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Inhibition of Neointima Formation through DNA Vaccination for Apolipoprotein(a): A New Therapeutic Strategy for Lipoprotein(a)

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Lipoprotein(a) [Lp(a)] is an unique lipoprotein consisting of the glycoprotein apolipoprotein(a) [apo(a)] in low-density lipoprotein. Although Lp(a) is a well-known independent risk factor for cardiovascular disease; however, there is no drugs to decrease plasma Lp(a) level. Thus, to inhibit the biological activity of Lp(a), we developed DNA vaccine for apo(a) by the targeting to the selected 12 hydrophilic amino acids in the kringle-4 type 2 domain of apo(a). Hepatitis B virus core protein was used as an epitope carrier to enhance the immunogenicity. Intramuscular immunization with apo(a) vaccine resulted in the significant inhibition of neointima formation in carotid artery ligation model using Lp(a) transgenic mice, associated with anti-apo(a) antibody and decrease in vascular Lp(a) deposition. Overall, this study provided the first evidence that the pro-atherosclerotic actions of Lp(a) could be prevented by DNA vaccine directed against apo(a), suggesting a novel therapeutic strategy to treat cardiovascular diseases related to high Lp(a).

Lipoprotein(a) [Lp(a)] is a unique plasma lipoprotein that consists of a cholesterol-rich low-density lipoprotein (LDL) particle with one molecule each of apolipoprotein B-100 (apoB) and apolipoprotein(a) [apo(a)], which are bound through a single disulfide bond[1]. Lp(a) is found only in humans, primates and hedgehogs. Apo(a) is a homolog of plasminogen[2] that contains 10 different types of plasminogen kringle-4-like repeats (kringle-4 types 1 through 10) and regions homologous to the kringle-5 and inactive protease regions[3]. Lp(a) is considered an independent cardiovascular risk factor because numerous studies have demonstrated the potent positive association between plasma Lp(a) levels and cardiovascular disease/coronary artery disease. Increased Lp(a) levels are believed to promote atherosclerosis via Lp(a)-derived cholesterol entrapment in the intima, inflammatory cell recruitment, and/or the binding of pro-inflammatory oxidized phospholipids[4]. Lipid-lowering agents such as statins have little or no effect on plasma Lp(a) levels[5]. Although niacin or estrogen might reduce plasma Lp(a) levels slightly, there is no specific agent to reduce plasma Lp(a)[6–8] or prevent Lp(a)-induced atherosclerosis.

To prevent cardiovascular events induced by Lp(a), we employed a vaccine strategy. Although vaccines are often used for infectious diseases and cancer, recent applications have expanded their use to treat common adult diseases, such as Alzheimer's disease or hypertension[9–11]. To induce both humoral and cellular immune responses, we chose plasmid DNA vaccine because the unmethylated CpG motifs in the plasmid DNA backbone have been considered to be 'built-in' adjuvants owing to their ability to activate the innate immune system by means of Toll-like receptor 9 (TLR9)[12]. In addition, recent evidence has suggested that the double-stranded structure of DNA, independently of the CpG motifs, possesses immunomodulatory effects. The present study demonstrated the inhibition of neointima formation through DNA vaccination for apo(a) in a carotid artery ligation model using Lp(a) transgenic mice.

Results

Production of anti-apo(a), but not anti-plasminogen, antibody after apo(a) DNA vaccination. We constructed our plasmid DNA to include the HBc (Hepatitis virus B core) protein because HBc is an epitope carrier protein and is able to self-assemble into icosahedral virus-like particles (VLPs) in heterologous expression systems[13]. Fig. 1a shows the plasmids that were constructed: pcDNA3.1-HBc (control vector) and pcDNA3.1-HBc-apo(a). We selected a 12-amino acid sequence (EAPSEQAPTEQR) from apo(a) as the targeted antigen. This sequence overlaps with the repeated sequence of the kringle-4 type 2 domain of apo(a) and is present multiple time in the repeated kringle-4 type 2 domain (Figs. 1b and 1c). Although apo(a) is highly similar to plasminogen (containing multiple copies of kringle-4, a single copy of kringle-5 and an inactive protease domain), the selected sequence was not highly homologous to plasminogen. The antigen sequence was a hydrophilic domain that was known as the potential B-cell epitope, as previously described[14]. First, FVB female mice, which do not express Lp(a) or apo (a),

were immunized with pcDNA3.1-HBc-apo(a) [apo(a)], pcDNA3.1-HBc [control] or saline through intramuscular administration using an electroporator three times every 2 weeks (Fig. 2a). Although FVB mice have no endogenous apo(a), the antigen of this DNA vaccine might have been recognized as a foreign substance. Titers of anti-apo(a) antibody were only observed in the apo(a) group (Fig. 2b, left). Based on an analysis of the IgG subtypes, we predicted that this immunization would lead to a Th1-biased immune response with predominantly IgG2a production (Fig. 2b, right). Six weeks after the third immunization, an additional immunization was given to the mice, which raised the titer of the anti-apo(a) antibody (Fig. 2c, left). This immunization might have also led to a Th1-biased immune response with predominantly IgG2a production (Fig. 2c, right). Importantly, anti-plasminogen antibody could not be detected after the immunizations (Fig. 2d) despite the high degree of homology between apo(a) and plasminogen, which indicated that the immunization had little effect on the fibrinolytic system.
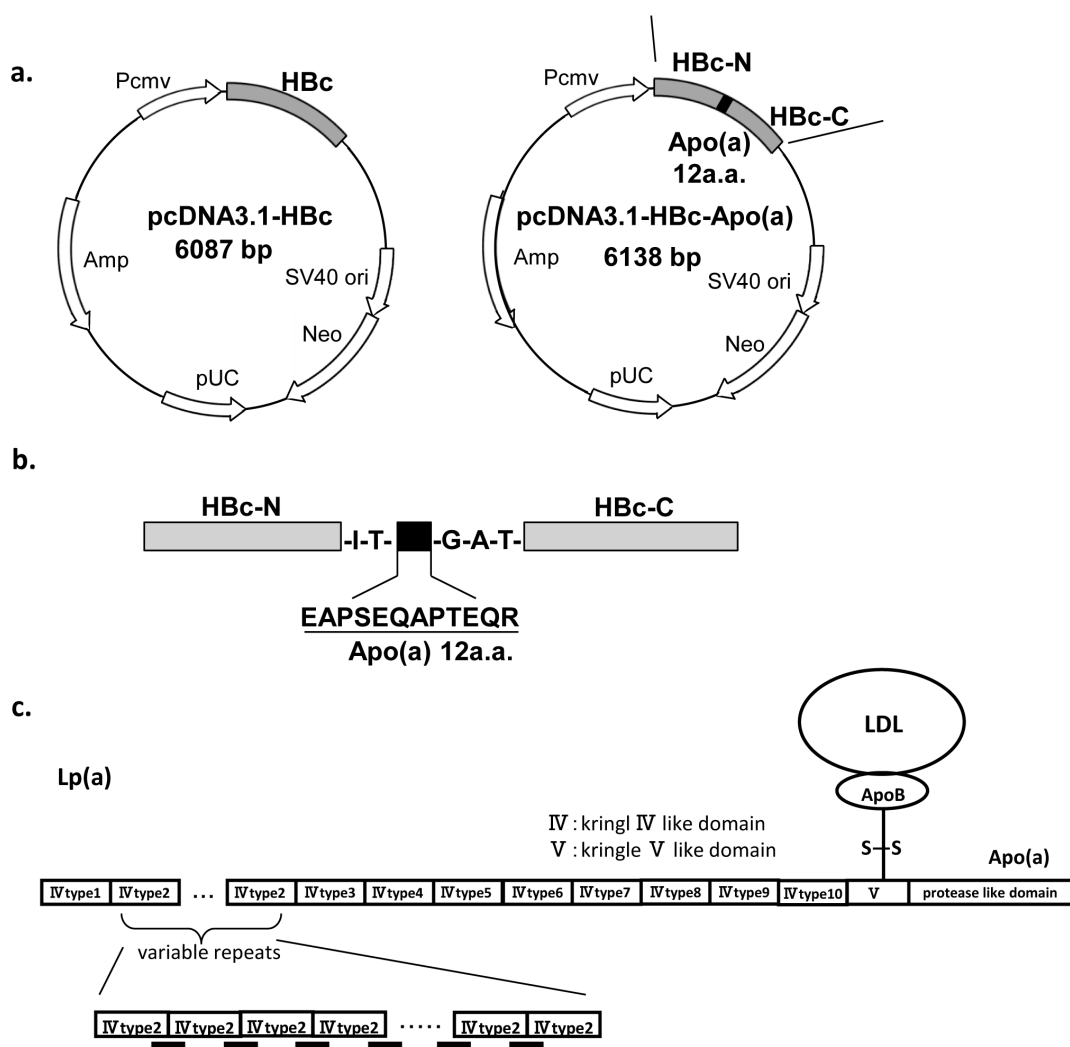

Figure 1 | Plasmid DNA construction for vaccination (a) Plasmid map of pcDNA3.1-HBc (control vector) and pcDNA3.1-HBc-apo(a) (vaccination vector). HBc indicates the full sequence of HBc. HBc-N indicates the N-terminus of HBc (1-80 a.a.), and HBc-C indicates the C-terminus of HBc (81-183a.a.). ISS indicates the CpG motifs, which consisted of four different motifs as described in the Materials and Methods section. (b) Detailed information of plasmid design the for apo(a) vaccine. Twelve amino acids (EAPSEQAPTEQR) as an antigen for apo(a) and linkers (the N-terminal I-T dipeptide linker and the C-terminal G-A-T tripeptide) were designed for in-frame fusion with apo(a) to allow flexibility in the conformation of apo(a) epitope when surface-exposed on the HBc particle. The apo(a) and the linkers are represented by single-letter codes. (c) Schema of Lp(a) to show the targeted antigen. Apo(a) principally consists of kringle IV-like domains (1-10), a kringle V-like domain, and repeated kringle IV type2 (IV type2) variable repeats. The black boxes indicate the antigen (EAPSEQAPTEQR), which overlaps the repeated sequence of the kringle IV type2 domain of apo(a) and is multiply present in the repeated kringle IV type2 domain.
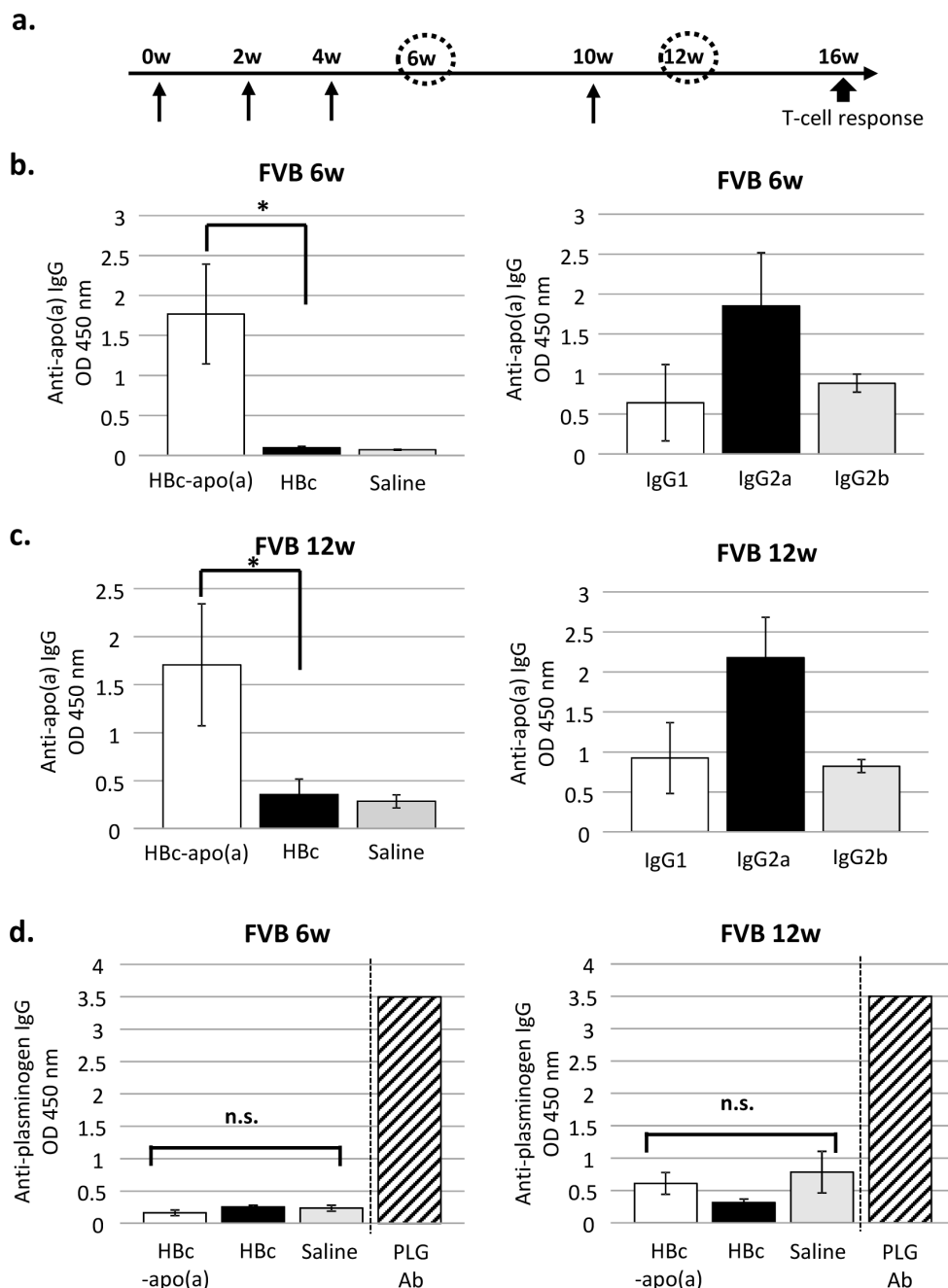
Figure 2 | DNA vaccination for apo(a) in FVB mice (a) Time course of DNA vaccination. Vaccination was initially performed using 8 week-old mice (0 w), and subsequent vaccinations were given at 2 weeks (2 w), 4 weeks (4 w), and 10 weeks (10 w) after the first vaccination. Antibody titers were quantified at 6 and 12 weeks after first vaccination, and T-cell activity was evaluated at 16 weeks after first vaccination. (b) and (c) Titers of anti-apo(a) antibodies at 6 and 12 weeks. Total IgG titers for apo(a) were increased only in mouse sera (100 dilution) from the apo(a) group (left panel). The IgG subtype distribution (IgG1, IgG2a or IgG2b) was also evaluated using subtype-specific IgG antibodies in mouse sera (100 dilution) from the apo(a) group (right panel). (d) Anti-plasminogen antibody titers in mice assayed by ELISA. Total IgG titers for plasminogen were evaluated in mouse sera (100 dilution) from the apo(a) group at 6 and 12 weeks after the first immunization, and anti-plasminogen antibody (PLG Ab) was used as a positive control.

To assess the safety and validity of the epitope [12 a.a. in apo(a)], we performed a T-cell proliferation assay and an ELISpot assay. In immunized female FVB mice, the T-cell proliferation assay showed that stimulation with apo(a) 12 a.a. did not induce the proliferation of splenocytes from immunized mice (Fig. 3a). Similarly, in the ELISpot assay, stimulation with apo(a) 12 a.a. induced the production of neither IFN-γ nor IL-4 (Fig. 3b). These data indicated that apo(a) 12 a.a. did not induce T-cell activation.

Inhibition of neointimal formation by DNA vaccine in Lp(a) transgenic mice. Because apo(a) is present only in humans, primates and hedgehogs, we used Lp(a) transgenic mice that were generated by crossing human apo(a) transgenic mice and human apoB transgenic mice[15–18] to test the biological effects of DNA vaccination for apo(a). In Lp(a) transgenic mice, serum Lp(a) levels were higher in female mice than in male mice[8]. Because bilateral ovariectomy in Lp(a) transgenic mice has been shown to
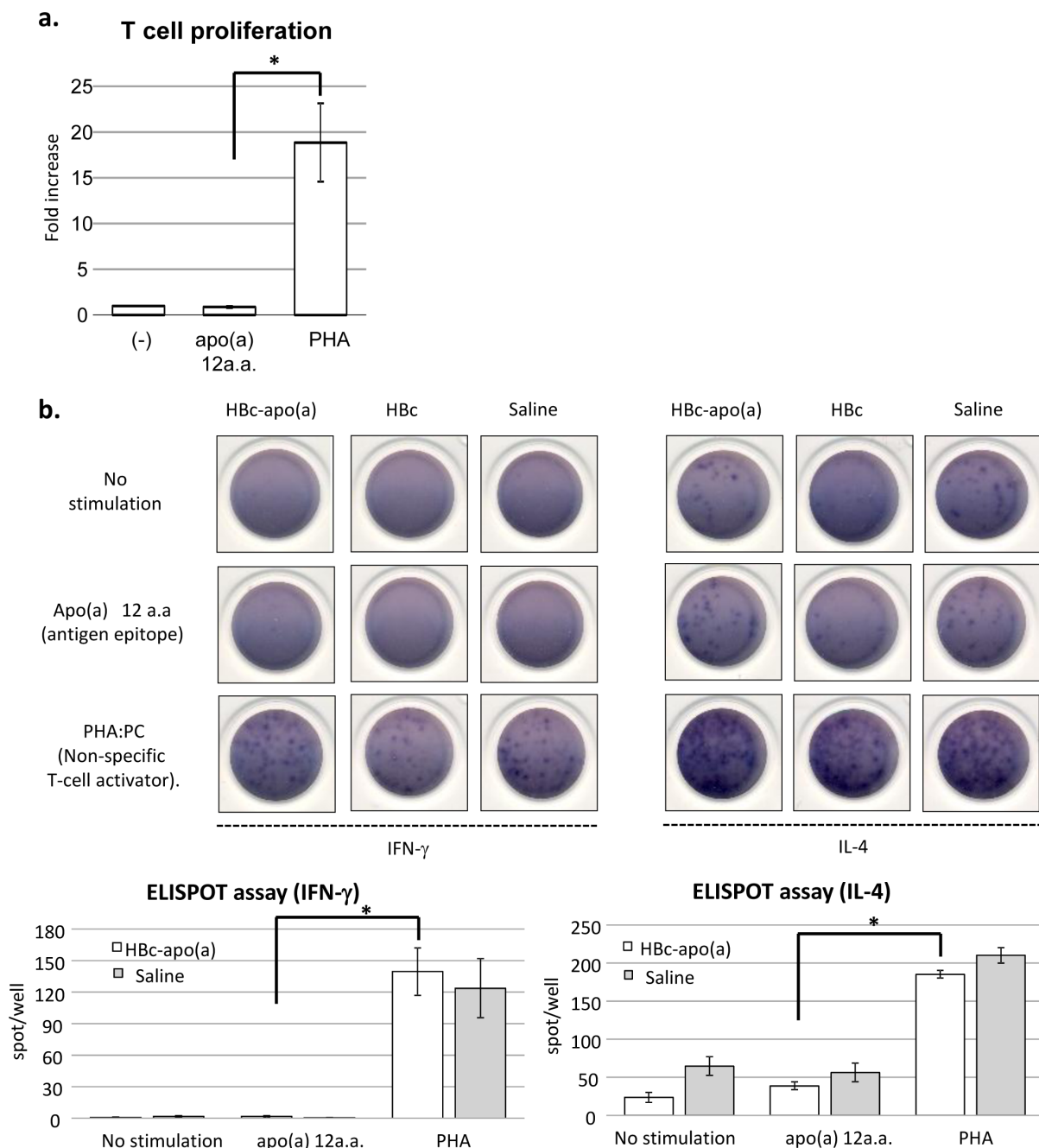
Figure 3 | T-cell responses to DNA vaccination in FVB mice. (a) T-cell proliferation assay by [³H] thymidine uptake. Cultured splenocytes from mice immunized with apo(a) vaccine plasmid DNA were stimulated or not with peptides containing the antigen sequence (apo(a) 12a.a.; EAPSEQAPTEQR). PHA was used as a positive control for non-specific T-cell activation. (b) ELISpot assay. Splenocytes were obtained from the apo(a), control or saline groups and stimulated or not with peptides containing the antigen sequence or PHA. The blue dot is the positive spot for IFN-γ (left panel) and IL-4 (right panel). (c) Quantification of ELISpot assay. Quantification was performed by counting the number of spots per well in each well. More than three wells per group were counted.

increase serum Lp(a) levels, we performed ovariectomy on our Lp(a) transgenic mice 2 weeks before the first immunization. The Lp(a) transgenic mice were immunized similarly to the FVB mice (Fig. 4a). Two weeks after the third and fourth immunizations, titers of anti-apo(a) antibody were only observed in the apo(a) group (Figs. 4b and 4c). Even in the Lp(a) transgenic mice, this immunization did not induce anti-plasminogen antibodies (Fig. 4d). The titers of anti-apo(a) antibodies maintained at a constant level at least up to until 44 weeks after the first immunization (Fig. 4e).

The neutralizing activity of the anti-apo(a) antibody induced by DNA vaccination for apo(a) was tested using a carotid artery ligation model in Lp(a) transgenic mice because flow cessation caused by ligation of the left common carotid artery leads to increased neointima formation. We previously reported that ovariectomized female Lp(a) transgenic mice significantly accelerated the neointima formation compared to that of un-ovariectomized female Lp(a) transgenic mice. Although there was no significant difference in the media thickness among the groups of Lp(a) transgenic mice that had been immunized with apo(a), control or saline (data not shown), the ratio of the neointima thickness to the media thickness was significantly lower in the apo(a) group than in the control or saline groups (Fig. 5a). Moreover, the expression of Lp(a) in the ligated vessels of
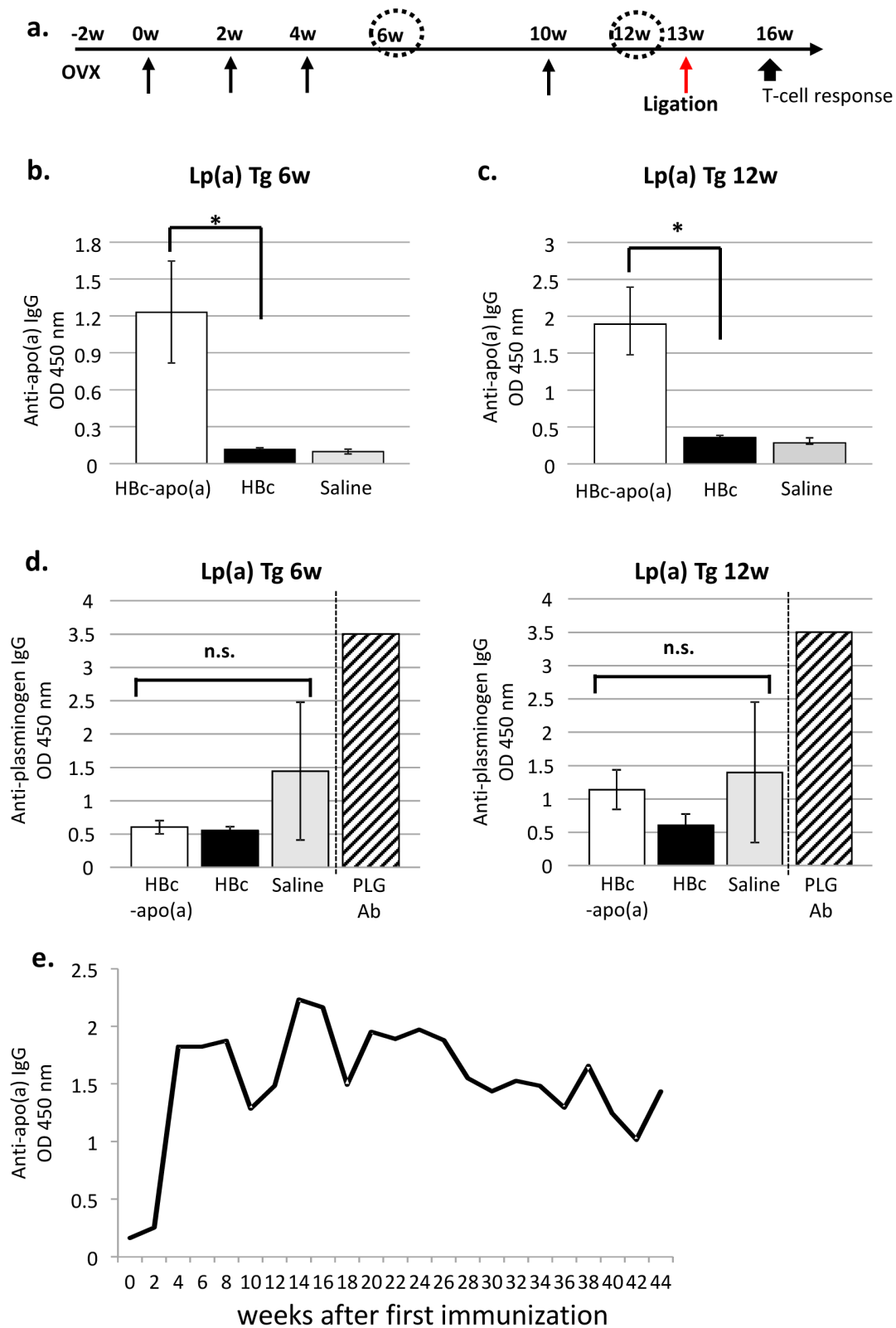
Figure 4 | DNA vaccination for apo(a) in Lp(a) transgenic female mice. (a) Time course of DNA vaccination. Female mice of Lp(a) transgenic mice were ovariectomized at 8 weeks of age. After 2 weeks, the initial DNA vaccination was performed at 10 weeks of age (0 w) and at 2 weeks (2 w), 4 weeks (4 w), 10 weeks (10 w) after the first vaccination. Antibody titers were quantified at 6 and 12 weeks after the first vaccination, and the T-cell activity was evaluated at 16 weeks after first vaccination. (b) and (c) Titers of anti-apo(a) antibodies at 6 and 12 weeks. The total IgG titers for apo(a) were increased only in mouse sera (100 dilution) from the apo(a) group. (d) Anti-plasminogen antibodies in mice assayed by ELISA. The total IgG titers for plasminogen were evaluated in mouse sera (100 dilution) from the apo(a) group at 6 and 12 weeks after the first immunization, and anti-plasminogen antibody (PLG Ab) was used as a positive control. (e) The titer of anti-apo(a) antibodies from 0 to 44 weeks after immunization.
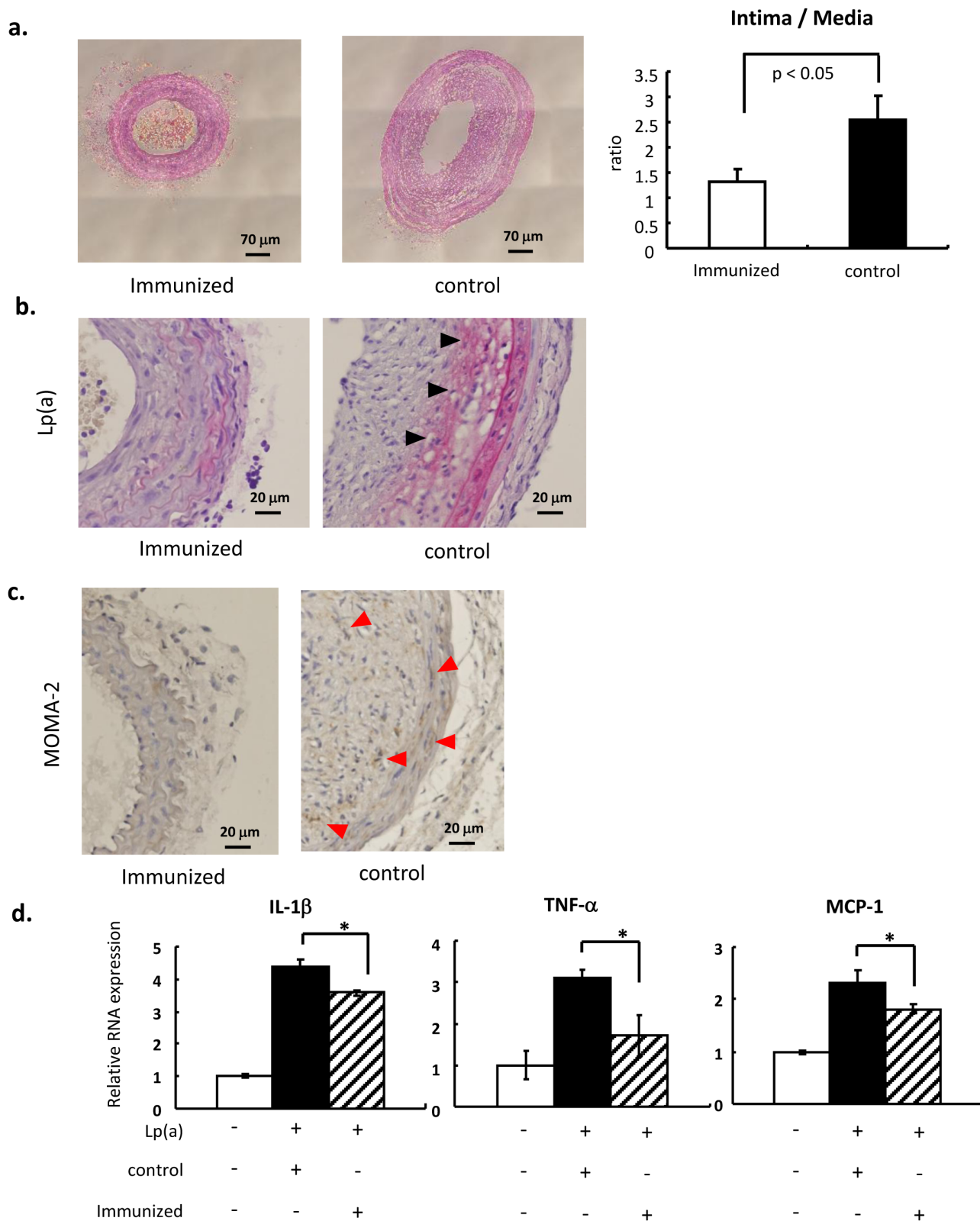
Figure 5 | Carotid artery ligation model in Lp(a) transgenic mice. (a) Carotid artery ligation was performed at 13 weeks after the first immunization, and the vascular remodeling was evaluated at 16 weeks through hematoxylin and eosin staining on the ligated vessels. Neointima formation was reduced in immunized mice [apo(a) group] compared with control (saline and control). The ratio of the neointima to the media was calculated after quantifying the areas of both layers. The bar graph shows the ratio of the neointima to the media in immunized mice and controls. (b) Immunostaining with anti-Lp(a) antibody. Lp(a) deposition (pink with black arrow) in ligated vessels was observed only in the control group. (c) Immunostaining with anti-MOMA-2 antibody. Macrophage (brown with red arrow) migration was detected only in the control group. (d) The expression of the inflammatory cytokines IL-1β, TNF-α and MCP-1 was analyzed using real-time PCR in macrophages that had differentiated from THP-1 cells in the presence of sera from immunized (apo(a) vaccination) or control group (saline and control) mice. *$P < 0.05$.

Lp(a) transgenic mice was assessed using immunohistochemistry because the deposition of Lp(a) has been observed in thickened blood vessel. Notably, the deposition of Lp(a) in the ligated vessels was markedly decreased in the apo(a) group compared with the control group (Fig. 5b). Furthermore, the migration of macrophages was also decreased in the ligated vessels of the apo(a) group compared with the control group, as assessed by immunohistochemistry for a macrophage marker with an anti-MOMA-2 antibody (Fig. 5c). Finally, to further evaluate the neutralizing activity of apo(a) vaccine-induced antibody, the expression of IL-1β, TNF-α and MCP-1 induced by Lp(a) was quantified in macrophages that were differentiated from THP-1 cells using sera from the mice. The serum from mice that had immunized with the apo(a) vaccination significantly inhibited the expression of IL-1β, TNF-α and MCP-1 compared with the serum from control mice. These results suggested that the apo(a) vaccination attenuated the pro-atherosclerotic action of Lp(a) through the induction of neutralizing anti-apo(a) antibodies.

Similarly to the FVB mice, in the immunized Lp(a) transgenic mice, the T-cell proliferation assay showed that stimulation with apo(a) 12 a.a. did not induce the proliferation of splenocytes from immunized mice (Supplement Fig. 1). In the ELISpot assay, stimulation with apo(a) 12 a.a. induced the production of neither IFN-γ nor IL-4 (Supplement Fig. 2). These data also indicated that apo(a) 12 a.a. did not induce T-cell activation.

Discussion

Lp(a) has been of central interest in vascular biology because numerous epidemiological studies have indicated that Lp(a) is an independent risk factor for cardiovascular diseases such as atherosclerosis and ischemic heart disease[19–24]. Indeed, previous studies with transgenic technology showed that mice expressing the apo(a) gene developed atherosclerosis[16,17,25]. Because of the high degree of homology between apo(a) and plasminogen, Lp(a) and apo(a) have been thought to enhance the proliferation of human vascular smooth muscle cells (VSMCs) in culture by inhibiting the activation of plasminogen to plasmin[26–28]. Thus, researchers have speculated that the removal of Lp(a) from the plasma may reduce coronary events. Therefore, research efforts (including our own) have tried to prevent atherosclerosis that is induced by high Lp(a) by decreasing apo(a) while avoiding any effects on plasminogen production. In fact, the prevention of restenosis has been reported by reducing Lp(a) levels through LDL apheresis[29–31], although performing apheresis in all patients with high Lp(a) levels is not practical. However, in this study we focused on a different strategy to neutralize the biological activity of Lp(a) and/or apo(a) through the development of a DNA vaccine against apo(a) using the HBc system. As the targeted antigen, we selected 12 amino acids of the kringle-4 type 2 repeated domain of apo(a) that did not share sequence homology with plasminogen. This 12-amino acid motif was incorporated into the hydrophilic domain of the B-cell epitope, which has been used previously to induce an immune response. We expected that the antibody that was produced by this construct could potentially bind to not only free apo(a) in the plasma but also to apo(a) associated with apoB in LDL. If free apo(a) were captured by the anti-apo(a) antibody, vaccination against apo(a) might inhibit neointima formation by reducing the deposition of Lp(a) in the ligated vessel or neutralization apo(a) or Lp(a).

Interestingly, apo(a) DNA vaccination markedly decreased the neointima formation induced by artery ligation, which was associated with a decrease in the vascular accumulation of Lp(a) in Lp(a) transgenic mice. Unexpectedly, the serum Lp(a) levels were not decreased in the present study (data not shown) even though the vaccine against apo(a) successfully produced neutralizing antibodies against Lp(a). Similar findings have been previously reported for another vaccine: angiotensin II vaccination did not decrease angiotensin II levels, although it significantly decreased high blood pressure[11]. Alternatively, it is possible that the ELISA used in this study could not distinguish free Lp(a) and antibody-bound Lp(a) because the detecting antibody targets a different epitope than the induced antibody. Further study will be necessary to answer this question.

Importantly, DNA vaccines have several advantages, including the ability to induce a wide range of immune responses. For example, DNA vaccines activate TLR9 and mediate cellular responses through CpG motifs within the DNA sequences without adjuvants[12]. In this study, titers of anti-apo(a) antibody were only observed in the apo(a) group, and this immunization led to a Th1-biased immune response. Vaccination against self-antigens has been recently reported in the treatment of cancer[32], rheumatoid arthritis[33], Alzheimer's disease[9,34–43], hypertension[11,44,45], and dyslipidemia[46]. The adverse effects of vaccination should be carefully considered, especially in hypertension and dyslipidemia, because safe and effective drug therapies have been already established. Therefore, it is important to avoid T-cell activation toward self-antigens and ensure reversibility of the inhibition against the target molecule. For example, a clinical trial in Alzheimer's disease patients was halted because the participants developed aseptic meningoencephalitis due to autoimmune responses[34,37–39]. To avoid autoimmune responses, we used the HBc system as a carrier because it has strong 'non-self' helper T cell epitopes. In this study, we succeeded in producing antibodies against the targeted sequence of apo(a) without T-cell activation toward apo(a).

Overall, this study provided the first evidence that the pro-atherosclerotic actions of Lp(a) could be prevented through DNA vaccination directed against apo(a) independent from plasminogen function, which suggests a novel therapeutic strategy for the treatment of cardiovascular diseases that are related to high Lp(a) levels. The selective blockade of apo(a) and Lp(a) is particularly attractive because the high degree of homology between apo(a) and plasminogen causes difficulty in the development of drugs against Lp(a). We anticipate that the modification of this apo(a) vaccine will increase its potential clinical utility for the treatment of Lp(a)-related cardiovascular diseases.

Methods

Animals. The experiments were approved by the Ethical Committee for Animal Experiments of the Osaka University Graduate School of Medicine. The mice had free access to water and food during the experimental periods. Female FVB mice were purchased from Charles River. Lp(a) transgenic mice were created by mating human apo(a) transgenic mice and human apoB transgenic mice[15–18]. Human apo(a) YAC transgenic mice were created through the insertion of a human apo(a) YAC that included the apo(a) gene, the 70 kb apo(a)-like gene, and 260 kb of genomic DNA (YAC DNA)[16]. Especially, the YAC apo(a) contains 12 kringle-4 like repeats; Lp(a) transgenic mice have apo(a) containing one kringle-4 type 1, type 3 through 10, and three krigle-4 type 2 domains followed by kringle-5-like domain and protease-like domain. Human apoB transgenic mice were created through the insertion of 76 kb of genomic DNA (P1 phagemid DNA) that contained the intact apoB gene[17]. The backgrounds of both mice were FVB mouse.

Construction of HBc-apo(a) fusion gene expression vector. We used the plasmid pcDNA3.1 (pcDNA3.1/V5-His-TOPO, Invitrogen) containing the cytomegalovirus promoter. The HBc gene was obtained by PCR and ligated into pcDNA3.1 [HBc]. The apo(a) 12 amino acid (a.a.) sequence (EAPSEQAPTEQR) with an N-terminal Ile-Thr dipeptide linker and a C-terminal Gly-Ala-Thr tripeptide extension was synthesized by PCR using the following oligonucleotides: PCR1, HBc-1 (5′-GCCATGGATAT-CGATCCTTATAAAGAATTCGGAGC-3′) as the forward primer and Lp(a)-1 (5′-GTTAACTTGGAAGATCCAGCTATCACTGAGGCTCCTTCCGAACA-AGCACCGACT-3′) as the reverse primer with and pPLc3 (BCCM/LMBP) as the template; PCR2, HBc-2 (5′-GGCCTCTCACTAACATTGAGATTCCCGAGA-TTGAGA-3′) as the forward primer and Lp(a)-2 (5′-TTCCGAACAAGCACCGA-CTGAGCAAAGGGGTGCTACTAGCAGGGACCTGGTAGTC-3′) as the reverse primer with pPLc3 as the template. The PCR products from PCR1 and PCR2 were use as the template of PCR3: HBc-1 as the forward primer and HBc-2 as the reverse primer. The PCR product from PCR3 was ligated into pcDNA3.1 [HBc-apo(a)].

Vaccination protocol. Female FVB or Lp(a) transgenic mice were vaccinated intramuscularly three times at 2-week intervals (8 weeks, 10 weeks, and 12 weeks old) with 60 μL of TE containing 120 μg of plasmid DNA or saline using an electric pulse generator with a pair of stainless steel needles that were 10 mm in length and 0.3 mm in diameter with a fixed distance between them of 3 mm (NEPA GENE). The

voltage remained constant at 70 V during the pulse duration. Three pulses at the indicated voltage followed by three more pulses of the opposite polarity were administered to each injection site at a rate of one pulse/s, with each pulse being 50 ms in duration. Six weeks after the third immunization (18 weeks old), an additional immunization was administered to the mice. The Lp(a) transgenic mice were bilaterally ovariectomized before the first immunization.

Measurement of apo(a) antibody in serum. Two weeks after both the third immunizations and last immunization, serum was collected from the immunized mice of all groups. Serum levels of apo(a)-specific antibodies in these mice were measured by ELISA. Briefly, ELISA plates were coated with 5 μg/mL apo(a) 12 a.a. peptide in carbonate buffer overnight at 4°C. The plates were blocked with PBST containing 3% skim milk at room temperature for 2 hours, and serial dilutions (1 : 100 to 1 : 312500) of serum samples from the immunized mice were added to the wells. Further, the plates were incubated overnight at 4°C, washed seven times with PBST, and HRP-conjugated mouse IgG (whole or each subtype) was added and incubated at room temperature for 3 hours. After four washes with PBST, 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich) was added. Production of the blue reaction product was stopped by adding 0.5 mol/L sulfuric acid, and the resulting end product was read at 450 nm.

T-cell proliferation assay. The T-cell proliferation assay was performed as previously reported. Syngeneic T cells (mouse splenocytes, 5×10^5 cells/well) were cultured with 10 μg/ml recombinant apo(a) 12 a.a. peptide, phytohemagglutinin (PHA, 50 μg/mL, as positive control) and medium separately, at 37°C in 5% CO_2 for 40 hours. Furthermore, 1 μCi of [³H] thymidine (Perkin Elmer) was added to each well for 8 hours. The cells were harvested, and the [³H] thymidine uptake (cpm) was determined using a MicroBeta 1450 Trilux scintillation counter (Wallac Oy). The stimulation index was expressed as the ratio of stimulated cells to non-stimulated cells.

Enzyme-Linked ImmunoSpot (ELISpot) assay. The ELISpot assay was carried out using the Mouse IFN-γ Development Module and the Mouse IL-4 Development Module for their respective targets (R&D Systems) according to the manufacturer's instructions. Briefly, 96-well plates for ELISpot (Millipore) were preincubated with anti-mouse IFN-γ or IL-4 antibodies overnight at 4°C and blocked with PBS containing 1% BSA and 5% sucrose for 2 hours at room temperature. Splenocytes from individual immunized mice (5×10^5 cells per well) were added to wells with 10 μg/ml recombinant apo(a) 12a.a. peptide, PHA (50 μg/mL as positive control) and medium separately and incubated at 37°C in 5% CO_2 for 48 hours. The plates were washed four times with PBST, incubated with biotinylated anti-mouse IFN-γ or IL-4 antibody overnight at 4°C and washed again with PBST three times. The ELISpot color module (R&D systems) was used for color development. Diluted streptavidin-AP concentrate with PBS containing 1% BSA complex was added into each well and incubated for 2 hours at room temperature. After washing with PBST and deionized water, a BCIP/NBT solution was added into each well, and the plates were incubated in the dark for 30 minutes at room temperature. The plates were washed with deionized water and air dried at room temperature. The colored spots were quantified manually using a dissecting microscope (Olympus).

Carotid artery ligation model. The carotid artery ligation model was employed using Lp(a) female transgenic mice as previously described[8]. One week after the last immunization, the left common carotid artery of female Lp(a) transgenic mice was exposed through a small midline incision in the neck, and the artery was completely ligated with 6-0 silk just proximal to the carotid bifurcation to disrupt blood flow. Following ligation of the common carotid artery, the vessel typically undergoes inflammatory changes and neointima formation.

Quantification of vascular remodeling was performed 3 weeks after carotid ligation. The left common carotid artery was removed, fixed in 4% paraformaldehyde, and equilibrated in PBS containing 10% sucrose, in PBS/20% sucrose and in PBS/30% sucrose. The samples were then embedded for rapid freezing. Cross sections were laid on slides and stained; some sections were frozen at −80°C. For evaluation of neointima formation, the slides were stained with hematoxylin and eosin (HE). Each of the five stained slides was quantified by measuring the area of neointima and media (ImageJ). Immunostainings with anti-Lp(a) and anti-MOMA-2 antibodies were visualized with VECTASTAIN ABC-AP and Vector Red (Vector Laboratories, Inc.).

Statistics. All of the results were expressed as the means ± S.E.M. The data were compared using Student's t-test or using ANOVA followed by Fischer's test for multiple comparisons. All of the statistical analysis was performed using StatView (SAS Institute, Inc.). Values of $P < 0.05$ were considered statistically significant.

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M.K., H.N., T.K. and R.M. designed research; M.K., H.K., F.N., M.S., H.K. and H.T. performed the experiments (Figs. 1–5, Supplement Figures 1, 2). M.K., H.N. and R.M. wrote the paper. All authors discussed and agreed on the results.

Additional information

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