Immunization using an Apo B-100 related epitope reduces atherosclerosis and plaque inflammation in hypercholesterolemic apo E (−/−) mice

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Abstract

Immune system modulates atherosclerosis and immunization using homologous LDL reduces atherosclerosis in hyperlipidemic animals. The nature of athero-protective antigenic epitopes in LDL remains unclear. We have recently identified nearly a 100 antigenic epitopes in human apo B-100 and in this study we evaluated the effects of immunization with two such epitopes on atherosclerosis in hypercholesterolemic apo E (−/−) mice. Male apo E (−/−) mice were immunized at 6–7 weeks of age with two different apo B-100 related peptide sequences using alum as adjuvant and mice immunized with alum alone served as controls. Peptide-2 immunization reduced aortic atherosclerosis by 40% and plaque inflammation by 80% compared to controls without a reduction in circulating cholesterol levels whereas Peptide-1 immunization had no effect. Peptide-2 immunization also reduced the progression of aortic lesions when mice were immunized at 16 weeks of age, suggesting the possibility of immuno-modulation in treating established atherosclerosis. The athero-protective effect of Peptide-2 immunization was absent in splenectomized mice but could be conveyed to non-immunized mice via adoptive transfer of splenocytes from peptide-2 immunized mice. In conclusion, immunization with a specific apo B-100 related peptide sequence reduces aortic atherosclerosis and plaque inflammation. Such acquired immunity and athero-protective effect appears to be mediated by splenocytes. These data demonstrate the feasibility of peptide based immunomodulating therapy for atherosclerosis.
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Atherosclerosis is a chronic arterial inflammatory disease that starts at young age with its usual clinical manifestation occurring later in life [1–3]. Cholesterol-lowering medications focused on LDL-lowering reduce morbidity and mortality from atherosclerotic vascular disease but adverse cardiovascular clinical events continue to occur despite LDL-lowering therapy. These facts highlight the need for additional preventive and therapeutic interventions for atherosclerosis.

The involvement of both the innate as well as adaptive immune system in atherogenesis is being increasingly recognized with evidence for both pro-atherogenic and athero-protective effects of the immune system [4]. Selective inhibition of pro-atherogenic immune responses and/or activation of athero-protective immune responses may provide novel additional approaches for inhibition of atherosclerotic vascular disease [5,6]. It has been known for sometime that immune response to oxidized LDL is evident in experimental as well as human atherosclerosis. Until a

few years ago the precise impact of such an immune response to oxidized LDL on atherosclerosis was unclear; however, several studies have now consistently demonstrated that immunization of rabbits or mice with homologous oxidized or MDA-modified LDL reduces atherosclerosis, suggesting that at least part of the immune response to antigens in oxidized LDL is athero-protective [7–10].

The precise identities of antigenic epitopes in native or oxidized LDL are not known. In an attempt to identify antigenic epitopes in apo B-100, the protein part of LDL, we designed a polypeptide library of 302 peptides containing peptide sequences spanning the length of apo B-100 out of which 100 peptide epitopes appeared to act as antigens [11]. In a previous study, immunization with a mixture of several native unmodified apo B-100 related peptide antigens reduced atherosclerosis in hyperlipidemic mice [12]. In our continuing efforts to further define the effects of immunization using apo B-100 related peptide antigens and unlike our previous study [12], we tested the efficacy and feasibility of immunization using single peptide as antigen in modulating atherosclerotic lesions in the present study. Furthermore, we also tested the effects of early and late immunization as well as the transferability of such peptide-based acquired immunity on modulating atherosclerosis.

Methods

Establishment of a polypeptide library of apo B-100 related sequences. The establishment of a polypeptide library has been previously reported [11]. Briefly, we synthesized 302 peptides spanning the entire sequence of human apolipoprotein B-100 (Accession No. P04114). Peptides were 20 amino acids long with a 5 amino acid overlap to cover all sequences at breaking points and numbered 1–302 starting at the N-terminal end of the protein. Out of these 302 peptides, 100 sequences were identified to which an immune response could be recognized using pooled human sera as previously reported [11]. In this report, we selected two such synthetic peptide sequences from the polypeptide library. The amino acid sequence of peptide-1 was EEEMLENVSLVCPKDATRFK and that of peptide-2, adjacent to peptide-1, was ATRFKHLRKYTYNYEAESSS. Peptide-1 and Peptide-2 had 70% and 95% sequence homology, respectively, to murine apo B protein (Accession No. AAH38263). Peptides were purchased from Euro-Diagnostica AB, Malmö, Sweden.

Antigen preparation. Peptide antigen was prepared using Imject SuperCarrier EDC kit (Pierce, Rockford, IL) according to manufacturer's instruction with minor modification. One milligram peptide in 500 µl conjugation buffer was mixed with 2 mg carrier in 200 µl deionized water. This mixture was then incubated with 1 mg conjugation reagent (EDC, 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide HCl) at room temperature for 2 h followed by dialysis against 0.083 M sodium phosphate, 0.9 M sodium chloride, pH 7.2, solution overnight at 4 °C. The dialyzed conjugate was diluted with Imject dry blend purification buffer to a final volume of 1.5 ml. Alum was used as immunoadjuvant and mixed with peptide conjugate with 1:1 dilution in volume. The amount of peptide in each immunization was 33 µg/100 µl per injection.

Animal protocol. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center. All mice were housed in an animal facility accredited by the American Association of Accreditation of Laboratory Animal Care, kept on a 12-h day/night cycle, and had unrestricted access to water and food. At the time of sacrifice, mice were anesthetized by inhalation of Enflurane. Mice were purchased from the Jackson Laboratories (Bar Harbor, ME).

I. Peptide immunization

For early immunization study, male apo E (−/−) mice, maintained on normal chow diet, received subcutaneous primary immunization with peptide-1 ($n = 10$), peptide-2 ($n = 10$) or alum alone ($n = 9$) at 6–7 weeks of age, followed by an intra-peritoneal booster 3 weeks later. Two weeks after booster, diet was switched to high cholesterol chow (0.15% cholesterol, 21% fat, TD 88137, Harlan-Teklad) and continued until sacrifice at the age of 25 weeks. Blood samples were collected by retro-orbital bleeding 2 weeks after booster and at the time of sacrifice. For late immunization study, male apo E (−/−) mice were kept on high cholesterol diet since 6–7 weeks of age. Since peptide-1 immunization did not reduce atherosclerosis (see Result below), peptide-1 was omitted in this part of the study. At 16 weeks of age, mice received subcutaneous primary immunization with peptide-2 or alum alone, followed by an intra-peritoneal booster 3 weeks later. Mice were sacrificed at age of 30 weeks. A separate group of mice were kept on high cholesterol diet and sacrificed at 16 weeks of age to serve as baseline control.

II. Transfer of splenocytes from immunized mice to naïve recipients

In another experiment, mice were immunized and kept on normal chow diet as described in early immunization. At 12 weeks of age, mice were splenectomized and kept alive afterward (donor groups). Splenocytes were then isolated using sterile techniques (see below). Naïve recipient mice, of the same age and on normal chow diet, received pooled splenocytes via tail vein injection. Both donor and recipient mice were then placed on a high cholesterol chow until sacrifice at the age of 25 weeks. Blood samples were collected by retro-orbital bleeding at the time of sacrifice.

Splenocyte isolation and transfer. Splenocytes were isolated as previously described [13]. Briefly individual spleen from donor mouse was sliced into 2 mm pieces and pressed through a sterile wire mesh (Cellector, Bellco Glass). Red blood cells were lysed and the cells were suspended in PBS/1% FBS (Gibco/BRL). Splenocytes were pooled, collected by centrifugation, and resuspended in PBS. Each recipient mouse received 7.5×10^7 splenocytes via tail vein injection. The number of donor mice equaled the number of recipient mice.

Tissue harvesting, sectioning, and en face preparation of descending aorta. After the heart and aortic tree were perfused with normal saline at physiological pressure, the heart and proximal aorta were excised and embedded in OCT compound (Tissue-Tek) and cryo-sectioned as described before [14,15]. Descending thoracic and abdominal aorta was cleaned, processed, and stained with Oil Red O to assess the extent of atherosclerosis with computer-assisted histomorphometry as described previously [15].

Immunohistochemistry and histomorphometry. The sections from aortic sinus were stained with MOMA-2 antibody (Serotec) for immunocytochemical identification of macrophages using standard protocol. Trichrome stain to assess collagen content and Oil Red O stain for plaque size and lipid content were done using standard protocol. Computer-assisted morphometric analysis was performed to assess histomorphometry as described previously [14].

Measurement of peptide-specific IgM and IgG or antibody against LDL. Antibody titer against immunized human peptide was measured utilizing the ELISA protocol described previously [12]. In brief, native synthetic peptides diluted in PBS, pH 7.4 (20 µg/ml), were absorbed to microtiter plate wells (Nunc MaxiSorp, Nunc, Roskilde, Denmark) overnight at 4 °C. After serial washing and blocking, mouse serum (1:100) in TBS-0.05% Tween 20 (TBS-T) was added to ELISA wells for 2-h incubation at room temperature and then overnight at 4 °C. After detection antibody was added, an alkaline phosphatase conjugated streptavidin (Sigma) was used for color development. The absorbance was read at 405 nm and presented as optical density (OD) unit after the background was subtracted. In some instances, pooled mouse serum (1:100) was incubated with various concentrations of nLDL or copper-oxidized LDL (CuoxLDL, 3, 10, 30 or 100 µg/ml) for 2 h at room temperature and then overnight at 4 °C for competitive ELISA. The samples were then added to ELISA plates for antibody determination as described above.

For determination of antibody titer against LDL, an ELISA against nLDL or CuoxLDL was developed. Briefly, human LDL (Biomedical Technologies, MA) was dialyzed against PBS overnight to remove EDTA and subsequently oxidized by incubating with $CuSO_4$ (5 μl of 5 μM $CuSO_4$ in 100 μl of 0.5 mg/ml LDL) for 24 h at 37 °C. ELISA plates were coated with 10 μg/ml nLDL or CuoxLDL under the same condition as described above. Mouse serum (1:100) was used for ELISA and detection antibody was HRP-conjugated goat anti-mouse IgG with ABTS as substrate. The ratio of absorbance reading from CuoxLDL divided by the absorbance reading from nLDL was calculated to quantify antibody titers to minimize non-specific binding to LDL as described previously [16]. Due to limited availability of serum, not all animals in each group were included for antibody titer determination.

RT-PCR. Total RNA from spleen was isolated using Trizol Reagent (Life Technologies). cDNA was prepared using Thermoscript RT system (Life Technologies). An aliquot of RT product was subjected to PCR after cycling conditions were optimized. The primer sequence used for the amplification of IFN-γ, IL-4, IL-5, IL-10, and β-actin had been published previously [17–19]. The densitometric analyses of IFN-γ, IL-4, IL-5, and IL-10 bands were standardized to the corresponding β-actin density.

Statistics. Data are presented as means ± STD. Statistical method used is listed in text or figure legend. Normally distributed data were analyzed by parametric method, whereas non-normally distributed data were analyzed by non-parametric method. A value of $p < 0.05$ was considered as statistically significant.

Results

Effect of peptide immunization on atherosclerosis

Early immunization group

At the time of sacrifice, mice immunized with peptide-2 weighed significantly more than the other 2 groups (38.7 ± 4.6 vs. 33.0 ± 4.5 and 34.0 ± 3.5 grams, respectively, $p < 0.05$) and their mean serum cholesterol levels tended to be the highest among the 3 groups although the differences were not statistically significant (Alum group: 1081 ± 386 mg/dl; peptide-1 group: 715 ± 386 mg/dl; peptide-2: 1425 ± 180 mg/dl). The extent of aortic atherosclerosis in the descending thoracic and abdominal aorta between alum group and peptide-1 group was similar; however, peptide-2 immunized mice, despite highest circulating cholesterol levels, showed a 40% reduction in the extent of aortic atherosclerosis compared to the control (Figs. 1A and B). Aortic sinus plaques of mice immunized with peptide-2 had significantly lower macrophage immunoreactivity (Figs. 1C and D) and significantly higher collagen content compared to controls (Figs. 1E and F). There was no difference in the aortic sinus plaque size among 3 groups (data not shown).

Immunization with peptide-1 or 2 significantly increased IgG titer against these peptide sequences at 2 weeks after booster, but titer was not different compared to Alum group at sacrifice (Table 1). Native LDL or copper oxidized LDL did not compete with the specific binding to peptides by competitive ELISA (data not shown). However, only peptide-2 immunization induced a persistently higher IgM titer both at 2 weeks after booster and at sacrifice (Table 1). The expression of splenic cytokines such as IFN-γ, IL-4 and IL-10 at sacrifice was significantly higher in peptide-1 or 2 immunization group when compared to Alum group, whereas there was no significant difference in IL-5 expression (Table 2). These data indicated peptide immunization generated both humoral and cellular immune responses in immunized mice.

Since peptide-2 immunization reduced aortic atherosclerosis, we further characterized the antibody responses to LDL in mice immunized with peptide-2. Two weeks after booster immunization, the IgG titer against oxLDL was similar (Alum group 1.8 ± 0.6, $n = 7$; peptide-2 group 1.4 ± 0.4, $n = 10$), whereas the IgG titer was significantly higher in peptide-2 immunized group at the time of sacrifice (Alum group 1.9 ± 0.3, $n = 7$; peptide-2 group 2.6 ± 0.7, $n = 10$; $p < 0.05$ by t test).

Late immunization group

We further tested whether peptide-based immunization was able to retard atherosclerotic progression if immunization was started after disease process had already begun and animals had been exposed to hypercholesterolemic pro-atherogenic auto-antigens. The rationale underlying this approach is to test whether this concept is feasible for "therapeutic" use since most of patients with clinically manifested atherosclerotic vascular disease have had atherosclerosis for some time. Hence, we tested this strategy in older hypercholesterolemic mice. Peptide-2 immunization resulted in a significant 42% overall reduction in the progression of aortic atherosclerosis compared to Alum group (Fig. 2), despite comparable levels of circulating cholesterol (16 week control: 1292 ± 252 mg/dl, $n = 8$; Alum group: 929 ± 440 mg/dl, $n = 9$; peptide-2 group: 1033 ± 371 mg/dl, $n = 10$).

Similar to the early immunization study, late immunization with peptide-2 had a significantly higher IgM titer against peptide-2 when compared to Alum group at sacrifice (1.003 ± 0.161 OD unit vs. 0.787 ± 0.109 OD unit, $n = 5$ each group, $p = 0.038$ by t test). There was no difference in IgG titer against peptide-2 between these 2 groups.

The levels of IgG (Alum group 4.9 ± 2.3, $n = 8$; peptide-2 group 4.2 ± 2.2, $n = 8$) or IgM (Alum group 6.2 ± 1.7, $n = 8$; peptide-2 group 5.3 ± 1.6, $n = 8$) titer against copper oxidized LDL between peptide-2 immunized mice and mice from Alum group at sacrifice were similar.

Effect of splenocyte transfer from peptide-2 immunized mice on atherosclerosis

While adoptive transfer of splenic B-cells from non-immunized donor apoE (−/−) mice could reduce atherosclerosis in non-splenectomized recipients [20], it is not known whether acquired immunity from immunization can be transferred to confer athero-protection in non-splenectomized recipients. Both splenectomized donor and recipient groups were sacrificed at 25 weeks of age. Plasma cholesterol levels at sacrifice did not differ among the donor groups; whereas unimmunized mice receiving splenocytes from peptide-2 immunized mice had lower
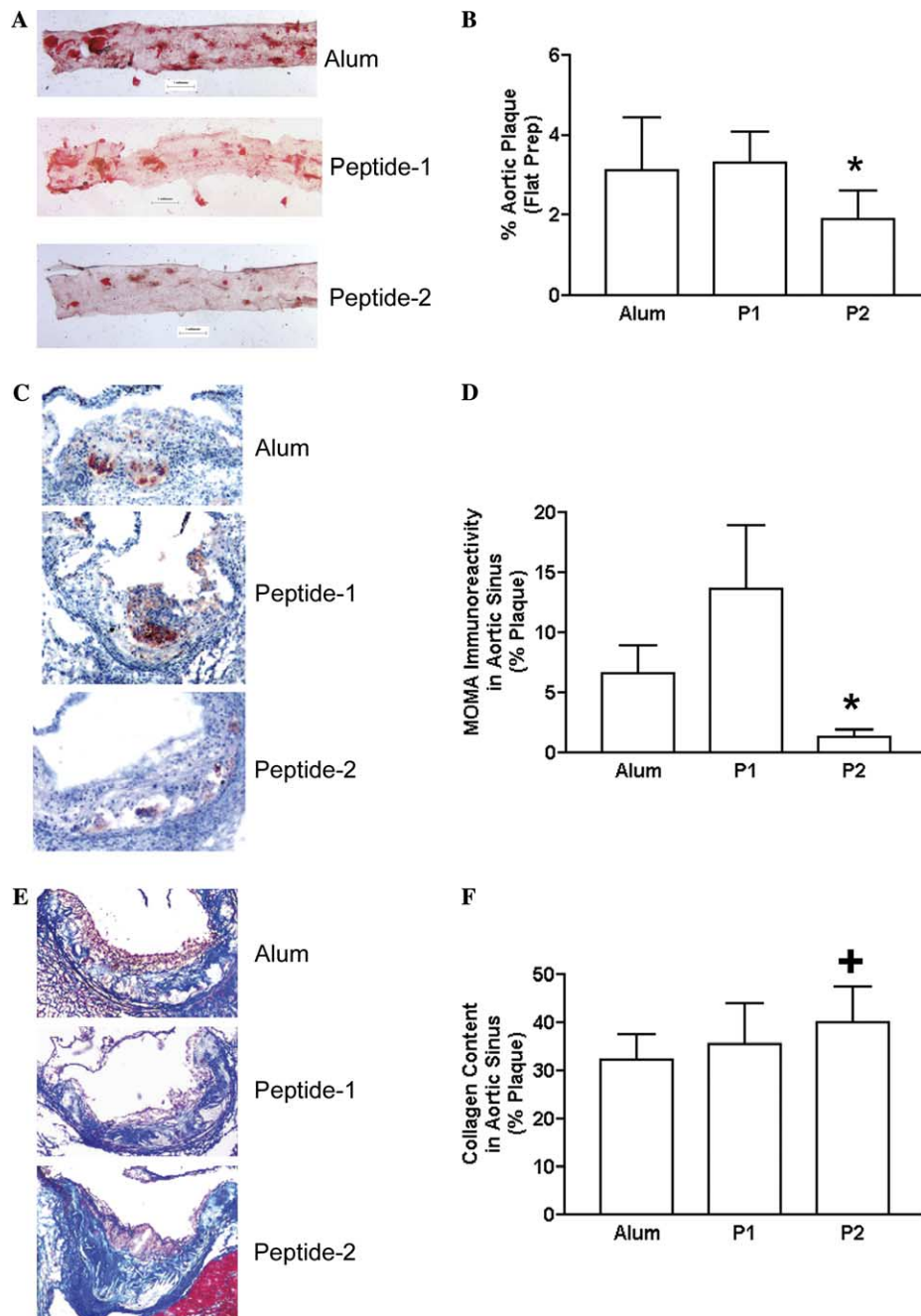
Fig. 1. In the early immunization study, the extent of aortic atherosclerosis was assessed by oil red o staining of en face preparation from mice. Representative picture from each group is shown in (A) and the bar graph in (B) compared the extent of aortic atherosclerosis among the groups studied. The extent of macrophage immunoreactivity in the aortic sinus was assessed by MOMA immunostaining. Representative picture from each group is shown in (C) and the bar graph in (D) compared the extent of immunoreactivity among the groups studied. The collagen content in the aortic sinus was assessed by Trichrome staining. Representative picture from each group is shown in (E) and the bar graph in (F) compared the collagen content among the groups studied. "*" $p < 0.05$ compared to Alum and peptide-1 group by ANOVA followed by Newman–Keuls multiple comparison test. "+" $p < 0.05$ compared to Alum group by Kruskal–Wallis test followed by Dunn's multiple comparison test.

cholesterol levels compared to unimmunized mice receiving splenocytes from alum or peptide-1 immunized mice (Table 3).

Among the donor mice, splenectomy abolished the athero-protective effect of peptide-2 immunization (Table 3). However, unimmunized mice receiving splenocytes from peptide-2 immunized mice had a 60% reduction in the extent of aortic atherosclerosis, in comparison to unimmunized mice receiving splenocytes from alum- or peptide-1 immunized mice (Table 3). Although Peptide-2 recipient mice had a lower level of plasma cholesterol level compared to the other groups, there is no linear relationship between plasma cholesterol level and the amount of aortic atherosclerotic lesions measured on flat prep, suggesting the

Table 1
IgM and IgG titers (OD unit) against human peptide sequence at 2 weeks after booster and at sacrifice

	2 weeks after booster	p value	At sacrifice	p value
IgG titer				
Alum group	0.001 ± 0.003	$0.052^{\#,\&}$	0.126 ± 0.136	NS
Peptide-1 group	0.099 ± 0.149		0.073 ± 0.189	
Alum group	0.033 ± 0.016	$0.038^{\#}$	0.077 ± 0.063	NS
Peptide-2 group	0.055 ± 0.026		0.087 ± 0.040	
IgM titer				
Alum group	0.702 ± 0.405	NS	0.354 ± 0.152	NS
Peptide-1 group	0.545 ± 0.235		0.312 ± 0.221	
Alum group	0.151 ± 0.286	0.015^{*}	0.070 ± 0.132	0.008^{*}
Peptide-2 group	0.259 ± 0.102		0.159 ± 0.115	

$N = 10$ in each group; "#" by unpaired t test; "&" one outlier exists in peptide-1 group and p value becomes 0.0005 if the outlier is removed from analysis "*" by Mann–Whitney test. ELISA plates used to determine the IgG or IgM titer for Alum group were coated differently with peptide-1 or peptide-2 respectively as controls for peptide-1 or peptide-2 group.

Table 2
Densitometric analysis of splenic cytokine expression at sacrifice

	Alum group	Peptide-1 group	Peptide-2 group
INF-r/β-actin ratio	32.1 ± 21.4	$84.8 \pm 8.8^{*}$	$83.0 \pm 6.4^{*}$
IL-10/β-actin ratio	8.8 ± 6.3	$29.6 \pm 13.9^{*}$	$29.0 \pm 5.1^{*}$
IL-4/β-actin ratio	5.2 ± 4.8	$18.6 \pm 7.6^{*}$	$17.5 \pm 4.0^{*}$
IL-5/β-actin ratio	100.2 ± 22.2	90.3 ± 32.8	99.2 ± 10.6
	$(n = 7)$	$(n = 6)$	$(n = 6)$

$N = 4$ in each group otherwise indicated.
* $p < 0.05$ compared to alum group by ANOVA followed by Newman–Keuls multiple comparison test.
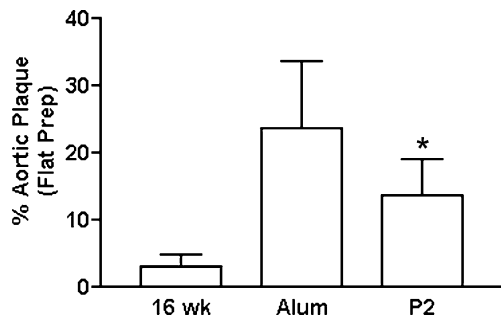

Fig. 2. Comparison of the extent of aortic atherosclerosis as assessed by oil red O staining of en face preparation among groups studied in the late immunization experiment. Both Alum and peptide-2 immunized mice were sacrificed at 30 weeks. "*" $p < 0.05$ compared to 16-week control and Alum group by ANOVA followed by Newman–Keuls multiple comparison test.

reduction of aortic atherosclerosis is independent of the plasma cholesterol level (Fig. 3).

Due to limited availability of mouse serum, pooled serum was used for the determination of IgG or IgM titers against peptide-2 in the donor or splenocyte recipient mice. There was no difference in the levels of peptide-2 IgM (Alum group: 1.091 OD unit vs. peptide-2 group: 1.028

Table 3
Effect of splenectomy and splenocytes transfer on plasma cholesterol levels, body weight, and extent of atherosclerotic lesions in aorta or aortic sinus from donor (after splenectomy) and splenocyte recipient groups

	Plasma cholesterol (mg/dl)	BW (g)	Aorta (%)	Aortic sinus (mm^2)
Donor group				
Alum ($n = 5$)	1081 ± 364	29.7 ± 4.0	2.6 ± 0.8	0.416 ± 0.151
Peptide-1 ($n = 4$)	1339 ± 613	34.2 ± 2.5	4.7 ± 1.1	0.455 ± 0.097
Peptide-2 ($n = 9$)	910 ± 459	35.2 ± 5.8	3.0 ± 1.7	0.446 ± 0.175
Recipient group				
Alum ($n = 15$)	1148 ± 555	35.3 ± 4.0	5.6 ± 2.9	0.437 ± 0.133
Peptide-1 ($n = 4$)	1629 ± 374	32.1 ± 3.7	5.0 ± 2.9	0.458 ± 0.053
Peptide-2 ($n = 9$)	$560 \pm 239^{*}$	30.7 ± 7.6	$2.1 \pm 1.8^{*}$	0.330 ± 0.133

* $p < 0.05$ compared to alum and peptide-1 recipient group by ANOVA followed by Newman–Keuls multiple comparison test.
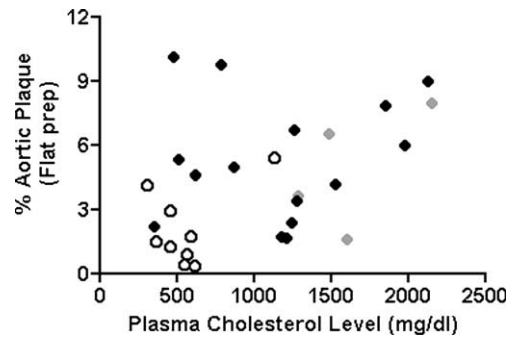

Fig. 3. Scatter plot demonstrating the lack of linear relationship between circulating cholesterol levels and the extent of aortic atherosclerosis on en face preparation from recipient mice. Dark circle, alum group; gray circle, peptide-1 group; and open circle, peptide-2 group.

OD unit) or IgG (Alum group: 0.492 OD unit vs. peptide-2 group: 0.461 OD unit) titers between Alum donors or peptide-2 donors at sacrifice. However, the level of IgM titer against peptide-2 was higher in the mice that received splenocytes from peptide-2 immunized donors when compared to mice that received splenocytes from Alum immunized donor mice (peptide-2 group: 0.807 OD unit vs. alum group: 0.739 OD unit). IgG titer was lower in the mice that received splenocytes from peptide-2 immunized donors (peptide-2 group: 0.392 OD unit vs. alum group: 0.469 OD unit). There was no difference in the IgG or IgM titer against oxLDL between the Alum and peptide-2 immunized mice in the donor groups or in the recipient groups (data not shown).

Discussion

Our study demonstrates the following novel points that have not been reported before: (i) immunization of apo E null mice at 6–7 weeks of age using a single specific unmodified human apo B 100-related peptide sequence (peptide-2) as an antigen significantly reduces aortic atherosclerosis and plaque macrophage immunoreactivity; (ii) peptide-2 immunization also retarded the progression of aortic

atherosclerosis when immunization was started after atherosclerosis was established; (iii) such acquired peptide-based athero-protective immunity could be adoptively transferred through splenocytes to unimmunized mice.

The specificity of the effects of peptide-2 immunization is supported by the lack of athero-protection by immunization with a different peptide sequence (peptide-1) even though both peptide-2 and peptide-1 bear high homology to mouse apoB. The protective effects of certain peptide sequences and not others suggest that only specific apoB-100 related peptide sequences provoke an athero-protective immune response [11,12]. This peptide-based immunization strategy is potentially advantageous over the use of homologous oxLDL or native LDL as antigen because of the ease of synthesis and standardization of peptide purity and avoidance of isolating LDL and its attendant risks for contamination.

It is unclear why only one of the two peptides tested was atheroprotective even though both were highly homologous to the mouse apoB-100. We do not think this distinction is due to differences in antigen presentation because both peptides elicited IgG responses. This difference in athero-protective effect could lie in the ability of peptide-2 to induce an IgM response. This speculation is based on recent observations that IgM isotype antibody provided athero-protection when several different antigenic epitopes were used for immunization. For example, immunization with *Pneumococcal* extract using Freund's adjuvant induced high IgM titer against oxLDL with high cross-reactivity to phosphorylcholine and moderate IgG_{3a} response against oxLDL [21]. In the same study mice receiving Freund's adjuvant alone also showed an IgM response to oxLDL with much lower titer and without cross-reactivity to phosphorylcholine and minimal IgG_{3a} response against oxLDL. Yet, both strategies reduced atherosclerotic lesion size. Recently immunization with MDA-LDL was shown to induce not only adaptive humoral immunity against MDA-LDL but also innate immune response with high IgM titer against phosphorylcholine epitopes, all of which appear to be important in modulating atherosclerosis [22]. Our observation that only peptide-2 immunization elicited a sustained IgM antibody response at sacrifice further suggested that the antibody isotype elicited by immunization could be important. On the other hand, experimental evidence exists that IgG antibody against modified LDL could also be important in mediating athero-protection [9,23,24]. Further passive immunization experiments using different isotypes of antibody against the same (or different) antigenic epitope is needed to differentiate the efficacy of IgM vs. IgG in modulating atherosclerosis.

It is unlikely that changes in circulating cholesterol levels play a major role in the effects observed since peptide-2 immunization group had the highest cholesterol levels compared to peptide-1 or Alum immunized mice and yet only peptide-2 immunization reduced atherosclerosis and plaque inflammation. Splenectomy was also previously shown to increase atherosclerosis without changing cholesterol

level [20]. Similarly activation of an immune response in other studies, utilizing antigens such as MDA-LDL, native LDL, and *Pneumococcus*, was also shown not to significantly alter circulating cholesterol levels [9,21]. Based on these reports, there does not seem to be a direct association between reduction of atherosclerosis and plasma cholesterol level. However, we did observe a reduced cholesterol level among unimmunized mice receiving splenocytes from peptide-2 immunized mice for reasons that are unclear. This finding is not unique in our experiment. Caligiuri et al. [20] also reported adoptive transfer of splenic B or T-cells reduced circulating cholesterol levels in non-splenectomized recipient. In our study, further analysis revealed there was no direct relationship between circulating cholesterol level and aortic lesion reduction, suggesting athero-protection by adoptive transfer of splenocytes from peptide-2 immunized mice is independent of plasma cholesterol level. However, we cannot completely rule out the possibility that at least some of the effect observed during adoptive transfer experiments may have been related to changes in circulating cholesterol levels.

Activation of a cellular and humoral immune response by peptide immunization was demonstrated in this study. It is generally hypothesized that the immune response elicited by LDL immunization is MHC-II mediated and T-cell dependent. Since LDL is a heterogeneous molecule, it is likely that multiple peptidic epitopes are presented by the MHC-II molecule. The exact nature of these epitopes is not known. In a previous study, we demonstrated that several peptide sequences from apo B-100 are able to induce an athero-protective immune response in hyperlipidemic mice. The current report concurs with that study. Whether these peptide sequences are the same as the ones that are presented by antigen presenting cells after endogenous LDL is processed is not known. In the current study, antibodies generated against peptide-2 did not cross-react with LDL (native or modified) and had a different time-course profile compared to antibodies against LDL. This suggests that peptide-2 may not necessarily be one of the spontaneously generated epitopes presented to the immune system during immune response to endogenous oxidized LDL. An alternative explanation is that peptide-2 sequence was destroyed during ex vivo oxidation of LDL, or is not exposed on oxLDL.

Atherosclerosis is a chronic inflammatory arterial disease and generates immune responses against endogenous auto-antigens. It is possible that the spontaneously elicited immune response is not sufficient enough to clear the offensive antigens, a situation well known in chronic infection (such as hepatitis C or HIV infection). During its natural course, the specific immune response may become exhausted [25]. Hence, boosting the immune response by immunization (in our case—peptide-2) could re-expand immune cells that are responsible for athero-protection. In the late immunization experiment, our observation that peptide-2 immunization reduced progression of atherosclerosis in mice under a hypercholesterolemic pro-atherogenic

condition with long exposure to auto-antigens is consistent with this view and suggest the concept that "therapeutic" role of immunization is feasible.

Our finding that splenectomy abolished the beneficial athero-protective effects of immunization and splenocytes from immunized mice conveyed an athero-protective effect to unimmunized mice is consistent with a report suggesting the athero-protective role of spleen [20]. Splenectomy at young age enhances spontaneous atherogenesis and is accompanied by a reduction of IgG antibody titer against MDA-modified LDL. Such effects in splenectomized mice can be reversed by reconstitution with syngeneic splenocytes or B cells [20]. This observation, together with our findings, suggests that spontaneously developed immunity against modified LDL or "acquired" immunity against a specific epitope plays an important role in modulating atherogenesis. The spleen has been shown to be important in lipid and LDL metabolism [26–28], and is also known to be involved in the clearance of blood-born pathogens through immune-complex formation. The rapid removal of glycated LDL immune-complex by reticuloendothelial system [29] and the formation of IgM/apoB complex after MDA-LDL immunization [22] is consistent with this view. However, a recent report demonstrated that autoantibodies to oxLDL failed to alter the clearance of oxLDL [30]. These discrepant findings could be due to the difference in the epitopes to which the antibody react, or in the class of antibody (IgM vs. IgG), or both. In this study, we did not address which specific cell types from splenocyte isolates (T-cells vs. B-cells) are responsible for the adopted athero-protective effect of peptide-2 immunization. However, several studies have suggested that splenic B-cells could be the cell type responsible for the observed beneficial effect [20,31].

Removal of apoB containing immune complexes may not be the only mechanism because in spontaneous atherosclerosis splenic B lymphocyte transfer into splenectomized mice compensated for the atherosclerosis-enhancing effect from splenectomy [20]. An alternative explanation could be that there are specialized B cells that are important in mounting immune response to immunogens. The role of specialized B cells has been shown in another model of immunization [32]. It is possible that in our splenectomized mice the benefit of immunization was negated upon removal of such specialized cell types. However, the contribution of such specialized B cells in reducing atherosclerosis in our splenocyte recipient mice may be small since all recipient mice had intact spleens and yet only mice receiving splenocytes from peptide-2 immunized mice had reduction in atherosclerosis.

Together with other studies of LDL immunization and our previous report on immunization using mixtures of multiple peptides, this study further strengthens the concept that immune modulation therapy can produce athero-protective effects. The current peptide-based strategy provides not only a potentially novel therapeutic approach but also an important tool to dissect possible significant epitopes of LDL (specifically apoB-100) that can modulate atherogenesis. Additional evaluation of this potentially promising new approach to favorably modulate atherosclerosis is warranted.

Acknowledgments

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