

Treatment with apo B peptide vaccines inhibits atherosclerosis in human apo B-100 transgenic mice without inducing an increase in peptide-specific antibodies

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Abstract. Fredrikson GN, Björkbacka H, Söderberg I, Ljungcrantz I, Nilsson J (Malmö University Hospital, Lund University, Lund; and Malmö University, Malmö; Sweden). Treatment with apo B peptide vaccines inhibits atherosclerosis in human apo B-100 transgenic mice without inducing an increase in peptide-specific antibodies. *J Intern Med* 2008; **264**: 563–570.

Objectives. Autoantibodies to apolipoprotein (apo) B-100 peptides are present in human plasma and have been shown to be associated with decreased cardiovascular risk. The present study aimed to determine if apo B-100 peptide vaccines are atheroprotective in mice expressing human apo B-100 and if the effectiveness of the vaccines is influenced by the level of pre-existing peptide-specific autoantibodies.

Design. LDL receptor^{-/-}/human apo B-100 transgenic mice were immunized with native human apo B-100 peptides p45 or p210 at 6, 9 and 11 weeks and the extent of atherosclerosis determined by *en face* Oil Red O staining of the aorta at 25 weeks. Autoantibody levels were determined by enzyme-linked

immunosorbent assay, and RNA expression in the spleen was assessed by real time PCR.

Results. Control mice had high levels of autoantibodies against p210 but only low levels against p45. Immunization with native p45 and p210 reduced atherosclerosis by 66% ($P < 0.02$) and 59% ($P = 0.06$), respectively. The atheroprotective effect of apo B peptide immunization occurred in the absence of an increase in peptide-specific IgG, but was associated with an increase in IgM recognizing native and copper-oxidized LDL.

Conclusions. Immunization with apo B peptide-based vaccines inhibits atherosclerosis in mice expressing human apo B-100 suggesting that they can interact with their target as expressed in humans. The protective effect is independent of the pre-existing level of apo B peptide autoantibodies and can occur without activating an increase in peptide-specific antibodies suggesting that atheroprotection can be mediated by cellular immune responses.

Keywords: antibodies, apolipoprotein B-100, atherosclerosis, peptide, vaccine.

Introduction

Accumulation and oxidation of LDL particles in the arterial intima is believed to play an important role in

the development of atherosclerosis [1]. Oxidized LDL becomes targeted by adaptive immune responses resulting in generation of oxidized LDL-specific autoantibodies and T cells [2–5]. Modulation of these immune responses by immunization with oxidized LDL has been found to significantly reduce the

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development of atherosclerosis in hypercholesterolemic animals suggesting that it may be possible to develop therapeutic vaccines [6–10]. We have recently identified a number of native and malondialdehyde (MDA)-modified peptide sequences in the LDL apolipoprotein (apo) B-100 as major targets for the autoimmune responses involved in the atherosclerotic process [11]. Immunization of hypercholesterolemic mice with some of these apo B peptides significantly reduces the development of atherosclerosis [12, 13]. The most effective peptides identified in these studies correspond to the apo B-100 amino acids 661–680 (p45) and 3136–3155 (p210). Immunization of apo E^{-/-} mice with MDA-modified p45 is associated with a 50-fold increase in Th2-specific p45 immunoglobulins (IgG1) and a 48% decrease in the development of atherosclerosis [13]. Treatment of hypercholesterolemic mice with human recombinant MDA-p45 specific IgG recognizing oxidized but not native LDL has been shown to reduce the development of atherosclerosis to the same extent as active immunization [14]. Furthermore, MDA-p45 specific IgG treatment induces regression of existing lesions suggesting that antibodies against oxidized LDL epitopes are atheroprotective [15].

Epidemiological studies have also linked autoantibody responses against p45 and p210 to cardiovascular disease in man. Interestingly, these studies suggest that autoantibodies against the native peptide are associated with decreased cardiovascular risk [16] whilst autoantibodies against MDA-modified peptides reflects disease severity [11, 17] indicating that in a clinical situation it may be preferable to induce immune responses against native rather than MDA-modified apo B-100 peptides.

A confounding factor in the active apo B-100 peptide immunization studies [12, 13] is that human peptide sequences were used to immunize mice and that the sequence homology for these peptides between mouse and man is 85–95%. Accordingly, it cannot be excluded that the inclusion of nonhomologous amino acids is of importance for the immunogenicity and effectiveness of the vaccine. In the present study we have immunized LDL receptor^{-/-} mice expressing

human apo B-100 with native p45 and p210. Questions of relevance for development of an apo B peptide-based atherosclerosis vaccine are addressed: (i) does the atheroprotective effect of apo B immunization observed in previous studies depend on the presence of nonhomologous amino acids making the vaccine less effective in a situation where a completely homologous peptide is used? and (ii) is the autoimmune response against the p45 and p210 sequences of apo B-100 in LDL receptor^{-/-}/human apo B-100 transgenic mice similar to those observed in humans? This information is of importance for the further development of a novel immune modulating therapy for cardiovascular disease.

Methods

Animals

Male LDL receptor^{-/-} mice that express human apoB-100 on a C57Bl/6 background were kindly provided by Jan Borén, Gothenburg University. The mice ($n = 7–10$ per group) were given a first injection (100 μ L per injection and mouse) with the native peptides conjugated to the carrier or carrier alone at 6 weeks of age and booster injections 3 and 5 weeks later. The human apo B-100 peptide sequences used composed of amino acids 661–680 (IEIGL EGKGF EPTLE ALFGK; p45) and 3136–3155 (KTTKQ SFDLS VKAQY KKNKH; p210). The homology between the human and mouse sequences is 95% for p45 and 90% for p210 [Accession no.: P04114 (human) and XP_137955 (mouse)]. Each injection contained 50 μ g of the native peptide and 50 μ g of the carrier cationized bovine serum albumin (cBSA) dissolved in 0.083 mol L⁻¹ sodium phosphate 0.9 mol L⁻¹ NaCl pH 7.2, according to the manufacture's protocol (No. 77652, Pierce, Rockford, IL, USA) and with Alum (aluminum hydroxide, Pierce) included in all injections as adjuvant. The selection of the apo B-100 peptides was based on studies demonstrating the presence of IgM and/or IgG levels against these peptides in man. At 25 weeks of age, the mice were killed and the tissue collected as previously described [12, 13]. The Animal Care and Use Committee approved experimental protocol used in the study.

Staining of the descending aorta

En face preparations of the descending aorta were washed in distilled water, dipped in 78% methanol and stained for 40 min in 0.16% Oil-Red-O dissolved in 78% methanol/0.2 mol L⁻¹ NaOH as previously described [18]. The cover slides were mounted with a water-soluble mounting media L-550A (Histolab, Göteborg, Sweden). Lipids are stained red, which makes the plaques bordeaux-coloured. Stained area (Bordeaux-coloured) and total aortic areas were quantified blinded by microscopy and computer-aided morphometry (Olympus Micro Image, Hamburg, Germany).

RNA isolation and quantitative real-time PCR

Spleens were harvested and put in RNA later (Ambion, Austin, TX, USA) before storing them at -20 °C. After thawing on ice, tissue was disrupted in the presence of equal volumes of RLT buffer (Qiagen, Valencia, CA, USA) and phenol : chloroform (1 : 1) using a FastPrep FP120 instrument (BIO 101, Carlsbad, CA, USA) and Lysing Matrix D beads (Qbiogene, Carlsbad, CA, USA). After disruption and phenol : chloroform extraction, total RNA was isolated using RNeasy columns (Qiagen). Contaminating DNA was degraded by RQ1 DNase I (Promega, Madison, WI, USA) and RNA integrity was analysed by formaldehyde-agarose gel electrophoresis. Purified total RNA was reverse transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative mRNA expression was calculated from standard curves, constructed by serial dilution of gel-purified PCR products and normalized to 18S rRNA expression.

Peptide ELISA

The same native peptide for the immunization was used for coating (20 µg mL⁻¹ in phosphate-buffered saline pH 7.4) of microtiter plates (Nunc MaxiSorp, Nunc, Roskilde, Denmark) by overnight incubation at 4 °C. The detection of deposit antibodies recognizing the

peptides, involved both biotinylated goat anti-mouse IgM or IgG antibodies (Jackson ImmunoResearch, West Grove, PA, USA), as well as alkaline phosphatase conjugated rat anti-mouse IgG1 or IgG2a antibodies (Pharmingen, BD Bioscience, Erembodegem, Belgium), incubated for 2 h at room temperature. Mean values were calculated after subtraction of background absorbance. Data regarding the specificity and variability of the enzyme-linked immunosorbent assay (ELISA) have been published previously [11].

Serum cholesterol, triglyceride and oxidized LDL analysis

Total plasma cholesterol and plasma triglycerides were quantified with colourimetric assays, Infinity™ Cholesterol and Triglyceride (Sigma, St Louis, MO, USA). Oxidized LDL was measured using a commercially available ELISA kit (Mercordia, Uppsala, Sweden) in EDTA plasma.

Statistical analysis

Data are presented as mean ± SD. Analysis of the data was carried out using the Mann-Whitney two-tailed test. Statistical significance was considered at the level less than equal to 0.05.

Results

Presence of autoantibodies against apo B peptides, native and modified LDL in control mice

Analysis of plasma from control mice demonstrated that the expression of autoantibodies against the apo B-100 p45 and p210 differed markedly from each other (Fig. 1). Autoantibody levels against the native p45 peptide were low. In contrast, these mice had very high levels of autoantibodies against native p210 (Fig. 1). Both IgG1 and IgG2a against native p210 were present at high levels. Taken together these observations demonstrate that LDL receptor^{-/-}/human apo B-100 transgenic mice have an autoimmune response against the P210 apo B epitope and that this is a mixed Th1/Th2 response. Control mice also expressed IgM and IgG recognizing

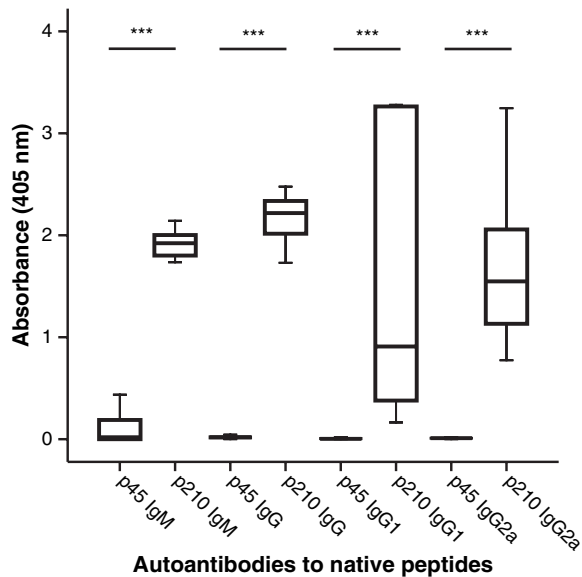


Fig. 1 Autoantibodies in plasma of LDL receptor^{-/-}/human apo B-100 transgenic control mice. Mice were immunized with carrier and adjuvant alone. Autoantibodies directed to native p45 or p210 were detected using the peptide enzyme-linked immunosorbent assay. Box plots demonstrate median, twenty-fifth and seventy-fifth percentiles and whiskers show the highest and lowest values. *** $P < 0.001$, $n = 10$.

MDA-LDL, whereas the levels of autoantibodies against native and Cu-oxidized LDL were low (Table 1). IgG against MDA-LDL were primarily IgG1 suggesting a predominance of Th2 immune responses.

Effect of immunization on antibody levels against apo B peptides, native and modified LDL

There was no increase in peptide-specific IgG in p45 immunized mice (Table 1). However, immunization with p45 was found to result in a fivefold increase in IgM recognizing native LDL and a threefold increase in IgM recognizing copper-oxidized LDL, but did not influence the level of IgG or IgM against MDA-LDL (Table 1). Accordingly, immunization with p45 activated expression of native and oxidized LDL IgM but did not influence the expression of peptide-specific IgG. An increased expression of IgM recognizing native and copper-oxidized LDL was also observed in mice immunized with p210 (Table 2). This was

Table 1 Antibodies against p45, native LDL, MDA-LDL and copper-oxidized LDL in mice immunized with cBSA (control) or p45

	cBSA	p45
IgM		
p45	0.09 ± 0.15	0.16 ± 0.15
nLDL	0.06 ± 0.06	0.30 ± 0.26***
MDA-LDL	1.24 ± 0.47	1.26 ± 0.46
Cu oxLDL	0.12 ± 0.15	0.36 ± 0.31*
IgG		
p45	0.02 ± 0.01	0.02 ± 0.01
nLDL	0.03 ± 0.06	0.01 ± 0.02
MDA-LDL	0.36 ± 0.23	0.27 ± 0.18
Cu oxLDL	0.05 ± 0.06	0.04 ± 0.05
IgG1		
p45	0.01 ± 0.01	0.01 ± 0.01
nLDL	0.03 ± 0.04	0.03 ± 0.03
MDA-LDL	0.19 ± 0.26	0.17 ± 0.22
Cu oxLDL	0.04 ± 0.06	0.04 ± 0.04
IgG2a		
p45	0.01 ± 0.01	0.01 ± 0.01
nLDL	0.02 ± 0.02	0.01 ± 0.01
MDA-LDL	0.05 ± 0.04	0.05 ± 0.02
Cu oxLDL	0.03 ± 0.03	0.03 ± 0.03

nLDL, native LDL; Cu oxLDL; copper-oxidized LDL; MDA-LDL, malondialdehyde LDL; cBSA, cationized bovine serum albumin; IgG, immunoglobulins.

* $P < 0.05$, *** $P < 0.005$ versus cBSA using the Mann-Whitney two-tailed test.

associated with a down-regulation of p210-specific IgG and IgG2a as well as the low-grade IgG2a response against copper-oxidized LDL.

Effect of immunization on atherosclerosis

Immunization with p45 reduced the development of atherosclerosis in LDL receptor^{-/-}/human apo B-100 transgenic mice by 66% ($P = 0.016$, Fig. 2). In mice immunized with p210, atherosclerosis was found to be reduced by 59% ($P = 0.059$). Cholesterol levels were similar in all groups, whilst triglyceride levels were lower in mice immunized with p45 and p210 (Table 3). The plasma level of oxidized LDL did not differ between control and immunized mice. Analysis of gene expression in the spleen did not identify any

Table 2 Antibodies against p210, native LDL, MDA-LDL and copper oxidized LDL in mice immunized with cBSA (control) or p210

	cBSA	P210
IgM		
p210	1.89 ± 0.19	2.00 ± 0.23
nLDL	0.06 ± 0.06	0.21 ± 0.10***
MDA-LDL	1.24 ± 0.47	1.34 ± 0.36
Cu oxLDL	0.12 ± 0.15	0.37 ± 0.18**
IgG		
p210	2.14 ± 0.25	1.60 ± 0.44***
nLDL	0.03 ± 0.06	0.02 ± 0.02
MDA-LDL	0.36 ± 0.23	0.38 ± 0.13
Cu oxLDL	0.05 ± 0.06	0.05 ± 0.05
IgG1		
p210	1.60 ± 1.35	1.27 ± 1.04
nLDL	0.03 ± 0.04	0.04 ± 0.03
MDA-LDL	0.19 ± 0.26	0.27 ± 0.18
Cu oxLDL	0.04 ± 0.06	0.06 ± 0.05
IgG2a		
p210	1.71 ± 0.82	1.04 ± 0.73*
nLDL	0.02 ± 0.02	0
MDA-LDL	0.05 ± 0.04	0.07 ± 0.05
Cu oxLDL	0.03 ± 0.03	0.01 ± 0.01*

nLDL, native LDL; Cu oxLDL; copper-oxidized LDL; MDA-LDL, malondialdehyde LDL; cBSA, cationized bovine serum albumin; IgG, immunoglobulins.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ versus cBSA using the Mann-Whitney two-tailed test.

significant differences in the expression of interferon- γ , interleukin-10 (IL-10), FoxP3, CCR4 (Th2 marker) or TIM3 (Th1 marker) between control and immunized mice, except for a slight increase of interferon- γ in the p210 immunized group ($P = 0.02$).

Discussion

Our results demonstrate that pilot vaccines containing the human apo B-100 p45 and p210 using Alum as adjuvant and cBSA as carrier inhibit the development of atherosclerosis in LDL-receptor-deficient mice expressing human apo B-100 by more than 50%. Unexpectedly, they also show that this can be achieved in absence of an increase in peptide-specific IgG. Several lines of evidence have previously provided support for

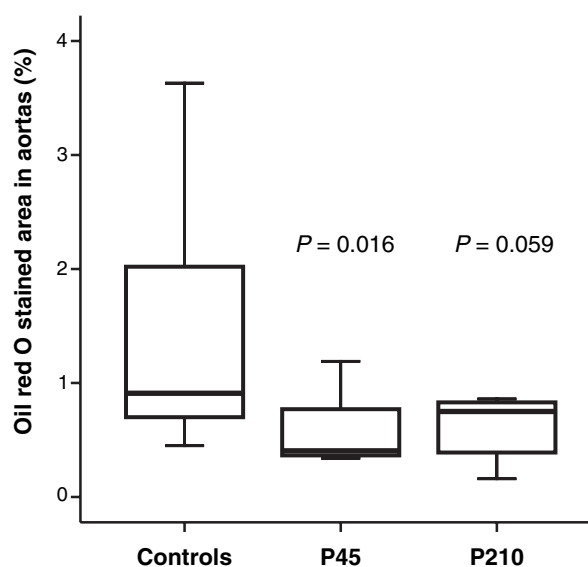


Fig. 2 Stained plaque area in aortas from immunized LDL receptor^{-/-}/human apo B-100 transgenic mice. Mice were immunized with carrier and adjuvant alone (controls) or with native peptide p45 or p210. Plaque areas were assessed by Oil Red O staining of *en face* mounts of the descending aorta. Values represent stained area in percent of total area. Box plots demonstrate median, twenty-fifth and seventy-fifth percentiles and whiskers show the highest and lowest values. Given P -values versus controls.

Table 3 Plasma cholesterol, triglyceride and oxidized LDL levels in control and immunized mice

	cBSA	p45	p210
Cholesterol (mg mL ⁻¹)	6.2 ± 2.9	5.7 ± 1.6	6.9 ± 2.0
Triglycerides (mg mL ⁻¹)	4.5 ± 2.1	2.8 ± 0.5*	2.7 ± 0.9*
Oxidized LDL (Units L ⁻¹)	192 ± 59	179 ± 44	202 ± 33

cBSA, cationized bovine serum albumin.

* $P < 0.05$ versus cBSA using the Mann-Whitney two-tailed test.

an atheroprotective role of apo B-100 antibodies. Atheroprotection following immunization of apo E^{-/-} mice with MDA-p45 is associated with a 50-fold increase in specific IgG1 [13]. Treatment of hypercholesterolemic mice with recombinant MDA-p45 IgG1 significantly reduces the development of atherosclerosis [14] and can also induce the regression of existing lesions [15]. Zhou *et al.* [10] have described a close association between the IgG response to immunization with MDA-LDL and the inhibition of atherosclerosis in

apo E^{-/-} mice. Moreover, prospective clinical studies have shown that high levels of IgG against p45 are associated with decreased risk for development of acute cardiovascular events in man [16]. Although these studies collectively provide strong support for a protective effect of apo B antibodies, the present observations suggest that they do not mediate apo B vaccine-induced atheroprotection in LDL receptor^{-/-}/human apo B-100 transgenic mice. Activation of regulatory T cells represents one alternative mechanism through which these vaccines may function. Dendritic cells that present antigens to T cells in a noninflammatory environment or in absence of a simultaneous activation of toll-like receptors normally induce T cell anergy or activation of regulatory T cells resulting in a tolerogenic response [19, 20]. It is likely that similar processes can occur also when immunizations are performed using adjuvants that do not interact with toll-like receptors such as Alum [21]. The protective role of regulatory T cells in atherosclerosis has been convincingly documented in a number of studies [22–24] and van Puijvelde *et al.* [25] recently demonstrated that oxidized LDL-specific CD4⁺/CD25⁺/Foxp3⁺ regulatory T cells contribute to atheroprotection after oral immunization with oxidized LDL. The decrease in specific IgG following immunization with p210 in the present study is well in line with this notion. However, we were unable to detect any increase in Foxp3 or IL-10 gene expression in the spleens of immunized mice. Clearly, further studies are required to determine if immunization with apo B peptide-based vaccines in humans should aim at increasing peptide-specific IgG or whether alternative immune mechanisms should be targeted.

This study also demonstrates that the previously observed atheroprotective effect of human apo B peptide-based vaccines in apo E^{-/-} mice does not depend on the presence of nonhomologous amino acids in the peptides. As LDL receptor^{-/-}/human apo B-100 transgenic mice express the same LDL protein as humans and have a lipoprotein phenotype similar to that characteristic for familial hypercholesterolemia [26] the present findings add support to the possibility that this type of vaccines may be effective also in humans. However, there are some limitations to the present model that should be considered. There is

evidence that human apo B expressed by rodent hepatocytes adopt a different conformation or undergoes different post-translational modifications than apo B expressed by human hepatocytes [27]. Moreover, although these mice express LDL particles with human apo B-100 it is not known whether the modifications taking place in the mouse arterial tissue are identical to those taking place in man. It is also not known if the immune response to oxidized LDL is the same in mice and man. Another possible confounding factor that needs to be considered is that human apo B^{+/+}/LDL receptor^{-/-} mice also express some mouse apo B [20, 28]. However, human apo B is by far the most common LDL protein in these mice and is likely to be the major epitope in oxidized LDL. Moreover, as human apo B sequences are used for immunization it is likely that also the induced immune response will primarily target human apo B.

One approach to assess the validity of the present model for the human situation is to search for similarities in the autoimmune response to oxidized LDL epitopes between LDL receptor^{-/-}/human apo B-100 transgenic mice and humans. Control mice had high levels of IgG and IgM against native p210, and almost no autoantibodies against native p45. This expression pattern is very similar to that observed in humans suggesting that the immune response to these oxidized LDL epitopes are the same in mouse and man [17, 29]. Hypothetically, the differences in autoantibodies to different apo B peptides could help to identify the peptide that has the potential to be the most effective in a human vaccine. If the mode of action of the vaccine is activation of a protective Th2 response it would be preferable to use a peptide against which there are low levels of pre-existing autoantibodies, such as p45, whilst if the mode of action is down-regulation or modulation of an ongoing immune response, an antigen against which there is high autoantibody levels such as p210, would be more attractive as a peptide vaccine. However, the present findings showed that the level of pre-existing autoantibodies had little or no influence on the effect of peptide immunization. Moreover, as discussed above, the effect of immunization on atherosclerosis was not associated with any apparent change in

peptide-specific antibody pattern. While immunization with p210 resulted in down-regulation of peptide-specific IgG2a as well as low levels of IgG2a against copper-oxidized LDL, compatible with inhibition of an ongoing pro-inflammatory Th1 response, immunization with p45 had no effect at all on peptide or oxidized LDL antibody levels. One possible explanation could be that p45 and p210 work by different mechanisms. However, one common characteristic for both peptides was the stimulation of the expression of IgM recognizing native and copper-oxidized LDL. Induction of a similar antibody response by immunization with *Streptococcus pneumoniae* has previously been shown to reduce atherosclerosis in LDL-receptor-deficient mice [30]. There is also evidence that the atheroprotective effect of immunization with MDA-LDL [31], as well as that of treatment with antibody against OX40 ligand [32], is linked to the increase in IgM against oxidized LDL. In view of these observations, it cannot be excluded that the protective effect of immunization with apo B peptides also may be mediated by IgM-recognizing epitopes in oxidized LDL.

The mechanisms involved in development of immune responses against native apo B peptide sequences in oxidized LDL remains to be fully understood. Oxidation of LDL is known to be associated with a proteolytic cleavage of apo B into smaller peptide fragments [5]. One possible explanation to the development of native apo B peptide immunogenicity is that this fragmentation alters the 3-dimensional structure of certain peptide sequences within apo B in a way that they are no longer recognized as self. Another possibility is that this fragmentation exposes part of the apo B molecule that is not normally accessible to immune cells.

In summary, our findings demonstrate that immunization with pilot apo B peptide-based vaccines reduces atherosclerosis in mice expressing LDL with human apo B-100 and reveal a pattern of apo B autoantibody expression similar to that observed in man. These findings add further support to the idea that it may become possible to develop novel therapies for cardiovascular disease based on immune modulation by vaccines. They also demonstrate that such vaccines

can be effective also in the absence of changes in peptide-specific IgG.

Conflict of interest statement

Jan Nilsson is signed as one of the inventors on a pending patent application from Forskarpatent i Syd, Sweden for immunization therapy against atherosclerosis using apo B peptides.

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