T-cells to induce a strong cytolytic response towards antigen-presenting cells in atherosclerosis using a different peptide antigen in the form of an SLP may be of great value for atherosclerosis research.

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References

[1] S. Barquera, A. Pedroza-Tobias, C. Medina, L. Hernandez-Barrera, K. Bibbins-Domingo, R. Lozano, and A. E. Moran, *Global overview of the epidemiology of atherosclerotic cardiovascular disease,* Arch Med Res **46**, 328 (2015).

[2] G. K. Hansson and A. Hermansson, *The immune system in atherosclerosis,* Nat Immunol **12**, 204 (2011).

[3] J. Armitage, C. Baigent, E. Barnes, D. J. Betteridge, L. Blackwell, M. Blazing, L. Bowman, E. Braunwald, R. Byington, C. Cannon, M. Clearfield, H. Colhoun, R. Collins, B. Dahlöf, K. Davies, B. Davis, J. de Lemos, J. R. Downs, P. Durrington, J. Emberson, B. Fellström, M. Flather, I. Ford, M. G. Franzosi, J. Fulcher, J. Fuller, C. Furberg, D. Gordon, S. Goto, A. Gotto, H. Halls, C. Harper, C. M. Hawkins, W. Herrington, G. Hitman, H. Holdaas, L. Holland, A. Jardine, J. W. Jukema, J. Kastelein, S. Kean, A. Keech, A. Kirby, J. Kjekshus, G. Knatterud, R. Knopp, W. Koenig, M. Koren, V. Krane, M. J. Landray, J. LaRosa, E. Lonn, P. MacFarlane, S. MacMahon, A. Maggioni, R. Marchioli, I. Marschner, B. Mihaylova, L. Moyé, S. Murphy, H. Nakamura, A. Neil, C. Newman, R. O'Connell, C. Packard, S. Parish, T. Pedersen, R. Peto, M. Pfeffer, N. Poulter, D. Preiss, C. Reith, P. Ridker, M. Robertson, F. Sacks, N. Sattar, R. Schmieder, P. Serruys, P. Sever, J. Shaw, C. Shear, J. Simes, P. Sleight, E. Spata, L. Tavazzi, J. Tobert, G. Tognoni, A. Tonkin, S. Trompet, J. Varigos, C. Wanner, H. Wedel, H. White, J. Wikstrand, L. Wilhelmsen, K. Wilson, R. Young, S. Yusuf, and F. Zannad, *Efficacy and safety of statin therapy in older people: a meta-analysis of individual participant data from 28 randomised controlled trials,* The Lancet **393**, 407 (2019).

[4] P. M. Ridker, B. M. Everett, T. Thuren, J. G. MacFadyen, W. H. Chang, C. Ballantyne, F. Fonseca, J. Nicolau, W. Koenig, S. D. Anker, J. J. Kastelein, J. H. Cornel, P. Pais, D. Pella, J. Genest, R. Cifkova, A. Lorenzatti, T. Forster, Z. Kobalava, L. Vida-Simiti, M. Flather, H. Shimokawa, H. Ogawa, M. Dellborg, P. R. Rossi, R. P. Troquay, P. Libby, and R. J. Glynn, *Antiinflammatory therapy with canakinumab for atherosclerotic disease,* New England Journal of Medicine **377**, 1119 (2017).

[5] R. A. van Dijk, A. J. F. Duinisveld, A. F. Schaapherder, A. Mulder-Stapel, J. F. Hamming, J. Kuiper, O. J. de Boer, A. C. van der Wal, F. D. Kolodgie, R. Virmani, and J. H. N. Lindeman, *A change in inflammatory footprint precedes plaque instability: A systematic evaluation of cellular aspects of the adaptive immune response in human atherosclerosis,* Journal of the American Heart Association: Cardiovascular and Cerebrovascular Disease **4**, e001403 (2015).

[6] J. Gewaltig, M. Kummer, C. Koella, G. Cathomas, and B. C. Biedermann, *Requirements for CD8 T-cell migration into the human arterial wall,* Hum Pathol **39**, 1756 (2008).

[7] J. van Duijn, J. Kuiper, and B. Slutter, *The many faces of $CD8^+$ T cells in atherosclerosis,* Curr Opin Lipidol **29**, 411 (2018).

[8] J. van Duijn, E. Kritikou, N. Benne, T. van der Heijden, G. H. van Puijvelde, M. J. Kroner, F. H. Schaftenaar, A. C. Foks, A. Wezel, H. Smeets, H. Yagita, I. Bot, W. Jiskoot, J. Kuiper, and B. Slutter, *CD8+ T-cells contribute to lesion stabilization in advanced atherosclerosis by limiting macrophage content and CD4 T-cell responses,* Cardiovasc Res **115**, 729 (2019).

[9] J. van Duijn, M. van Elsas, N. Benne, M. Depuydt, A. Wezel, H. Smeets, I. Bot, W. Jiskoot, J. Kuiper, and B. Slütter, *CD39 identifies a microenvironment-specific anti-inflammatory CD8$^+$ T-cell population in atherosclerotic lesions,* Atherosclerosis **285**, 71 (2019).

[10] E. Ammirati, F. Moroni, M. Magnoni, and P. G. Camici, *The role of T and B cells in human atherosclerosis and atherothrombosis,* Clinical & Experimental Immunology **179**, 173 (2015).

[11] G. N. Fredrikson, G. Berglund, R. Alm, J.-A. k. Nilsson, P. K. Shah, and J. Nilsson, *Identification of autoantibodies in human plasma recognizing an ApoB-100 LDL receptor binding site peptide,* Journal of Lipid Research **47**, 2049 (2006).

[12] B. Fagerberg, U. Prahl Gullberg, R. Alm, J. Nilsson, and G. N. Fredrikson, *Circulating autoantibodies against the apolipoprotein B-100 peptides p45 and p210 in relation to the occurrence of carotid plaques in 64-year-old women,* PloS one **10**, e0120744 (2015).

[13] W. Palinski, K. Tangirala Rajendra, E. Miller, G. Young Stephen, and L. Witztum Joseph, *Increased autoantibody titers against epitopes of oxidized LDL in LDL receptor–deficient mice with increased atherosclerosis,* Arteriosclerosis, Thrombosis, and Vascular Biology **15**, 1569 (1995).

[14] J. T. Salonen, H. Korpela, R. Salonen, K. Nyyssonen, S. Yla-Herttuala, R. Yamamoto, S. Butler, W. Palinski, and J. L. Witztum, *Autoantibody against oxidised LDL and progression of carotid atherosclerosis,* The Lancet **339**, 883 (1992).

[15] S. Stemme, B. Faber, J. Holm, O. Wiklund, J. L. Witztum, and G. K. Hansson, *T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein,* Proceedings of the National Academy of Sciences of the United States of America **92**, 3893 (1995).

[16] A. Hermansson, D. F. Ketelhuth, D. Strodthoff, M. Wurm, E. M. Hansson, A. Nicoletti, G. Paulsson-Berne, and G. K. Hansson, *Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis,* The Journal of Experimental Medicine **207**, 1081 (2010).

[17] K.-Y. Chyu, X. Zhao, P. C. Dimayuga, J. Zhou, X. Li, J. Yano, W. M. Lio, L. F. Chan, J. Kirzner, P. Trinidad, B. Cercek, and P. K. Shah, *CD8$^+$ T cells mediate the atheroprotective effect of immunization with an ApoB-100 peptide,* PLoS ONE **7**, e30780 (2012).

[18] P. C. Dimayuga, X. Zhao, J. Yano, W. M. Lio, J. Zhou, P. M. Mihailovic, B. Cercek, P. K. Shah, and K. Y. Chyu, *Identification of ApoB-100 peptide-specific CD8$^+$ T cells in atherosclerosis,* J Am Heart Assoc **6**, e005318 (2017).

[19] H. Hogenesch, *Mechanism of immunopotentiation and safety of aluminum adjuvants,* Front Immunol **3**, 406 (2012).

[20] M. J. Copland, M. A. Baird, T. Rades, J. L. McKenzie, B. Becker, F. Reck, P. C. Tyler, and N. M. Davies, *Liposomal delivery of antigen to human dendritic cells,* Vaccine **21**, 883 (2003).

[21] N. Benne, J. van Duijn, J. Kuiper, W. Jiskoot, and B. Slutter, *Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines,* J Control Release **234**, 124 (2016).

[22] M. Maji, S. Mazumder, S. Bhattacharya, S. T. Choudhury, A. Sabur, M. Shadab, P. Bhattacharya, and N. Ali, *A lipid based antigen delivery system efficiently facilitates MHC class-i antigen presentation in dendritic cells to stimulate CD8(+) T cells,* Scientific reports **6**, 27206 (2016).

[23] A. K. Varkouhi, M. Scholte, G. Storm, and H. J. Haisma, *Endosomal escape pathways for delivery of biologicals,* Journal of Controlled Release **151**, 220 (2011).

[24] E. M. Varypataki, N. Benne, J. Bouwstra, W. Jiskoot, and F. Ossendorp, *Efficient eradication of established tumors in mice with cationic liposome-based synthetic long-peptide vaccines,* Cancer Immunol Res **5**, 222 (2017).

[25] J. Heuts, E. M. Varypataki, K. van der Maaden, S. Romeijn, J. W. Drijfhout, A. T. van Scheltinga, F. Ossendorp, and W. Jiskoot, *Cationic liposomes: A flexible vaccine delivery system for physicochemically diverse antigenic peptides,* Pharmaceutical Research **35**, 207 (2018).

[26] K. Chen, Z. Wu, M. Zang, C. Wang, Y. Wang, D. Wang, Y. Ma, and C. Qu, *Immunization with glypican-3 nanovaccine containing TLR7 agonist prevents the development of carcinogen-induced precancerous hepatic lesions to cancer in a murine model,* American journal of translational research **10**, 1736 (2018).

[27] J. Hamzah, J. G. Altin, T. Herringson, C. R. Parish, G. J. Hämmerling, H. Donoghue, and R. Ganss, *Targeted liposomal delivery of TLR9 ligands activates spontaneous antitumor immunity in an autochthonous cancer model,* The Journal of Immunology **183**, 1091 (2009).

[28] E. M. Varypataki, K. van der Maaden, J. Bouwstra, F. Ossendorp, and W. Jiskoot, *Cationic liposomes loaded with a synthetic long peptide and poly(I:C): a defined adjuvanted vaccine for induction of antigen-specific T cell cytotoxicity,* The AAPS journal **17**, 216 (2014).

[29] R. Vita, S. Mahajan, J. A. Overton, S. K. Dhanda, S. Martini, J. R. Cantrell, D. K. Wheeler, A. Sette, and B. Peters, *The immune epitope database (IEDB): 2018 update,* Nucleic Acids Res **47**, D339 (2019).

[30] M. Nielsen, C. Lundegaard, P. Worning, S. L. Lauemoller, K. Lamberth, S. Buus, S. Brunak, and O. Lund, *Reliable prediction of T-cell epitopes using neural networks with novel sequence representations,* Protein Sci **12**, 1007 (2003).

[31] J. Sidney, E. Assarsson, C. Moore, S. Ngo, C. Pinilla, A. Sette, and B. Peters, *Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries,* Immunome Res **4**, 2 (2008).

[32] H. Horig, A. C. Young, N. J. Papadopoulos, T. P. DiLorenzo, and S. G. Nathenson, *Binding of longer peptides to the H-2Kb heterodimer is restricted to peptides extended at their C terminus: refinement of the inherent MHC class I peptide binding criteria,* J Immunol **163**, 4434 (1999).

[33] C. Pierides, A. Bermudez-Fajardo, G. N. Fredrikson, J. Nilsson, and E. Oviedo-Orta, *Immune responses elicited by ApoB-100-derived peptides in mice,* Immunol Res **56**, 96 (2013).

[34] C. Oussoren, J. Zuidema, D. J. Crommelin, and G. Storm, *Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. influence of liposomal size, lipid compostion and lipid dose,* Biochim Biophys Acta **1328**, 261 (1997).

[35] T. Nakanishi, J. Kunisawa, A. Hayashi, Y. Tsutsumi, K. Kubo, S. Nakagawa, M. Nakanishi, K. Tanaka, and T. Mayumi, *Positively charged liposome functions as an efficient immunoadjuvant in inducing cell-mediated immune response to soluble proteins,* J Control Release **61**, 233 (1999).

[36] Y. Ma, Y. Zhuang, X. Xie, C. Wang, F. Wang, D. Zhou, J. Zeng, and L. Cai, *The role of surface charge density in cationic liposome-promoted dendritic cell maturation and vaccine-induced immune responses,* Nanoscale **3**, 2307 (2011).

[37] E. D. Quakkelaar and C. J. Melief, *Experience with synthetic vaccines for cancer and persistent virus infections in nonhuman primates and patients,* Adv Immunol **114**, 77 (2012).

[38] R. R. French, H. T. Chan, A. L. Tutt, and M. J. Glennie, *CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help,* Nat Med **5**, 548 (1999).

[39] M. S. Bijker, S. J. van den Eeden, K. L. Franken, C. J. Melief, R. Offringa, and S. H. van der Burg, *CD8+ CTL priming by exact peptide epitopes in incomplete freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity,* J Immunol **179**, 5033 (2007).

[40] S. K. Maynard, J. D. Marshall, R. S. MacGill, L. Yu, J. A. Cann, L. I. Cheng, M. P. McCarthy, C. Cayatte, and S. H. Robbins, *Vaccination with synthetic long peptide formulated with CpG in an oil-in-water emulsion induces robust E7-specific CD8 T cell responses and TC-1 tumor eradication,* BMC Cancer **19**, 540 (2019).

[41] R. E. Toes, R. Offringa, R. J. Blom, C. J. Melief, and W. M. Kast, *Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction,* Proc Natl Acad Sci U S A **93**, 7855 (1996).

[42] S. Tandrup Schmidt, C. Foged, K. S. Korsholm, T. Rades, and D. Christensen, *Liposome-based adjuvants for subunit vaccines: Formulation strategies for subunit antigens and immunostimulators,* Pharmaceutics **8** (2016), 10.3390/pharmaceutics8010007.

[43] F. Faure, A. Mantegazza, C. Sadaka, C. Sedlik, F. Jotereau, and S. Amigorena, *Long-lasting cross-presentation of tumor antigen in human DC,* Eur J Immunol **39**, 380 (2009).

[44] V. Turk, V. Stoka, O. Vasiljeva, M. Renko, T. Sun, B. Turk, and D. Turk, *Cysteine cathepsins: from structure, function and regulation to new frontiers,* Biochim Biophys Acta **1824**, 68 (2012).

[45] T. Ruckrich, J. Brandenburg, A. Cansier, M. Muller, S. Stevanovic, K. Schilling, B. Wiederanders, A. Beck, A. Melms, M. Reich, C. Driessen, and H. Kalbacher, *Specificity of human cathepsin s determined by processing of peptide substrates and MHC class II-associated invariant chain,* Biol Chem **387**, 1503 (2006).

[46] D. S. Chu, R. N. Johnson, and S. H. Pun, *Cathepsin b-sensitive polymers for compartment-specific degradation and nucleic acid release,* J Control Release **157**, 445 (2012).

[47] A. Gistera, M. L. Klement, K. A. Polyzos, R. K. W. Mailer, A. Duhlin, M. C. I. Karlsson, D. F. J. Ketelhuth, and G. K. Hansson, *Low-density lipoprotein-reactive T cells regulate plasma cholesterol levels and development of atherosclerosis in humanized hypercholesterolemic mice,* Circulation **138**, 2513 (2018).

[48] E. Hailman, H. S. Lichenstein, M. M. Wurfel, D. S. Miller, D. A. Johnson, M. Kelley, L. A. Busse, M. M. Zukowski, and S. D. Wright, *Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14,* The Journal of Experimental Medicine **179**, 269 (1994).

[49] A. J. Staal-van den Brekel, M. A. Dentener, A. M. Schols, W. A. Buurman, and E. F. Wouters, *Increased resting energy expenditure and weight loss are related to a systemic inflammatory response in lung cancer patients,* Journal of Clinical Oncology **13**, 2600 (1995).

[50] F. Laugerette, M. Alligier, J.-P. Bastard, J. Drai, E. Chanséaume, S. Lambert-Porcheron, M. Laville, B. Morio, H. Vidal, and M.-C. Michalski, *Overfeeding increases postprandial endotoxemia in men: Inflammatory outcome may depend on LPS transporters LBP and sCD14,* Molecular Nutrition & Food Research **58**, 1513 (2014).

[51] J. M. Moreno-Navarrete, X. Escoté, F. Ortega, M. Serino, M. Campbell, M.-C. Michalski, M. Laville, G. Xifra, E. Luche, P. Domingo, M. Sabater, G. Pardo, A. Waget, J. Salvador, M. Giralt, J. I. Rodriguez-Hermosa, M. Camps, C. I. Kolditz, N. Viguerie, J. Galitzky, P. Decaunes, W. Ricart, G. Frühbeck, F. Villarroya, G. Mingrone, D. Langin, A. Zorzano, H. Vidal, J. Vendrell, R. Burcelin, A. Vidal-Puig, and J. M. Fernández-Real, *A role for adipocyte-derived lipopolysaccharide-binding protein in*

inflammation- and obesity-associated adipose tissue dysfunction, Diabetologia **56**, 2524 (2013).

[52] H. Grasberger, X. De Deken, O. B. Mayo, H. Raad, M. Weiss, X.-H. Liao, and S. Refetoff, *Mice deficient in dual oxidase maturation factors are severely hypothyroid,* Molecular endocrinology (Baltimore, Md.) **26**, 481 (2012).