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Liposome-based vaccines for immune modulation: from antigen selection to nanoparticle design

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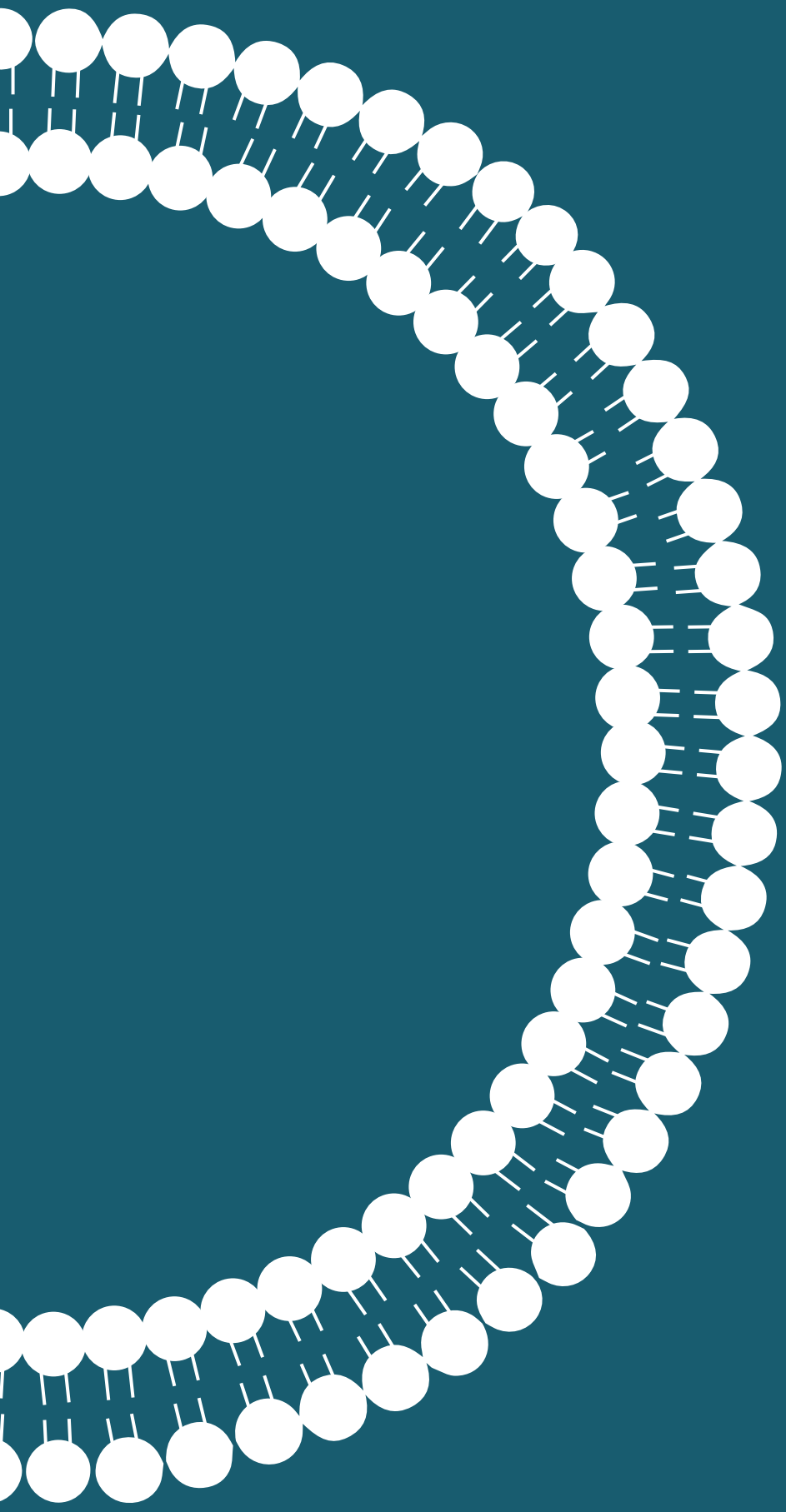

Chapter 3

Immunopeptidomics analysis of human atherosclerosis plaques identifies antigenic drivers of atherosclerosis

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ABSTRACT

Atherosclerosis has an auto-immune component driven by self-reactive T and B cells. Identifying their antigenic drivers may lead to new diagnosis and treatment approaches. Here, we aim to identify immunogenic T cell epitopes derived from atherosclerosis-relevant proteins such as ApoB100 by studying the repertoire of peptides presented by HLA in human plaques.

We used immunopeptidomics to identify peptides presented by HLA-DR molecules in 51 plaques from patients that underwent endarterectomy surgery. We selected a set of 20 peptides derived from ApoB100 and studied the presence and cytokine profile of ApoB100-specific $CD4^+$ T cells in peripheral blood mononuclear cells (PBMCs) from atherosclerosis patients. Results revealed significant $CD4^+$ T cell activation in response to these ApoB100 peptides in 22.4% of the patients, and this T cell response correlated positively with plaque vulnerability. Furthermore, the cytokine profile of these cells was characterized by production of IL-10 and IL-17A but no IFN-γ.

Keywords

immunopeptidomics, PBMCs, antigen-specific T cells, ApoB100

INTRODUCTION

Atherosclerosis is an inflammatory disease of the arteries, and it is the main underlying cause of cardiovascular disease (CVD). Initially thought to be primarily a disease related to lipid metabolism, growing evidence has highlighted the important role of the immune system in the development of the disease.

The development of monoclonal antibody technology and immunohistochemistry allowed to study the cellular composition of atherosclerotic plaques. Initial studies showed the presence of activated $CD8^+$ and $CD4^+$ T lymphocytes and their distribution in human plaques, with about 20% of cells present in the shoulder region and the fibrous cap being T cells [1, 2]. More recently, the development of single cell sequencing technologies has allowed to identify the presence of clonally expanded T cells in atherosclerotic plaques, indicating an antigen-specific T cell response and an auto-immune component to this disease[3]. The interaction between the T cell receptor (TCR) and its cognate human leukocyte antigen (HLA)/peptide complex triggers the activation and proliferation of T cells, giving rise to a clonally expanded population of T cells with the same TCR. Furthermore, our lab has shown the increased expression of CD69 in $CD4^+$ T cells in the plaque compared to circulation. This suggests recent antigen-specific T cell activation in the plaque and confirms the importance of antigen-specific $CD4^+$ T cell immunity in atherosclerosis[4].

Elucidating the antigen specificity of these interactions is important for the development of novel biomarkers for disease progression and novel therapeutic approaches such as therapeutic vaccines[5]. Previous work has shown that T cells in the plaque can proliferate and produce IFN-γ in response to native and oxidized LDL[6]. These data point towards the potential role of ApoB100, which is the main protein in LDL particles, as a driver of autoimmunity in atherosclerosis. Indeed, ApoB100 epitopes have been discovered using *in silico* approaches and ApoB100-specific $CD4^+$ T cells have been detected in patients and associated with severity of CVD[7]. Here we propose a direct approach to identify ApoB100 epitopes that may drive $CD4^+$ T cell expansion in the lesion, by performing immunopeptidomics on human atherosclerotic plaques derived from patients that underwent endarterectomy surgery. Using this approach, we established a peptide pool that allows detection of ApoB100-specific $CD4^+$ T cells in PBMCs from non-HLA typed patients. Importantly, we show that the extent of the antigen-specific T cell responses associates with stability of atherosclerotic plaques.

MATERIALS & METHODS

Materials

Peptides used for proliferation experiments were synthesized in-house by solid-phase peptide synthesis (SPPS). Peptides used for CD40L expression experiments were custom-made by Genscript (Rijswijk, Netherlands). RPMI 1640 culture medium was obtained from Lonza (Basel, Switzerland). Fetal Bovine Serum (FBS) was purchased from Sigma-Aldrich (Zwijndrecht, Netherlands), penicillin/streptomycin from Fisher Scientific (Landsmeer, Netherlands) and L-glutamine was purchased from VWR (Amsterdam, Netherlands). CellTrace™ CFSE cell proliferation kit was obtained from ThermoFisher Scientific (MA, USA), concanavalinA was purchased from Invivogen (Toulouse, France), tetanus toxoid (*Clostridium tetani*) and *Staphylococcus* enterotoxin A (*Staphylococcus aureus*) were obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). Recombinant human IL-2 was purchased from Roche (Mannheim, Germany).

Fluorescently labelled antibodies for flow cytometry antiCD3-PE (OK3), antiCD8-BrilliantViolet510 (SK1) and antiCD40L-APC (24-31) were purchased from Biolegend (CA, USA). AntiCD4-eFluor450 (OKT4) and Fixable Viability Dye APC-eFluor780 were obtained from eBioscience (ThermoFisher Scientific, MA, USA). AntiCD40 anti-human blocking antibody (HB14) was purchased from Miltenyi Biotec (Leiden, Netherlands).

LEGENDplex™ HU Th Cytokine Panel (12-plex) kit for quantification of IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, IFN-γ and TNF-α was purchased from Biolegend (CA, USA).

Methods

Patient population

For immunopeptidomics, human carotid and femoral atherosclerotic plaques were obtained from 51 anonymous patients that underwent primary endarterectomy surgery at the Haaglanden Medical Center, Westeinde, The Hague, The Netherlands (Cohort 1). Samples were handled in compliance with the “Code for Proper Secondary Use of Human Tissue”.

For the T cell proliferation assay, whole blood was collected from 17 patients that underwent carotid endarterectomy surgery at the Haaglanden Medical Center, Westeinde, The Hague, The Netherlands (Study approval number: 17-046, protocol number NL57482.098.17) (Cohort 2). For the CD40L expression experiment, whole blood and atherosclerotic plaques were obtained from 58 patients that underwent carotid endarterectomy surgery at the Haaglanden Medical Center, Westeinde, The Hague, The Netherlands (Study approval number: Z19.075, protocol number

NL71516.058.19). Both studies, all patients provide informed consent and were approved by the Medical Ethics Committee of the HMC, conforming to the principles outlined in the Declaration of Helsinki. All blood samples were collected by venipuncture prior to surgery. Atherosclerotic plaque specimens were obtained from primary endarterectomy surgeries, and restenotic plaques were excluded due to their different plaque composition as compared to primary atherosclerotic plaques[8]. Informed consent was obtained from all patients involved in cohort 2 and 3. Healthy volunteers were recruited from the Leiden Academic Center for Drug Research (Leiden, The Netherlands) and Sanquin Research (Amsterdam, The Netherlands) and provided informed consent.

Histology assessment

The culprit segment (5mm) of the atherosclerotic plaque (Cohort 3) was fixed in Shandon Zinc Formal-Fixx (Dilution 1:5; ThermoFisher) for 24h and subsequently stored in 70% Ethanol until further use. Plaque samples were graded in a semiquantitative scale as previously described[9]. Briefly, plaque samples were embedded in paraffin and subsequently sectioned in 5μm thick sections using a microtome. Sections were stained with Movat's pentachrome staining and three sections of each plaque were analysed for plaque features according to the semiquantitative scoring systems of AtheroExpress biobank[10] and the Oxford Plaque Study[11].

The plaque vulnerability score of each patient was calculated as the average of the scores for necrotic core, calcification, foam cell content, cholesterol crystals, neovascularization and inflammatory cell content.

All patients underwent a CT-scan with arterial contrast pre-operatively. The degree of stenosis of the carotid arteries was measured using the formula of the North American Symptomatic Carotid Endarterectomy Trial (NASCET):

$$\%\ internal\ carotid\ artery\ (ICA)\ stenosis = \left(1 - \frac{Narrowest\ ICA\ diameter}{Diameter\ normal\ distal\ cervical\ ICA}\right) \times 100$$

Isolation of HLA-peptide complexes by affinity chromatography

For the pilot feasibility study, a single plaque sample from one patient was used (Cohort 1). For the extended study plaque samples from 50 patients (Cohort 1) were pooled together and processed for immunopeptidomic analysis.

Carotid or femoral plaques were cut into small pieces and a total of 2.4 g of plaque material was processed further. Lysis buffer was added (50 mM Tris-Cl pH 8.0, 150

mM NaCl, 5 mM EDTA, 0.5% Zwittergent 3-12 (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate) and protease inhibitor (Complete, Roche Applied Science)) and the solution was probe sonicated on ice, followed by shaking on ice for 2 hours. The lysate was centrifuged for 10 min at 2500 × g and for 45 min at 31,000 x g to remove nuclei and other insoluble material, respectively. Next, lysates were passed through a 0.5 ml CL-4B Sepharose column to preclear the lysate. The cleared lysate was passed through a 0.2 ml column containing 2.5 mg anti-HLA-DR (B8.11.2) IgG coupled to protein A Sepharose[12]. The antibody columns were washed with 1 ml of lysis buffer, 1 ml of low salt buffer (20 mM Tris-Cl pH 8.0, 120 mM NaCl), 1 ml of high salt buffer (20 mM Tris-Cl pH 8.0, 1 M NaCl), and finally with 1 ml of low salt buffer. Peptides were eluted with 1 ml of 10% acetic acid and purified on a 10kDa filter (Microcon, Millipore). The filtrate was diluted with 2 ml of 0.1% TFA and purified by SPE (Oasis HLB, Waters) using 20% and 30% acetonitrile in 0.1% trifluoroacetic acid (TFA) to elute the peptides.

Liquid chromatography-Mass spectrometry

Lyophilized peptides were dissolved in 95/3/0.1 (v/v/v) water/acetonitrile/formic acid (FA) and analysed in an online C18 nanoHPLC MS/MS consisting of an Ultimate3000nano gradient HPLC system (Thermo, Bremen, Germany), and an Exploris480 Mass Spectrometer (Thermo). Fractions were injected onto a cartridge precolumn (300 µm × 5 mm, C18 PepMap, 5 μm, 100 Å), and eluted using a homemade analytical nano-HPLC column (50 cm × 75 µm; Reprosil-Pur C18-AQ 1.9 μm, 120 Å) (Dr. Maisch, Ammerbuch, Germany). The gradient was run for 120 minutes from 2% to 40% solvent B (20/80/0.1 water/acetonitrile/FA v/v/v). The nano-HPLC column was drawn to a tip of □5 µm that acted as the electrospray needle of the MS source. The mass spectrometer was operated in data-dependent MS/MS mode for a cycle of 20 MS/MS scans, with a HCD collision energy at 30 V and recording of the MS2 spectrum in the orbitrap, with a quadrupole isolation width of 1.2 Da. A resolution of 60,000 was used in the master scan (MS1), the scan range used was 300-1500, at standard AGC target at a maximum fill time of 50 ms. The lock mass correction on the background ion used was m/z = 445.12. Precursors were dynamically excluded after n=1 with an exclusion duration of 45 s, and with a precursor range of 20 ppm. Charge states 1-3 were included. Singly charged precursors were selected from the m/z in the range 800-1400, doubly charged precursors were selected from the m/z in the range 400-1000, and triply charged precursors were selected from the m/z in the range 300-900. For MS2 the first mass was set to 110 Da, with an MS2 scan resolution of 30,000 at an AGC target of 100% with a maximum fill time 'auto'.

For post-analysis, raw data were converted to peak lists using Proteome Discoverer version 2.1 (Thermo Electron), and subsequently submitted to the Uniprot *Homo sapiens* minimal database (20596 entries), using Mascot v2.2.07 (www.matrixscience.com) for peptide identification. Mascot searches were done with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and no enzyme was specified. Methionine oxidation and cysteinylation of cysteine were set as variable modifications. The false discovery rate was set < 1% and peptides with mascot ion scores < 35 were discarded.

The candidate peptides were synthesised, and the MS spectra of the synthetic peptides were compared to the MS spectra of their counterpart eluted from the plaque samples in order to confirm their identity.

Peptide synthesis

Peptides of the ApoB100 peptide pool 7 (PP7) used for the feasibility pilot study were synthesized in-house by Fmoc solid-phase peptide synthesis using the microwave-assisted automated peptide synthesizer Liberty Blue (CEM). Peptide synthesis was performed at 0.1mmol scale using a Tentagel S-RAM resin. After synthesis, peptides were acetylated in the C-terminus and cleaved from the resin using 95% TFA, 2.5% triisopropylsilane (TIPS) and 2.5% water. The resulting peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) using a Kinetic Evo C18 column. Peptides were detected by absorbance at 220nm using an SPD-10AVP UV/Vis detector. Fractions were collected and analysed by liquid chromatography-mass spectrometry (LC-MS) to confirm peptide purity and identity. Purified peptides were freeze-dried and stored at -20°C until use. Peptides of the ApoB100 PP20 were synthesized by Genscript.

In silico prediction of peptide binding affinity for HLA-DR isotypes

The immune epitope database (IEDB) was used to predict the binding affinity of ApoB100 peptides identified in the immunopeptidomic analysis. The prediction was performed using the consensus approach that combines NN-align, SMM-align, CombLib and Sturniolo. If no predictor was available for a particular MHC class II molecule, NetMHCIIpan was used. Prediction was run for all 14 HLA-DR alleles available in IEDB.

T cell proliferation experiments

To assess T cell proliferation in response to the ApoB100 peptide pool, we used cryopreserved white blood cell (WBC) samples from patients that underwent carotid endarterectomy surgery (Cohort 2). Whole blood obtained from patients was lysed twice with ACK lysis buffer and cryopreserved as previously described[4]. For the assay, cryopreserved samples were defrosted, counted, and incubated overnight at

37°C and 5% CO_2 at a cell density of $1x10^6$ cells/ml. Next, cells were labelled with 0.5μM CFSE and again incubated overnight at 37°C and 5% CO_2. For stimulations, 200,000 cells per well were seeded in U-bottom 96-well plate and stimulated with either ApoB100 peptides, ConcanavalinA (ConA) or complete RPMI-1640 medium (RPMI-1640 supplemented with 10% (v/v) HI-FCS, 2mM L-glutamine, 100U/mL penicillin/streptomycin, 20μM β-mercaptoethanol). Human IL-2 was added to all conditions at a concentration of 20U/mL. Cells were stimulated for 5 days followed by 5 days of culture for a total of 10 days. Cell culture medium was refreshed every 2 days. On day 10, cells were labelled with anti-CD3, anti-CD4, anti-CD8 antibodies and the fixable viability dye eFluor780. Cell proliferation was assessed by the dilution of the CFSE label using flow cytometry in a Cytoflex S.

CD40L expression experiment

PBMCs were isolated from whole blood samples (Cohort 3) and cryopreserved until further use as previously described[4]. PBMCs were thawed, counted, and seeded in U-bottom 96-well plates at 500,000 cells per well. PBMCs were stimulated with ApoB100 peptide pool (5ug/mL each peptide), 2 ng/mL *Staphylococcus* enterotoxin B (SEB) or complete RPMI-1640 medium control. The peptide pool PepMix™ Human actine (PM-ACTS, JPT Peptide Technologies GmbH, Berlin, Germany) was also used as negative control at 5ug/mL of each peptide in the pool. Anti-CD40 blocking antibody was included in all conditions at 1μg/mL. PBMCs were incubated with stimulants for 18h and subsequently stained for flow cytometry with anti-CD3, anti-CD4, anti-CD8, anti-CD40L antibodies and fixable viability dye. Samples were measured using the Cytoflex S flow cytometer. For each patient, the stimulation index was calculated as:

$$\text{Stimulation index} = \frac{\%\ \text{CD4}^+\text{CD40L}^+\ \text{T cells in peptide pool stimulation condition}}{\%\ \text{CD4}^+\text{CD40L}^+\ \text{T cells in medium control}}$$

Cytokine profile by multiplex ELISA

To determine the cytokine profile of stimulated PBMCs (Cohort 3), 500,000 cells per well were seeded in U-bottom 96-well plate and stimulated for 3 days with ApoB100 peptide pool (5 μg/mL each peptide) or complete RPMI-1640 medium as control. During stimulation cells were incubated at 37°C and 5% CO_2. The level of IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, IFN-γ and TNF-α in the conditioned medium was measured using bead-based multiplex ELISA LegendPlex (Biolegend, USA). Assay was performed according to manufacturer´s instructions. Samples were measured using the Cytoflex S flow cytometer and data was analysed using LegendPlex software.

Statistics

Normal distribution of the data was assessed using Anderson-Darling test, D'Agostino & Pearson test, Shapiro-Wilk test and Kolmogorov-Smirnov test. Statistical difference between peptide-stimulated and unstimulated samples was analysed using two-tailed Wilcoxon matched-pairs signed rank test. Statistical correlation between variables was analysed using Pearson's correlation test. P-values lower than 0.05 were considered significant. Analyses were performed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software, California, USA).

RESULTS

ApoB100-derived peptides eluted from one plaque induced CD4+ T cell proliferation in patients WBC

Immunopeptidomic aims to identify the set of peptides presented by HLA molecules in a given sample. To assess the feasibility of this technique for the identification of immunogenic epitopes from ApoB100, we studied the immunopeptidome of a single plaque sample from one patient. Briefly, the plaque sample was processed to isolate HLA/peptide complexes and peptides were subsequently eluted from the HLA molecules and identified by LC-MS/MS (Figure 1). The analysis identified 7 peptides derived from ApoB100 presented by HLA-DR molecules. An *in silico* analysis of the predicted binding affinity of these peptides for the most common HLA-DR alleles showed medium/high affinity (IC_{50} < 1000nM) for a wide range of alleles (Supplementary Figure 1).

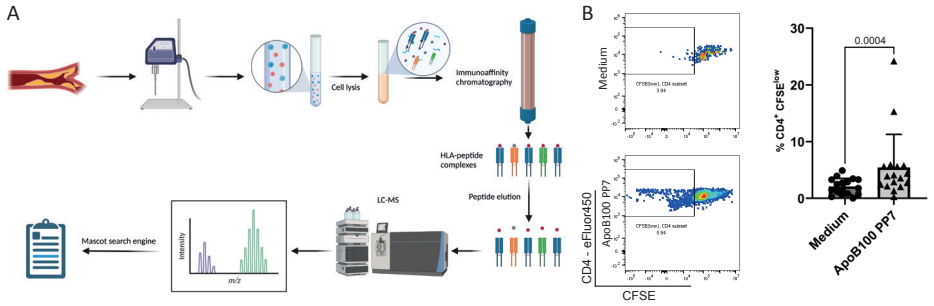

Figure 1. Immunopeptidomics experimental approach and feasibility study. (A) Immunopeptidomic workflow from a cryopreserved atherosclerosis plaque sample. Endarterectomy plaques were cut in small pieces and sonicated in lysis buffer. Subsequently, the HLA-peptide complexes were isolated by immunoaffinity chromatography. Peptides were eluted from the HLA molecules and LC-MS was used to separate and identify the peptides in the sample. Finally, the MS spectra of each potential peptide were assigned to a peptide sequence by searching the human proteome database with the Mascot™ engine. (B) Representative flow cytometry plots of proliferating $CD4^+$ T cells from lysed blood in response to stimulation with preliminary ApoB100 peptide pool PP7 or medium control and dot plot (n=17) of the percentage of proliferating $CD4^+$ T cells in each condition. P-value determined using two-tailed Wilcoxon matched-pairs signed rank test.

These 7 peptides were used to stimulate WBC samples from CVD patients. In order to have a better chance to detect a potentially rare $CD4^+$ T cell population, we used a proliferation-based assay to expand the antigen-specific population. CFSE-labelled WBC samples were stimulated with this ApoB100 peptide pool (ApoB100 PP7) for 5 days in the presence of recombinant human IL-2, followed by 5 days of cell culture without peptides. Flow cytometry was used to determine $CD4^+$ T cell proliferation based on the dilution of the CFSE label after cell division (Figure 1B). The results showed an increase in the proliferation of $CD4^+$ T cells in the peptide pool condition compared to the medium control (p-value < 0.05) (Figure 1B). These results show that the immunopeptidomic approach can be applied for the identification of relevant immunogenic antigens presented in plaque samples.

Expanded analysis identified 13 potentially immunogenic ApoB100-derived peptides presented by HLA-DR

After the positive results from the pilot feasibility study, we used the same immunopeptidomic approach to elucidate the repertoire of peptides presented by HLA-DR molecules in 50 endarterectomy samples. A total of 2988 unique peptides were eluted from HLA-DR at a false discovery rate 1% and a mascot ion score > 35. Peptide length distribution, a common quality control check performed in

immunopeptidomic analysis[13], showed that indeed the length of the peptides eluted from HLA-DR was centred around 15 amino acids, with the typical length distribution for HLA class II peptides between 10 and 25 amino acids[14] (Figure 2A).

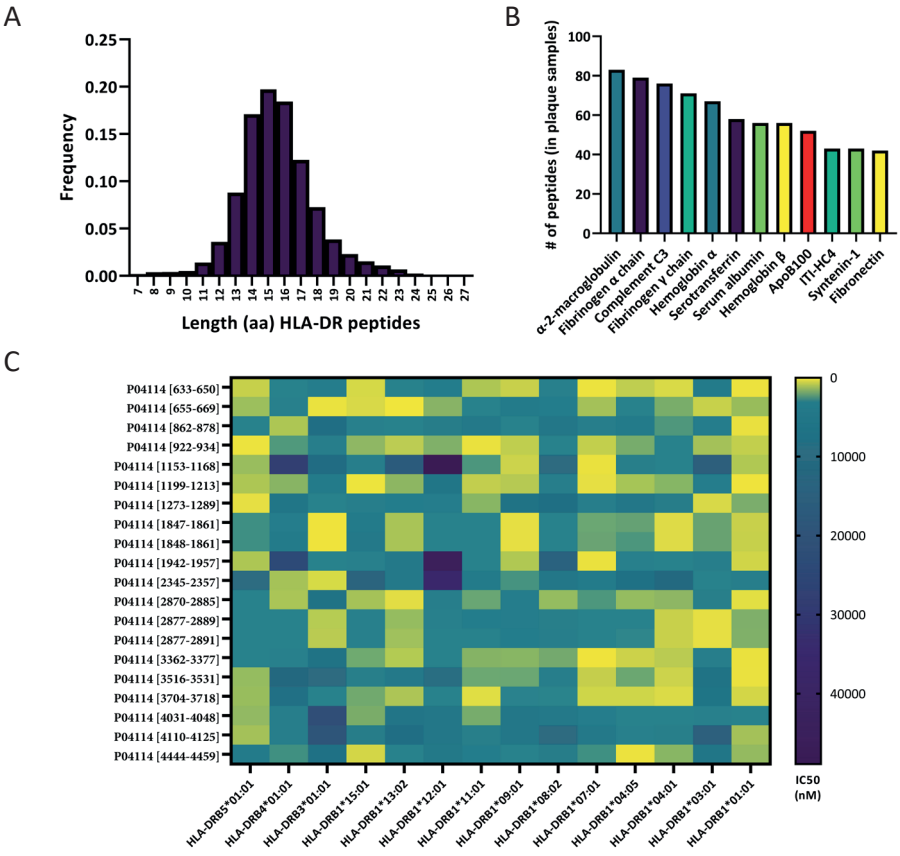

Figure 2. Immunopeptidomic analysis of n=50 atherosclerosis plaque samples. (A) Frequency distribution of the HLA_DR peptides lengths. (B) Number of peptides eluted from plaque samples grouped by source protein. (C) Heatmap representing the predicted binding affinity (IC50 values in nM) of 20 selected peptides to different HLA-DR alleles. P04114 is the UniProt accession number for Apolipoprotein B100, number between brackets represent the position of the epitope in the amino acid sequence of the protein.

Next, we grouped the peptides eluted from HLA-DR by their parent protein and ranked the original proteins by their contribution to the peptide repertoire (Figure 2B). The main protein contributors to the immunopeptidome of the plaque were α-2-macroglobulin, fibrinogen α chain and complement C3. Interestingly, ApoB100 was the ninth source of peptides by number, with 52 peptides derived from this protein. Adaptive immune responses against ApoB100 have been previously linked to

atherosclerosis plaque development [7, 15, 16], therefore we focus on peptides derived from this protein to study atherosclerosis-specific immune responses. The amino acid sequences of the 52 ApoB100-derived peptides were uploaded to the Immune Epitope Database (IEDB) to predict the binding affinity to 14 alleles of HLA-DR. Based on this predicted binding affinity, we selected 13 peptides that had a medium to high affinity (IC50 < 1000 nM) for a wide range of HLA-DR alleles (Figure 2C and Supplementary Figure 2). We combined the 7 peptides identified in the pilot study with these 13 peptides in a peptide pool (ApoB100 PP20) for further experiments. The amino acid sequence and position within the ApoB100 protein of each peptide in the ApoB100 PP20 can be found in Supplementary Table 1. The identity of the eluted peptides was confirmed by comparing the MS spectra of synthetic peptides and the peptides eluted from plaque samples (data not shown).

ApoB100 peptide pool induced CD4+ T cell activation in a subset of atherosclerosis patients

With the peptide pool covering a wide number of HLA-DR variants, we hypothesized that this pool could be used to detect ApoB100 specific $CD4^+$ T cells in CVD patients, regardless of their HLA type. In order to quantify the original ApoB100-specific $CD4^+$ T cell population in CVD patients, we used an activation induced marker (AIM) assay based on the expression of CD40L by recently activated $CD4^+$ T cells[17]. We stimulated PBMCs from a second cohort of patients with the ApoB100 PP20 peptide pool. Gating strategy for the flow cytometry data can be found on Supplementary Figure 3. For each patient we calculated the stimulation index. We observed a subgroup of patients (13/56, 22.4%) with a stimulation index equal or above the threshold of 2 (Figure 3A), suggesting this subgroup contained a detectable number of ApoB100 specific $CD4^+$ T cells in the circulation. We defined the threshold for the stimulation index based CD40L response to ApoB100 PP20 of PBMCs from healthy volunteers (Figure 3A). Stimulation of patients PBMCs with a negative control peptide pool derived from human actin resulted in significantly lower response (Figure 3A).

We next addressed whether the quantity of ApoB100 specific $CD4^+$ T cells is indicative of disease progression. As we have access to the endarterectomy material of PBMC donors, we scored these plaques based on the following histological features: necrotic core, calcification, foam cell, cholesterol crystals, inflammatory cells content and neovascularization (Figure 3B, Supplementary Table 2) and calculated a plaque vulnerability score as the average of these parameters. Next, we compared the stimulation index with the plaque vulnerability score, however the correlation did not reach statistical significance (Figure 3C) (p-value = 0.1). This correlation was significant (Pearson correlation coefficient (r) = 0.58, p-value = 0.049) in the subgroup of patients with higher stimulation index (Figure 3C). These data indicate that only

in patients with a significant CD4⁺ T cell response to ApoB100 the magnitude of the response correlate to the plaque vulnerability.

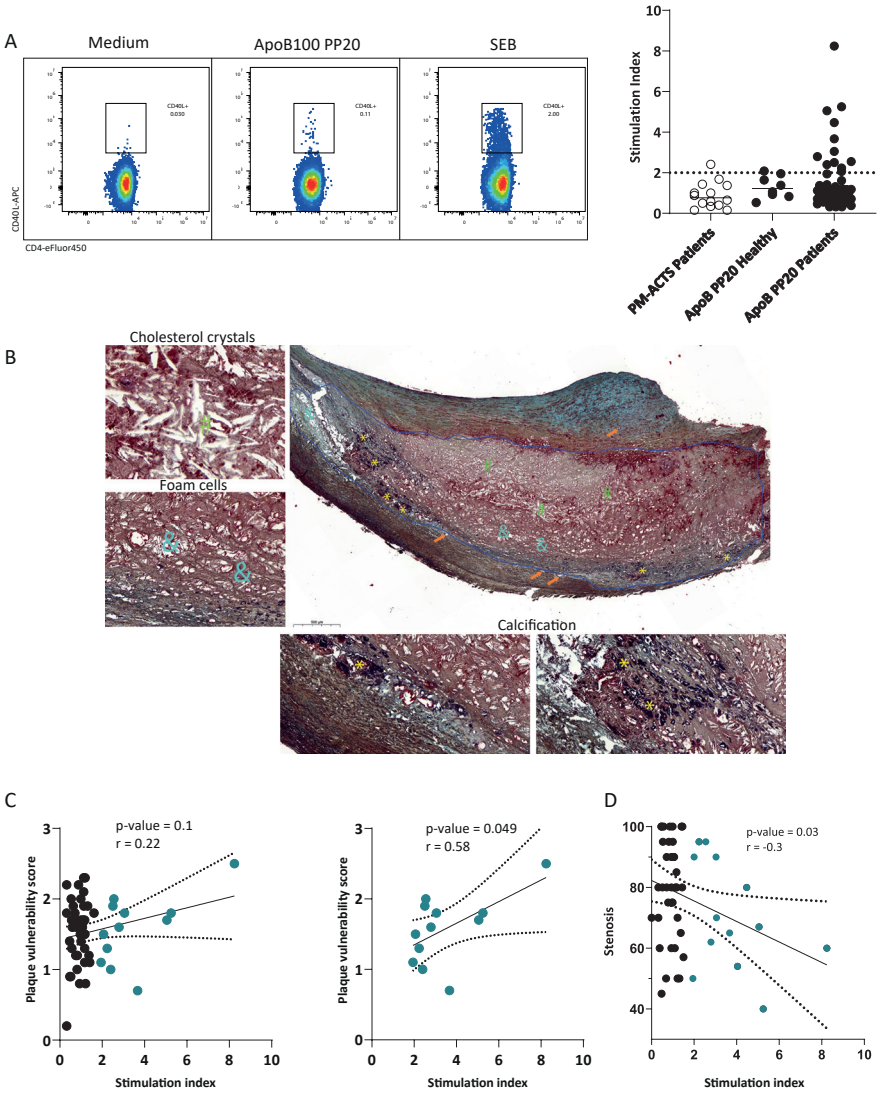

Figure 3. Expression of CD40L in CD4⁺ T cells from patient's PBMCs. (A) Representative flow cytometry plots of the gating of CD4⁺ CD40L⁺ cells in the live T cell population and dot plot with ApoB PP20 stimulation index per patient (n=58), PM-ACTS stimulation index per patient (n = 14) and ApoB PP20 stimulation index in healthy volunteers (n = 12). (B) Representative histology image of one of the plaque samples highlighting the different histological parameters taken into consideration to calculate the plaque vulnerability score. Blue line indicates the necrotic core, & depicts foam cells, # depicts cholesterol crystals, arrow and * indicate calcification. (C, left) Relationship between stimulation index and plaque vulnerability scores of all patients and (C, right) only subgroup with stimulation index ≥ 2. (D) Relationship between stimulation index and stenosis. Statistical correlation between plaque vulnerability scores and stimulation index was determined using Pearson's correlation test.

Cytokine profile of PBMCs stimulated with ApoB100 peptide pool showed a IL17-driven proinflammatory response

The relatively high background of the AIM assay may limit its sensitivity to detect very low frequency populations of ApoB100-specific $CD4^+$ T cells. Moreover, although the assay allows quantification of the number of ApoB100-specific $CD4^+$ T cells, it does not give insight into the phenotype of these cells. As previous work on ApoB100-specific $CD4^+$ T cell suggests, these cells can have atherogenic and atheroprotective functions based on their cytokine production[18, 19]. Therefore, to obtain sensitive and functional information about this antigen-specific $CD4^+$ T cell population we performed a multiplex bead-based ELISA assay to measure the cytokine profile of the PBMCs stimulated with ApoB100 PP20 peptide pool. For this, we stimulated patients PBMCs with either ApoB100 PP20 peptide pool or medium control for 3 days and performed multiplex ELISA with the conditioned medium. We observed that 31 out of 39 (79%) patients produced more IL-6 after peptide stimulation than their non-stimulated controls (Figure 4G), suggesting this protocol is more sensitive at detecting antigen-specific $CD4^+$ T cell responses than the AIM approach. We observed a trend toward increased IL-2 production in the ApoB100 peptide stimulated condition compared to control ($p=0.066$, Figure 4B) and significant increases in IL-10, IL-17A, IL-5, IL-6 and IL-9 in the peptide pool condition compared to control (Figure 4C, 4D, 4F, 4G and 4H). Interestingly, we did not observe significant differences in the levels of IFN-γ produced in the peptide pool condition and the control (Figure 4E), suggesting that ApoB100-specific $CD4^+$ T cells produce limited amounts of this Th1-related cytokine. While we do not observe a correlation between the level of individual cytokines and plaque vulnerability (Supplementary Figure 4), these data provide a clear indication that ApoB100-specific $CD4^+$ T cells contain different functional subsets of T cells.

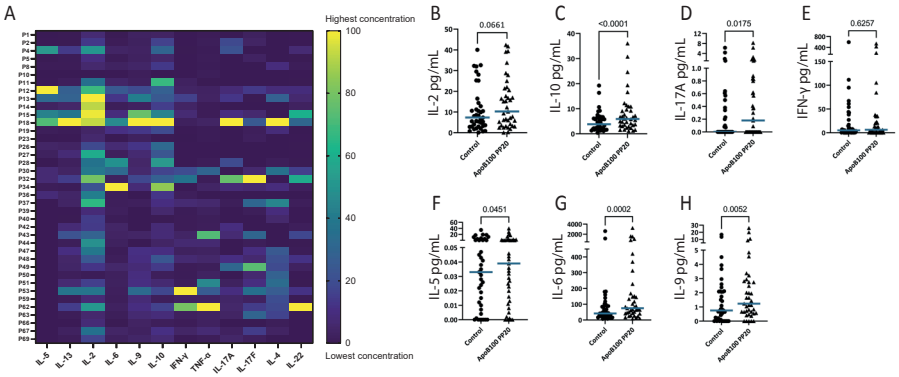

Figure 4. Cytokine profile of patient's PBMCs (n = 41) stimulated with ApoB100 PP20 peptide pool for 3 days. (A) Heatmap representing the concentration of 12 different cytokines (x axis) associated to T helper cell responses in conditioned medium of patients PBMCs (y axis). Data of each cytokine normalized to the highest concentration measured in all the patients. Concentration of IL-2 (B), IL-10 (C), IL-17A (D), IFN-γ (E), IL-5 (F), IL-6 (G) and IL-9 (H) in medium control and PBMCs stimulated with ApoB100 PP20. Blue bars in panels B-H represent median. P-values determined using two-tailed Wilcoxon matched-pairs signed rank test.

DISCUSSION

Several different approaches have been used in the last 20 years to try to identify ApoB100 immunogenic epitopes, mainly based on studies in mice and then translated to human[20] or by *in silico* screening of the ApoB100 sequence for strong binding motifs to the murine MHC-II isotype I-A^{b} [7, 21]. However, whether ApoB100-specific autoreactive CD4^{+} T cell can directly contribute to atherosclerosis development and plaque instability will be dependent on them being able to recognize their cognate antigens in the plaque. Here we use an immunopeptidomic approach to show that ApoB100 epitopes are presented in the atherosclerotic lesions, which provides an important rationale for ApoB100-specific CD4^{+} T cells as drivers of autoimmunity in atherosclerosis. To the best of our knowledge, none of the ApoB100 peptides identified and selected in this study has been reported before.

Proliferation-based experiments show that CD4^{+} T cells from blood of CVD patients respond to these peptides, suggesting the immunopeptidomic approach is a viable method for antigen discovery in atherosclerosis. Proliferation assays, however, have limitations regarding long culture times, unspecific proliferation as a result of cell death[22, 23] and the difficulty to precisely quantify the original population of antigen-specific T cells. The quantification of activation-induced markers such as CD40L on the other hand, allows the direct quantification of the original CD4^{+}

T cell population responding to the peptides. The stimulation of PBMCs from patients with the ApoB100 peptide pool identified in the immunopeptidomic analysis showed that a subgroup of 22% of patients had detectable levels of ApoB100-specific $CD4^+$ T cells. In this subgroup of patients, the stimulation index showed a positive correlation with histologically determined plaque vulnerability. However, when comparing the stimulation index with the percentage of maximal stenosis, a measurement of atherosclerosis plaque size, we observed a negative correlation, where patients with higher ApoB100-specific T cell responses showed lower levels of stenosis. Further research should be performed to determine the apparently opposing roles of ApoB100-specific T cells in plaque vulnerability and plaque size.

Multiple publications suggest the presence of ApoB100 specific $CD4^+$ T cells in atherosclerotic lesions[6, 7]. Moreover, a recent single-cell TCR sequencing study has shown the presence of clonally expanded, $CD69^+$ $CD4^+$ T cell populations in atherosclerotic lesions[4], suggesting the presence of antigen-specific T cell responses in the lesion. Future studies should determine whether the ApoB100-specific T cell responses observed in PBMCs are also present in the plaque. However, the methodology employed in this study, which is based on flow cytometry determination of activation markers, may not be sufficiently sensitive for this task. The identification of TCR sequences associated to the ApoB100 epitopes described here would allow to determine the presence of ApoB100-specific $CD4^+$ T cells in the lesion in a more sensitive manner. The identification of these TCRs in the single-cell sequencing data of human atherosclerotic plaques recently published by our lab would give a more comprehensive understanding on the characteristics of these antigen-specific T cells.

In order to obtain phenotypic information about the ApoB100-specific T cells, we also investigated the cytokines produced after ApoB100 stimulation. We observed an increased production of IL-10 and IL-17A but not in IL-2 or IFN-γ, the two signature cytokines of Th1 cells suggesting that these ApoB100-specific T cells present a Treg/Th17 phenotype. This data is in line with previous reports from Wolf et al. who showed the presence of ApoB100-specific $CD4^+$ T cells that evolve from an anti-inflammatory Treg phenotype in initial stages of atherosclerosis to a more Th17 phenotype in later stages[7]. We also observed an increase in the production of a plethora of cytokines such as IL-9, IL-5 and IL-6, associated to different $CD4^+$ T cell phenotypes[24, 25]. This suggest that there is not a unique phenotype in the ApoB100-specific T cell population, but rather a diverse set of T cell phenotypes within the population. Although our data reinforces the hypothesis that a shift from IL-10 to IL-17 production in ApoB100-specific $CD4^+$ T cells may be an important axis in the disease pathogenesis, we do not observe any correlations with plaque vulnerability. Cytokine levels, however, are difficult to interpret as we do not have

information on the cytokine production on a per cell basis, for which expansion of individual T cells coupled with TCR sequencing would be required.

The trend towards higher level of ApoB100-specific $CD4^+$ T cells in more vulnerable plaques should be explored further by increasing the number of patients in follow up studies and it can lead to novel biomarkers for atherosclerosis progression or status based on the level or phenotype of antigen-specific $CD4^+$ T cells.

Besides ApoB100 we also identified α-2-macroglobulin and fibrinogen alpha chain as the most prevalent proteins in the plaque's immunopeptidome. Both α-2-macroglobulin and fibrinogen are abundantly present in atherosclerosis lesions therefore their contribution to the immunopeptidome is not surprising. Fibrinogen has emerged as a candidate antigen driving autoimmune responses in rheumatoid arthritis[26] therefore high levels of antigen presentation of fibrinogen-derived peptides in atherosclerotic plaques might provide a link between atherosclerosis and rheumatoid arthritis[27]. Other candidate proteins have been proposed as potential targets of autoimmunity in atherosclerosis such as heat-shock proteins[28, 29]. In fact, proteins from the heat shock superfamily contributed with 18 peptides to the immunopeptidome. Therefore, this immunopeptidomic approach could be used to identify other important immunogenic epitopes from other proteins, which is key for the development of novel therapeutic strategies against atherosclerosis based on antigen-specific immune modulation.

CONCLUSIONS

In conclusion, here we show that immunopeptidomic-based analysis of atherosclerosis plaque tissue can be used to identify immunogenic epitopes of ApoB100. Using this approach, we identified 20 ApoB100 epitopes presented in human carotid and femoral atherosclerosis plaques and showed the presence of $CD4^+$ T cell responses against these peptides in PBMCs of CVD patients. These ApoB100-specific $CD4^+$ T cell responses showed a cytokine signature characterized by production of IL-17A, IL-10, IL-5, IL-6 and IL-9. Besides ApoB100, these data can be used to identify other immunogenic proteins and epitopes driving immune responses in atherosclerosis which is key for the development of new therapeutic strategies against CVDs.

AUTHORS CONTRIBUTIONS

FLV designed and performed experiments, analysed data and prepared and edited this manuscript and prepared figures. BS and PV designed the original concept and provided assistance regarding the experimental design. IS performed stimulation experiments, carried out peptide synthesis and aided with pilot studies. NSAC provided essential technical assistance with peptide synthesis. MJMJ, EH, MACD, LD, JM, MNBK processed patient material and performed PBMC isolation from patient´s blood samples. JAHMP, AW, HJS performed endarterectomy surgery and collected patients´ samples. RTNT performed peptide-HLA isolation from plaque samples, peptide elution and MS analysis. PHAQ, MRV performed the histology assessment of atherosclerosis plaque samples and contributed to the preparation of figures. AK, JK, IB provided valuable input during the preparation and edition of the manuscript.

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DATA AVAILABILITY STATEMENT

All non-patient related data is available upon request to the corresponding author (b.a.slutter@lacdr.leidenuniv.nl). Raw data of peptide discovery will be freely available from a repository after publication of this manuscript.

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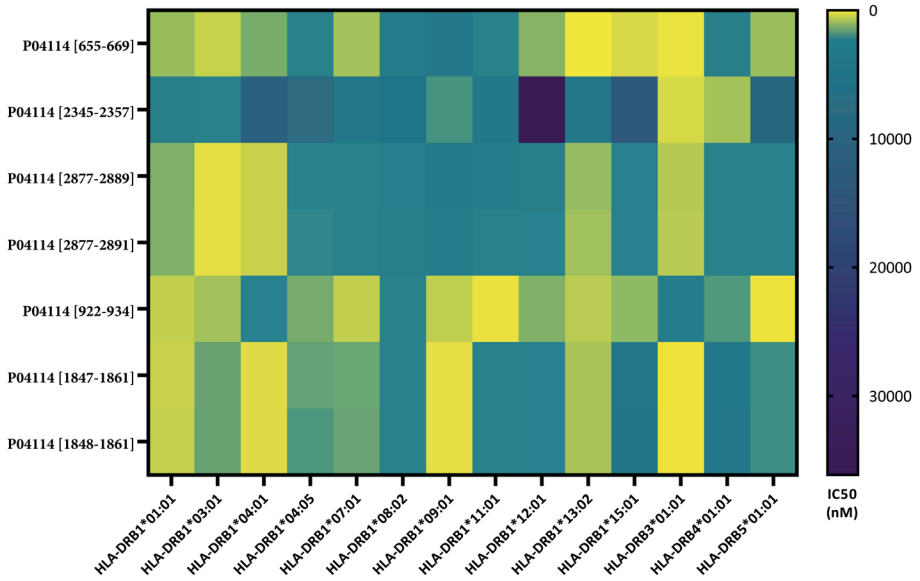
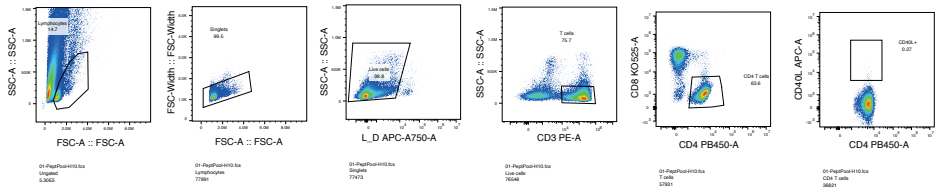
SUPPLEMENTARY DATA

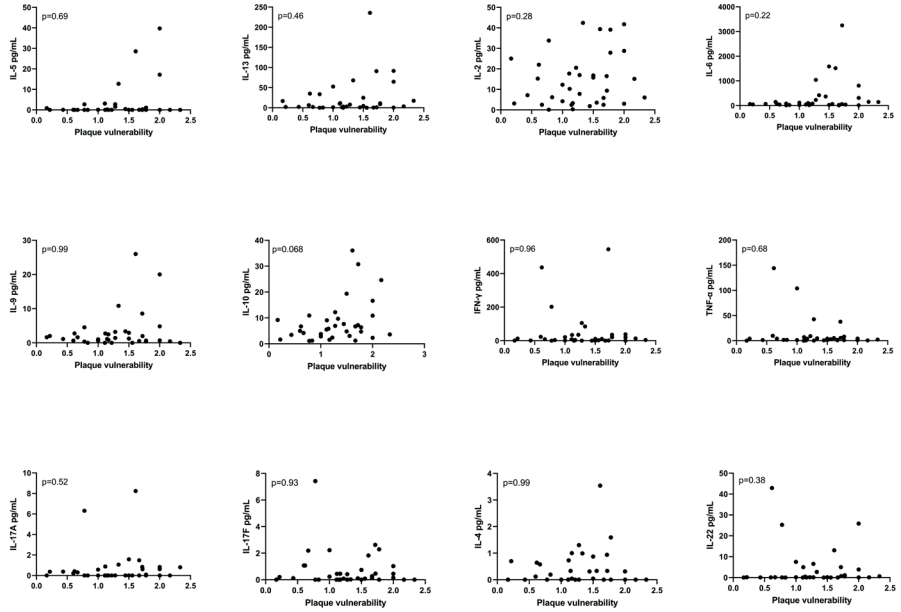


Supplementary Figure 1. Predicted binding affinity to HLA-DR alleles of ApoB100 peptides identified in feasibility study.

Supplementary Figure 2. Predicted binding affinity to HLA-DR alleles of ApoB100 peptides identified in immunopeptidomics of n=50 plaques.



Supplementary Figure 3. Gating strategy to define CD40L+ CD4 T cells in CD40L assay.



Supplementary Figure 4. Cytokine concentrations vs. plaque vulnerability score (n=30).

Supplementary Table 1. Amino acid sequence and position in ApoB100 protein (UniProt accession number = P04114) of peptides in ApoB PP20 peptide pool

ApoB PP20 peptide	Amino acid sequence	Position in ApoB100 protein
P1	IEGNLIFDPNNYLPK	P04114 [655-669]
P2	IERYEVDQQIQVL	P04114 [2345-2357]
P3	INNQLTLDSNTKY	P04114 [2877-2889]
P4	INNQLTLDSNTKYFH	P04114 [2877-2891]
P5	LKFIIPSPKRPVK	P04114 [922-934]
P6	LSASYKADTVAKVQG	P04114 [1847-1861]
P7	SASYKADTVAKVQG	P04114 [1848-1861]
P8	VSTAFVYTKNPNGYS	P04114 [3704-3718]
P9	VKLEVANMQAELVAKPS	P04114 [862-878]
P10	FSRNYQLYKSVSLPSLDP	P04114 [633-650]
P11	FSHDYKGSTSHHLVSR	P04114 [1942-1957]
P12	NNAEWVYQGAIRQIDD	P04114 [4110-4125]
P13	SNGVIVKINNQLTLDS	P04114 [2870-2885]
P14	IVAHLLSSSSSVIDAL	P04114 [3362-3377]
P15	SPDKKLTIFKTELRVRES	P04114 [4031-4048]
P16	DYPKSLHMYANRLLD	P04114 [1199-1213]
P17	SATAYGSTVSKRVAWH	P04114 [1153-1168]
P18	LHRNIQEYLSILTDPD	P04114 [4444-4459]
P19	EANTYLNSKSTRSSVK	P04114 [3516-3531]
P20	IPENLFLKSDGRVKYTL	P04114 [1273-1289]

Supplementary Table 2. Semiquantitative scoring of histological features of carotid plaques.

Feature	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Necrotic core	Not present	<20% of arterial area	20-40% of arterial area	40-70% of arterial area	>70% of arterial area
Calcification	Not present	<10% of arterial area	10-40% of arterial area	>40% of arterial area	-
Foam cell content	Not present	Few small size (5 cells) clusters	Multiple small or medium (10 cells) size clusters	One large size (15 cells) cluster or foam cells present around 70% of the arterial area	-
Cholesterol crystal content	Not present	<10% of necrotic core area	10-40% of necrotic core area	>40% of necrotic core area	-
Inflammatory cells content	Not present	Few small size clusters (50 cells) or a few cells scatter in the arterial area	Multiple small size clusters	One large size cluster (100 cells) or inflammatory cells scattered around 70% of the arterial area	-
Neovascularization	Not present	< 25 neovessels	25-50 neovessels	> 50 neovessels	-