ONLINE SUPPLEMENT

Materials and methods

Generation of recombinant constructs A, H, AH, AHC and AHHC

The construct A contains an epitope derived from human ApoB100 (peptide sequence: I^{688}EIGLEGKGFEPTLEALFGK^{707}, numbered including signal peptide) incorporated into the N-terminal of dendroaspin with a poly-glycine linker between ApoB peptide and dendroaspin. Schematic representation of the backbone of the dendroaspin structure is shown in Figure S1A [1]. Construct H incorporates an epitope of hHSP60 (peptide sequence: A^{153}ELKKQSKPVT^{163}) in dendroaspin loop III as a replacement of wild-type loop III sequence. Construct AH contains both ApoB and HSP60 epitopes located at the same sites as they were in constructs A and H respectively. Both construct AHC and construct AHHC have the same N-terminal sequence as in construct A as well as in construct AH, but with an additional *Cpn* sequence in a combination form derived from the major outer membrane protein (MOMP) of the *Cpn* (peptide sequence: G^{67}DYVFDRI^{74}) and polymorphic outer membrane protein (Omp) 5 of *Cpn* (peptide sequence: Q^{283}AVANGGAI^{291}). Construct AHHC contains an additional epitope derived from hHSP60 (peptide sequence: P^{303}GFGDNRKNQ^{312}) in dendroaspin loop II. Schematic representation of constructs is shown in Figure S1B.

The genes were synthesized by Genescript, USA Inc under confidential agreement and cloned into a PUC57 vector. A PCR was employed for producing the genes of constructs A, H, AH, AHC and AHHC converting a HindIII restriction site within PUC57 vector into an EcoRI site within pGEX-3X vector. The following primers were used: a forward primer with a BamHI cleavage site: 5'-GAA GGG ATC CAT ATC GAA GGT CGT ATC GAA ATC GGC-3' and a reverse primer with an EcoRI site: 5'-TCA TCA CGA GAA TTC TCA AAG GTT GCA TTT GTC AGA TTC-3' for genes in constructs A and AH; a

forward primer: 5'-GAA GGG ATC CAT ATC GAA GGT CGT CGT ATC TGC TAC-3' and a same reverse prime as above was used for gene of construct H; a forward primer 5'-GAA GGG ATC CAT ATC GAA GGT CGTA TCG AAA TCG GC-3' and a reverse primer: 5'-TCA TCA CGG GAA TTC TCA GAT AGC ACC ACC GTT AGC GAC AGC-3' were used for producing both genes of construct AHC and construct AHHC. For the PCR reactions, 100 μl of reaction mixture prepared in either *Taq* polymerase reaction buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.8; 1.5 mM $MgCl_2$; 0.1 % Triton X-100) or *Vent* polymerase reaction buffer [10 mM KCl; 10 mM $(NH_4)_2SO_4$; 20 mM Tris-HCl, pH 8.8; 2 mM $MgSO_4$; 0.1 % Triton X-100] containing ddNTP (400 μM), 300 ng of each oligonucleotide primer, 25–100 ng cDNA template and 1.5–2 units of either *Taq* or *Vent* DNA polymerase were placed in a Perkin-Elmer/Cetus Thermal Cycler (Norwalk, CT, USA). After initial denaturation of the DNA at 94^{o}C for 2.5 min, a cycle program was set: 40 cycles- 94^{o}C for 45 seconds, 70^{o}C for 2 minutes and 55^{o}C for 2 minutes. After PCR, 10 μl of each reaction mixture was electrophoretized in an agarose gel and visualized by ethidium bromide staining to check the yield. The PCR fragments were digested by EcoRI and BamHI and gene-cleaned to separate DNA templates from the newly synthesized genes, and cloned into the carboxyl terminus of the glutathione S-transferase (GST) gene in the restricted vector pGEX-3X (Amersham Biosciences UK Limited). The genes were transformed into an *E. coli* DH5α strain.

Dendroaspin contains four disulphide bridges and has an adaptable protein template that can be manipulated by the substitution and insertion of peptide sequences [2]. $hHSP60_{153\text{-}163}$ was inserted into loop III of dendroaspin (11 amino-acid residues) in constructs H, AH, AHC and AHHC. Loop III was selected as a site for substitution since it is solvent-exposed and located away from other loops; consequently, with the exception of construct A, the Arg-Gly-Asp (RGD)-tripeptide thought to be an integrin-binding site and its flanking sequence [3] were substituted by $hHSP60_{153\text{-}163}$ in all other

constructs. Although the RGD-tripeptide is not substituted in construct A, this tripeptide along with its flanking sequence do not contribute to lesion reduction, as immunization with a wild-type dendroaspin without coupled immunogen used was associated with the same grade of lesion as those in non-immunized controls. In addition, we introduced an ApoB peptide sequence into the N-terminus of constructs A, AH, AHC and AHHC and not into the loops since we considered that its 20 residue length would be too great within each loop. Similarly, we attached a combination of *Cpn* peptide (26 residues) onto the C-terminals of construct AHC and AHHC. This combination of *Cpn* peptide was proved to be immunogenic and showed cross-reactivity in preliminary studies (see *Figure 2* online supplement). In addition, we substituted loop II with $hHSP60_{303-312}$ epitope in construct AHHC to determine whether this modification has an additional effect on the immune response in reducing the atherosclerotic lesions. The genes of constructs were cloned into a DNA vector PGEX-3X as a glutathione S-transferase-fusion protein for the purpose of increasing protein yield as well as for affinity purification.

Transformation of *E. coli* DH5α and BL21

The pGEX-3X vector with insertions of the genes of constructs A, H, AH, AHC and AHHC were used to transform 50 μl of *E. coli* DH5α (for preparing plasmid DNA) or BL21 (for protein expression) competent cell line by incubation on ice for 30 minutes followed by a heat shock of 20 s at 37^{o}C. Following further 2-minute incubation on ice, the cells were shaken at 37^{o}C for 60 minutes in growth medium (1.0 ml) and were then plated. The positive colonies on the LB plate were screened by PCR. Presence of the correct coding sequence of gene was verified by dideoxy chain termination sequencing.

Protein expression

Protein expression in *E. coli* was performed as described previously [2]. In brief, bacterial culture conditions were set up as follows; the 2xYT/ampicillin medium (100 µg/ml) was inoculated with an overnight seed culture (1%, v/v), it was shaken at 37^{o}C until it reached an OD600 of 0.7. Isopropyl β-D-thiogalactoside (IPTG) was then added to a final concentration of 0.1 mM for induction. The cells were grown for additional 4 hours at a low temperature of 30°C and harvested by centrifugation.

Affinity and ion exchange chromatography

GST-fusion constructs A, H, AH AHC, and AHHC were prepared from the sonicated cells of *E. coli* by affinity chromatography using glutathione-Sepharose 4B columns followed by DE50 ion-exchange chromatography. Purified GST-fusion proteins were analyzed by SDS-PAGE for homogeneity (Figure S1C). The working concentration of the GST-fusion proteins was determined by a combination of protein estimation with the Micro BCATM-Protein Assay Reagent kit (Pierce) with BSA as an internal standard and protein estimation on SDS-PAGE (BSA as a standard) analyzed by a Bio-Rad gel scanner (GelDoc 2000).

Animal experiments:

Animals: Male Apob^{tm2Sgy}Ldlr^{tm1Her} J mice, The Jackson Laboratory) were maintained under standard husbandry conditions at the animal facility of the Department of Medical Microbiology and Immunobiology, University of Szeged and were given food and water ad libitum. Mice were used with a total of 8 groups (5 sample groups and three control groups) and 6 mice (5-6 weeks old) in each group. The experiment was repeated. For sample groups, immunizing antigens used were constructs A, H, AH, AHC and AHHC. For antigen injection, the "repetitive immunization multiple sites strategy" (RIMMS) was adopted [4,5]. Mice were inoculated 5 times at 2-3-day intervals. At each immunization mice received

20 μg protein combined with Aluminum hydroxide adjuvant (0.65 mg/mouse; Alum, Aluminum hydroxide gel from Sigma) in PBS with a final volume of 240 μl. This mixture was distributed into 8 sites, 30 μl for each site [5]. Group 1 received construct A, group 2: construct H, group 3: construct AH, group 4: construct AHC, group 5: construct AHHC, group 6-8 (control) were injected with the same dose of GST-dendroaspin mixed with Alum, Alum adjuvant, or Phosphate buffered saline (PBS), respectively. To study the cross-reaction between ApoB antigen and anti-*Cpn* peptide antibodies, or between *Cpn*-peptide and ApoB peptide antibodies, KLH-conjugated peptides were used. Mice were immunized with 20 μg KLH-peptide conjugates in combination with Alum and using KLH with Alum as a control. Peptides were synthesized and conjugated to KLH by Severn Biotech Ltd, UK. Two weeks after the first antigen injection (RIMMS), sera were collected for ELISA test.

The experiments were approved by the Animal Welfare Committee of the University of Szeged and conform with the Directive 2010/63/EU of the European Parliament.

Plasma-lipoprotein analysis

Plasma total cholesterol, triacylglycerol and high-density lipoprotein (HDL) were measured with a Moldular P800 assay system (Roche, Mannheim, Germany) through a service from the Department of Laboratory Medicine, University of Szeged, Hungary. Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula:

$$\text{LDL concentration (mmol/L)} = \text{total cholesterol} - (\text{HDL} + \text{triacylglycerol} \times 0.46).$$

Antibody response measurement

Blood samples were collected in heparinized capillaries by retro-orbital bleeding under pentobarbital sodium anesthesia at week two, and twelve weeks after the first injection of the antigens to test antibody production. The free ApoB-100 peptide, two different hHSP-60 peptides and *Cpn* peptide containing an N-terminal cysteine (synthesized by Severn Biotech Ltd, UK) were used in ELISA as antigens. Maleimide activated 96-well plates (Pierce, Thermo Fisher Scientific Inc., USA) were coated with these peptides individually and peptide-specific IgG was measured in the plasma of immunized mice according to the manufacturer's instructions. Horseradish peroxidase-conjugated α-mouse IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) was used as secondary antibody. 1:100 dilution of plasma samples was made before assaying for peptide-specific IgG.

Antibody cross-reaction measurement results

Immunization with KLH-conjugated ApoB and hHSP60 peptides was described earlier [5]. Sera from these experiments and sera of *Cpn* peptide-immunized mice were used for testing cross-reaction of peptide-specific antibodies. ApoB, hHSP60 and *Cpn* peptides, individually, induced high levels of peptide-specific IgG in mouse sera two weeks after the first immunization compared to the control group immunized with KLH alone. A low level of cross-reaction was observed when antisera to *Cpn* were used against ApoB peptide antigen (Figure S2A). High cross-reaction was observed when antisera to ApoB peptide were used against *Cpn* peptide antigen (Figure S2B). These results show cross-reactions between ApoB and *Cpn* peptides which may be due to molecular mimicry.

Tissue preparation

Twelve weeks after the first immunization, hearts with proximal aortas were harvested and mounted in OCT or paraffin, for immunohistochemical analyses and lesion measurement, respectively. The OCT-

embedded samples were frozen in the mounting medium (OCT compound, Tissue-Tek, Sakura Finetek, Europe) from which 5-μm thick sequential sections were taken using a Reichert-Jung Cryocut 1800 (Leica). The paraffin-embedded sections were prepared using a Leica Jung RM2055 microtome.

Spleens were dissected and one third of spleen of each mouse was embedded in OCT for immunohistochemical analyses, two third of the spleen and axial and inguinal lymph nodes were homogenized by pressing through a 70 μm nylon cell strainer and the cells were recovered in RPMI 1640 complete medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Morphometric analyses and quantitative measurements of atherosclerosis

Paraffin-embedded sections were cut serially at 8 μm intervals from the aortic sinus and mounted on slides. Prior to section staining, sections were deparaffinized in xylene and rehydrated in graded series of ethanol. For area measurements and morphometric analysis, slides prepared from formalin sections were stained with hematoxylin and eosin (HE) and elastin/van Gieson (Sigma) for histological evaluation using an Olympus U-ULH optical microscope (Olympus Optical Co. Ltd, Japan). Image-Pro Plus TM software version 4.0 (Media Cybernetics, Silver Spring, USA) was used to trace the external elastic lamina, internal elastic lamina and lumen in the sections of aortic root area to ascertain area of atherosclerotic lesions. The total aortic root area and lesion area were measured following which the ratio of total lesion area/total aortic root area was calculated and expressed as % of lesion in aortic root area.

The total cross-sectional aortic area (measured along the inner aortic perimeter) and lesion area were measured following which the ratio of total lesion area to total aortic lumen area was calculated and expressed as a percentage of lesion in the cross-section of the aortic root area.

Immunohistochemical analyses

Hearts with proximal aortas and spleens embedded in OCT were sectioned and sections with aortic sinus were fixed in methanol. Consecutive tissue sections were incubated in 0.2% TritonX-100/PBS for 1 hour then blocked with 1% BSA for 1 hour. The samples were stained with either purified hamster anti-mouse CD11c (eBioscience, Ltd., UK) or rat anti-mouse CD4 (BD Biosciences, UK), CD68, Foxp3 or TNF-α (BioLegend, USA) at 4°C overnight, washed in PBS and incubated with rabbit anti-rat IgG-FITC or goat anti-mouse IgG-TRITC (Sigma, UK). For IL-10 and for Foxp3 (BD Biosciences, UK), FITC-conjugated rat anti-mouse CD4 mAb and PE-labelled anti-mouse IL-10 and Foxp3 (BioLegend, San Diego, CA, USA) were used. All slides were counterstained with mounting medium containing DAPI (Vector Laboratories Inc., USA). Bright-field images were captured, scanned and overlaid using an Axiovert S100 TV immunofluorescence microscope (Zeiss, UK) equipped with Plan-NEOFLUAR objectives and a KTL/CCD-1300/Y/HS camera from Princeton Instruments, USA. Image-Pro Plus TM software version 4.0 was used to determine lesion area. The $CD68^+$, $CD11c^+$, $Foxp3^+$ and $CD4^+$ areas within the lesions and $CD4^+$ areas in spleen sections were measured with a microscope averaged in μm^2. Three sections from each animal (six animals from each group) were investigated.

Flow Cytometric Analysis

The $CD4^+$ T-cells from lymph nodes from recombinant construct- or GST-dendroaspin-immunized $Apob^{tm2Sgy}Ldlr^{tm1Her}$ J were used for detection of the level of Foxp3 expression using a Treg detection kit (MACS, catalog no. 130-094-165), according to the manufacturer's instructions. Cells were analyzed on a flow cytometer (Cytomics FC500; Bachman coulter, High Wycombe, UK).

Measurement of pro- and anti-inflammatory cytokines

Levels of murine cytokines IL-10, TGF-β, TNF-α, and IFN-γ were measured in plasma using ELISA kits following the manufacturer's instructions (R&D systems, Abingdon, UK). Levels of ConA-induced IL-10, TGF-β, TNF-α and IFN-γ in splenocyte cultures were measured. Briefly, 48-h spleen cell cultures of mice were stimulated with ConA (0, 1, 10,100 μg/ml). The cells were cultured for additional 24-h after adding ConA. Cytokine supernatants were determined by sandwich ELISA as recommended by the manufacturer. IL-10 and TNF-α levels in the lesions were quantified by immunohistochemical analyses and the ratio of IL-10 and TNF-α positive area/total lesion area, respectively was measured and calculated by using Image-Pro Plus TM, version 4.0.

$CD4^+$ T-cell proliferation assay

$CD4^+$ T-cells were purified from spleen cells of $Apob^{tm2Sgy}Ldlr^{tm1Her}$ J mice immunized with constructs A, H, AH, AHC and AHHC or with PBS (control) by using the magnetic-activated cell sorting (MACS) $CD4^+$ T-Cell Isolation Kit (Miltenyi Biotec) and used as responder cells. Constructs as antigens and γ-irradiated (30Gy) spleen cells from PBS-treated $Apob^{tm2Sgy}Ldlr^{tm1Her}$ J mice were used for stimulation. $CD4^+$ T-cells ($3x10^5$/well) were stimulated with γ- irradiated spleen cells ($2x10^5$/well) in the presence or absence of antigens (0.5 μM) for 72 hours at 37 °C in a 5% CO2, 90% air-humidified incubator. Eighteen hours before harvesting, 0.5 μCi of methyl-[^{3}H]-thymidine (Amersham Biosciences, Amersham, UK)/well were added into the cultures for the final 6 h. The cells were harvested and incorporated radioactivity was measured using a Micro 96 harvester (Skatron Instruments, Lier, Norway) and liquid scintillation counter (1205 BetaplateTM, Turku, Finland).

For antigen-specific regulatory function, $CD4^+CD25^+$ Treg cells were isolated from the spleen $CD4^+$ T-cells of $Apob^{tm2Sgy}Ldlr^{tm1Her}$ J mice immunized subcutaneously with the construct AHC. T-effector cells, $CD4^+CD25^-$ cells were isolated from the spleen $CD4^+$ T-cells of mice immunized with the construct AHHC. $CD4^+CD25^-$ cells (2×10^5) were co-cultured with $CD4^+CD25^+$ cells (2×10^5), in the presence of 2×10^4 irradiated (3000 rad) splenocytes (as a source of antigen-presenting cells) per well, and stimulated with 1 μM of either construct A or H or with GST control. After 3 days of culture, cells were pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham, Buckinghamshire, UK) for the last 18 hours of culture and then harvested, and the incorporation of [^{3}H]thymidine was determined using a liquid scintillation spectroscopy method (1205 BetaplateTM, Turku, Finland).

Statistical analyses

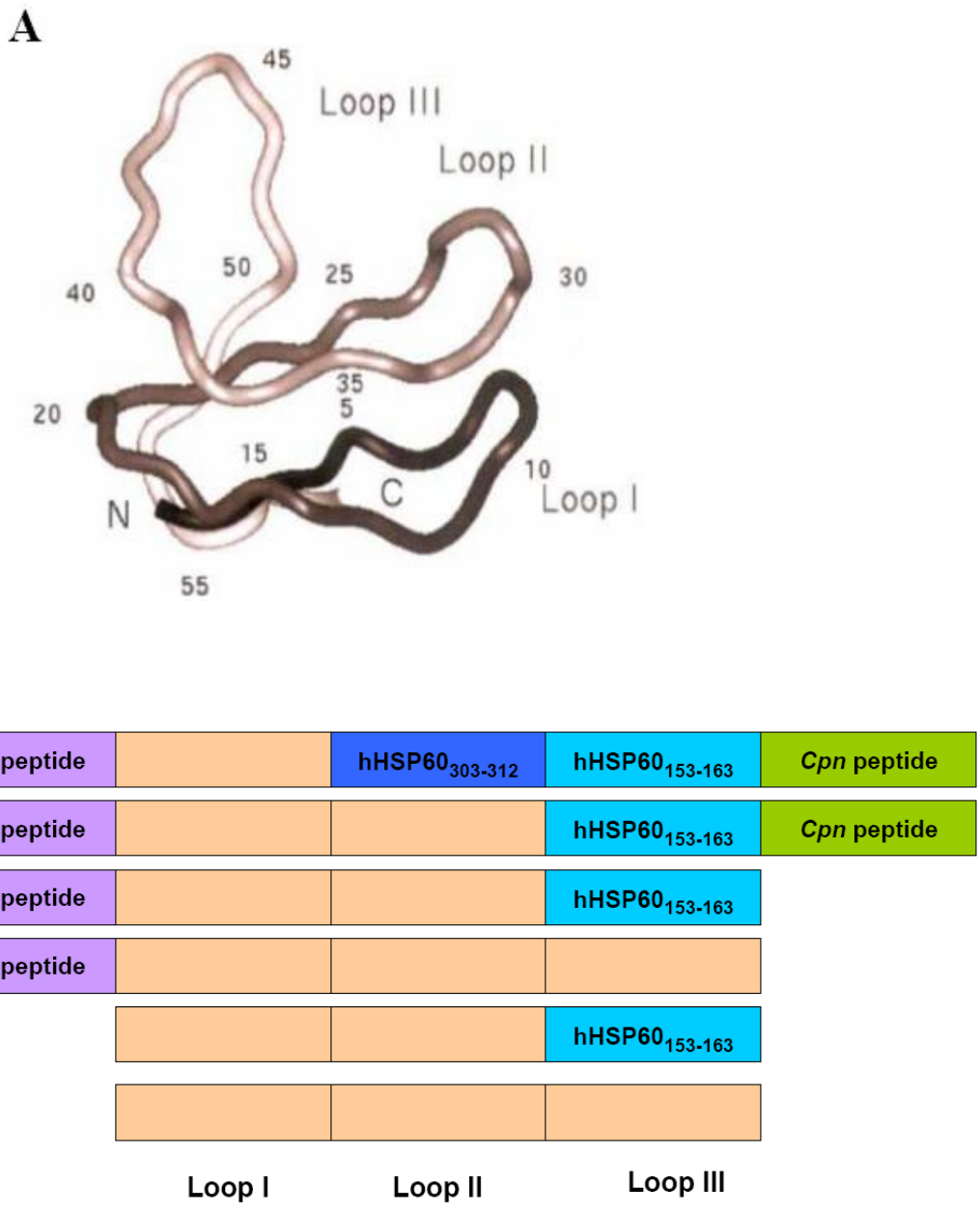
Data are reported as mean±standard error of the mean (±SEM), unless otherwise indicated. Figures were plotted using graph-pad Prism 5.01 and Sigma plot 9.0. For atherosclerotic lesion size, data were compared and intergroup differences were conducted using one-way ANOVA for multiple comparisons and post hoc bonferroni test. Others data were analyzed using Student's *t*-test (2-tailed analyses). Non-parametric distributions were analysized using Mann-Whitney *U* test for pairwise comparisons and the Kruskal-Wallis test for multiple comparisons. Differences between groups were considered significant at P values below 0.05.

References

1. Sutcliffe MJ, Jaseja M, Hyde EI, Lu X, Williams JA. Three-dimensional structure of the RGD-containing neurotoxin homologue dendroaspin. *Nat Struct Biol* 1994;**1**:802-807.

2. Lu X, Rahman S, Kakkar VV, Authi KS. Substitutions of proline 42 to alanine and methionine 46 to asparagine around the RGD domain of the neurotoxin dendroaspin alter its preferential antagonism to that resembling the disintegrin elegantin. *J Biol Chem* 1996;**271**:289-294.
3. Lu D, Chung KF, Xia M, Lu X, Scully M, Kakkar V. Integrin binding characteristics of the disintegrin-like domain of ADAM-15. *Thromb Haemost* 2006;**96**:642-651.
4. Kilpatrick KE, Wring SA, Walker DH, Macklin MD, Payne JA, Su JL, Champion BR, Caterson B, McIntyre GD. Rapid development of affinity matured monoclonal antibodies using RIMMS. *Hybridoma* 1997;**16**:381-389.
5. Lu X, Chen D, Endresz V, Xia M, Faludi I, Burian K, Szabo A, Csanadi A, Miczak A, Gonczol E, Kakkar V. Immunization with a combination of ApoB and HSP60 epitopes significantly reduces early atherosclerotic lesion in Apobtm2SgyLdlrtm1Her/J mice. *Atherosclerosis* 2010;**212**:472-480.

Figure S1


A
45
Loop III
Loop II
50
25
30
40
35
5
20
15
10
C
Loop I
N
55
B
AHHC
ApoB peptide
hHSP60303-312
hHSP60153-163
Cpn peptide
AHC
ApoB peptide
hHSP60153-163
Cpn peptide
AH
ApoB peptide
hHSP60153-163
A
ApoB peptide
H
hHSP60153-163
Den.
Loop I
Loop II
Loop III

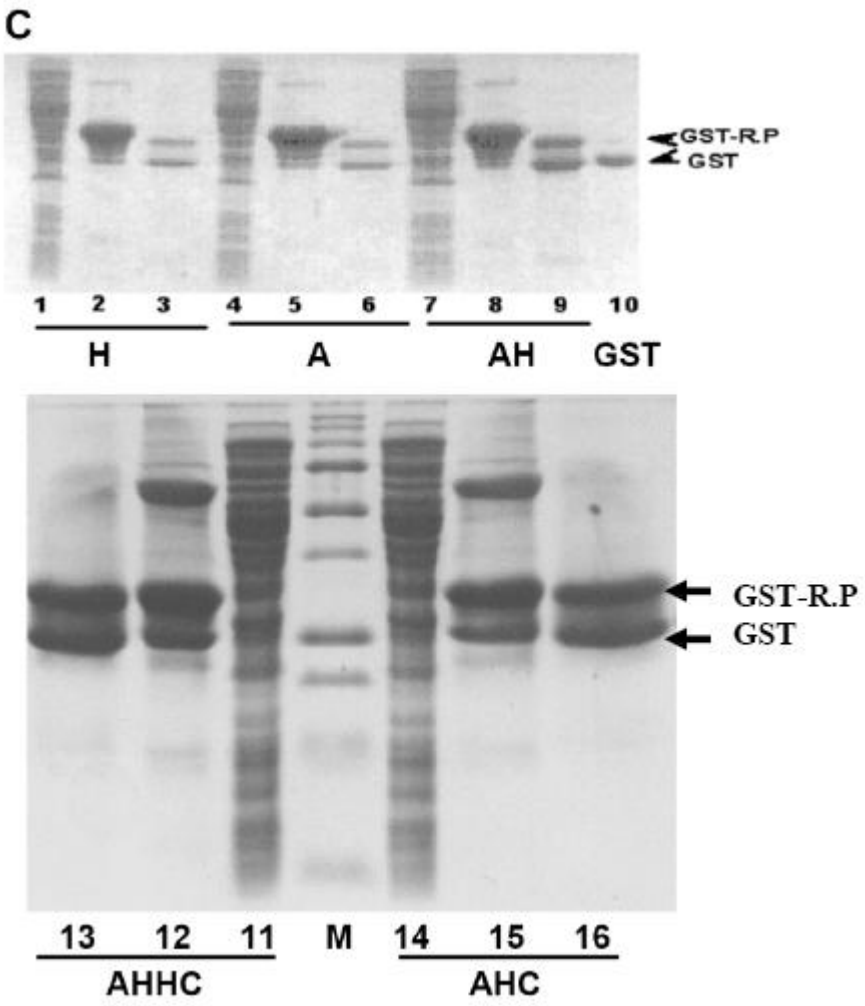


Fig. S1: Schematic representation of the backbone of the dendroaspin structure [1] (A). Schematic representation of alignment of constructs H, A, AH, AHC and AHHC and dendroaspin scaffold (B). SDS-PAGE analysis (15%, w/v gel) of protein purification (C). Column 1, 4, 7, 11 and 14 are supernatants before the affinity and ion exchange purification; column 2, 5, 8, 12 and 15 purified by affinity column; and 3, 6, 9, 10,13 and 16 were further purified by ion exchange column. R.P denotes recombinant protein; GST denotes glutathione-S-transferase which was used as a protein tag for protein expression and purification.

Figure S2

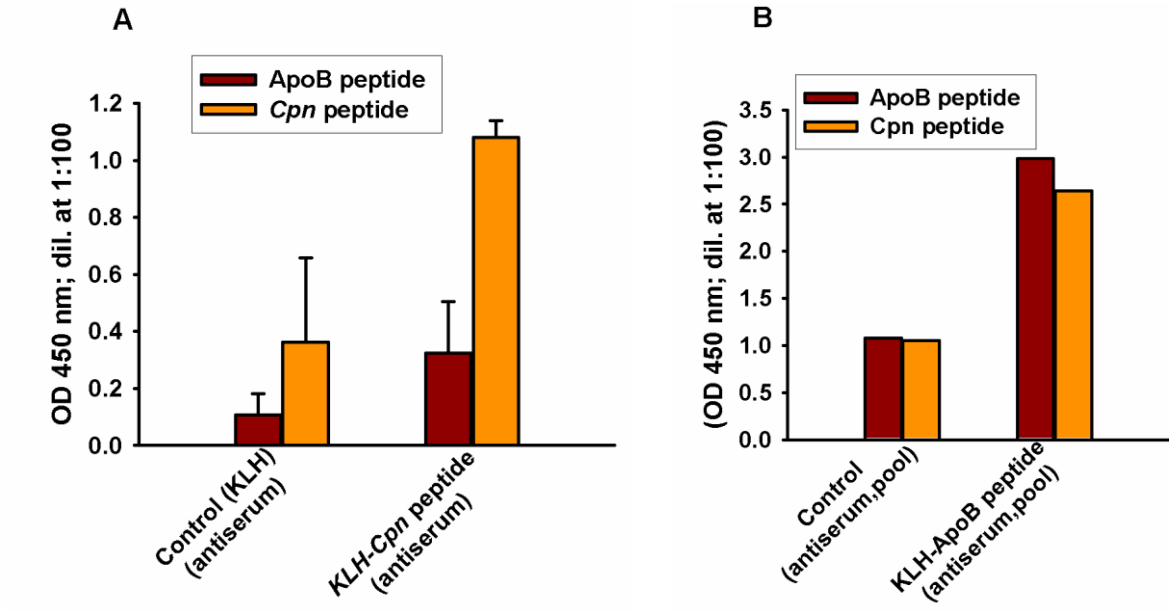


Figure S2 Cross-reactivity assays: peptide-induced specific IgG levels of pooled plasma samples from male mice at week 2 were measured by OD. The mean ODs obtained from plasma samples of Control mice (immunized with KLH) and sampling mice on ApoB peptide- and *Cpn* peptide-coated ELISA plates, respectively, dilution ratio: 1:100 as shown in Figure S2 A and B.

Figure S3

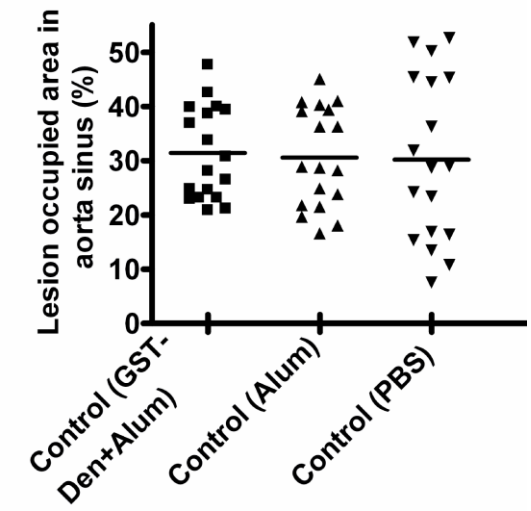


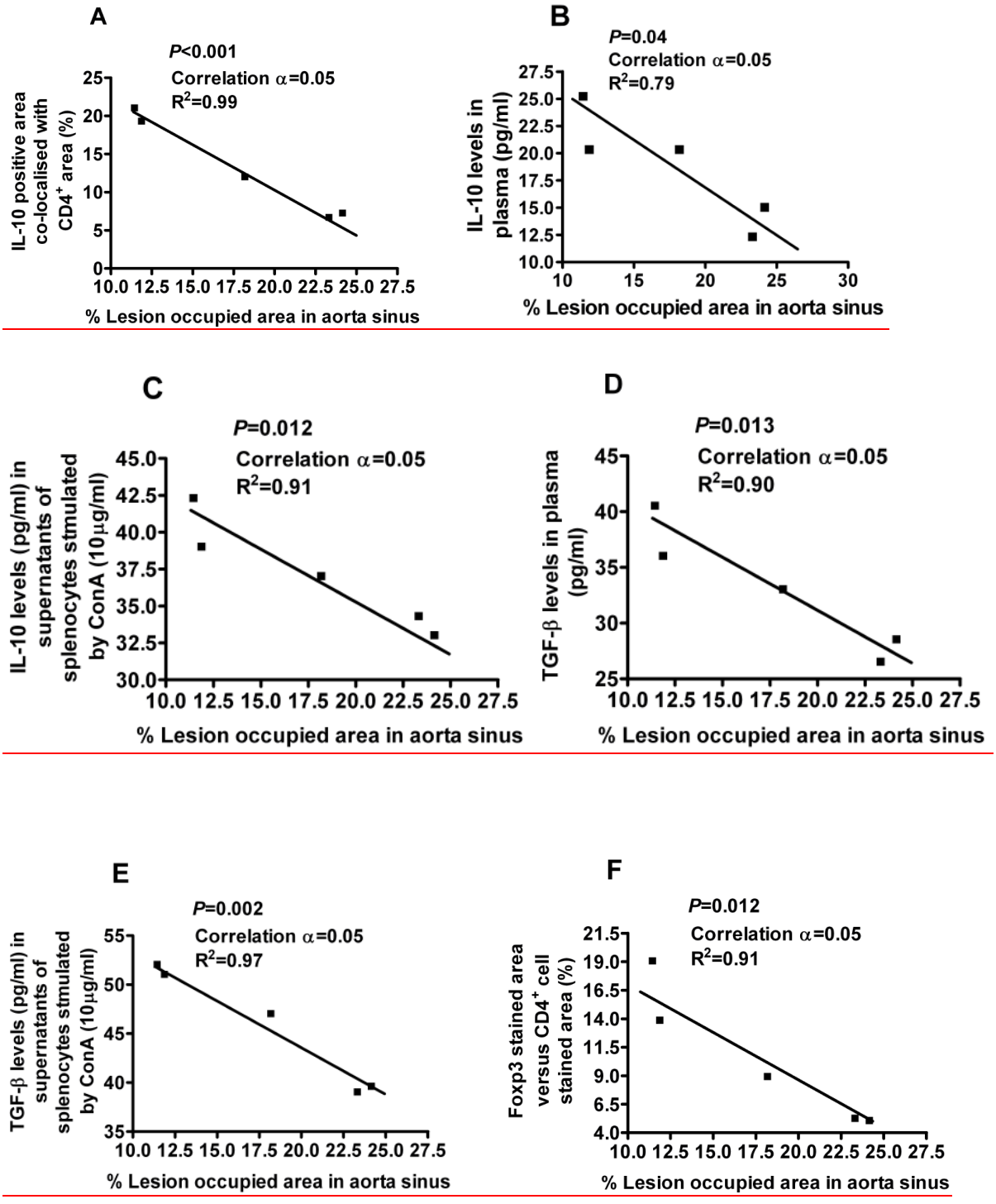
Figure S3: Photomicrograph of lesions observed in atherosclerotic aortas of mice in control groups as analyzed with elastin/van Gieson staining ((N=18sections).

Table S1

Lipid levels in pooled plasma

				Calculated values	Body weight at 10 weeks with feeding HFD
Groups	**Cholesterol (mmol/l)**	**Triglyceride (mmol/l)**	**HDL-cholesterol (mmol/l)**	**LDL-cholesterol (mmol/l)**	**(g)**
A	**34.57**	**2.52**	**5.24**	**28.18**	**21.73±1.29**
H	**31.39**	**2.14**	**4.61**	**25.81**	**20.35±0.60**
AH	**31.98**	**2.08**	**4.88**	**26.15**	**20.26±2.12**
AHC	**30.68**	**2.6**	**4.94**	**24.56**	**20.93±2.73**
AHHC	**34.54**	**2.79**	**5.12**	**28.15**	**20.38±1.06**
GST-den	**34.96**	**2.08**	**5.11**	**28.46**	**20.71±2.75**
PBS	**29.83**	**2.45**	**4.91**	**24.04**	**19.7±2.32**

Figure S4


A
P<0.001
Correlation α=0.05
R2=0.99
IL-10 positive area co-localised with CD4+ area (%)
% Lesion occupied area in aorta sinus
B
P=0.04
Correlation α=0.05
R2=0.79
IL-10 levels in plasma (pg/ml)
% Lesion occupied area in aorta sinus
C
P=0.012
Correlation α=0.05
R2=0.91
IL-10 levels (pg/ml) in supernatants of splenocytes stmulated by ConA (10μg/ml)
% Lesion occupied area in aorta sinus
D
P=0.013
Correlation α=0.05
R2=0.90
TGF-β levels in plasma (pg/ml)
% Lesion occupied area in aorta sinus
E
P=0.002
Correlation α=0.05
R2=0.97
TGF-β levels (pg/ml) in supernatants of splenocytes stmulated by ConA (10μg/ml)
% Lesion occupied area in aorta sinus
F
P=0.012
Correlation α=0.05
R2=0.91
Foxp3 stained area versus CD4+ cell stained area (%)
% Lesion occupied area in aorta sinus

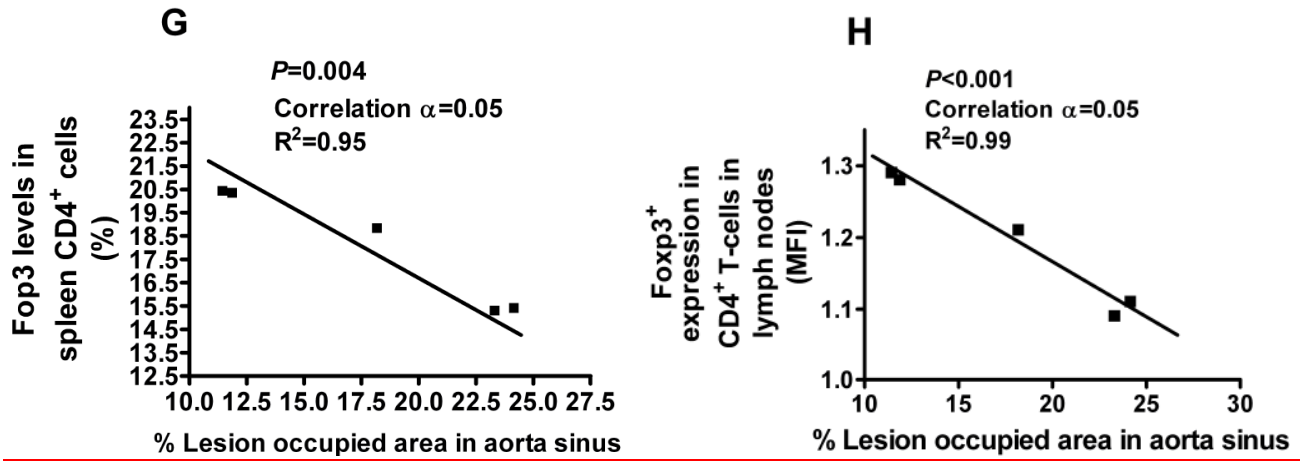


Figure S4: Statistical analysis of IL-10, TGF-β and Foxp3 expression levels in lesions, plasma samples and splenocyte supernatants in correlation with lesion occupied area in aorta sinus (plotted and analyzed using Prism version 5 software).

Figure S5

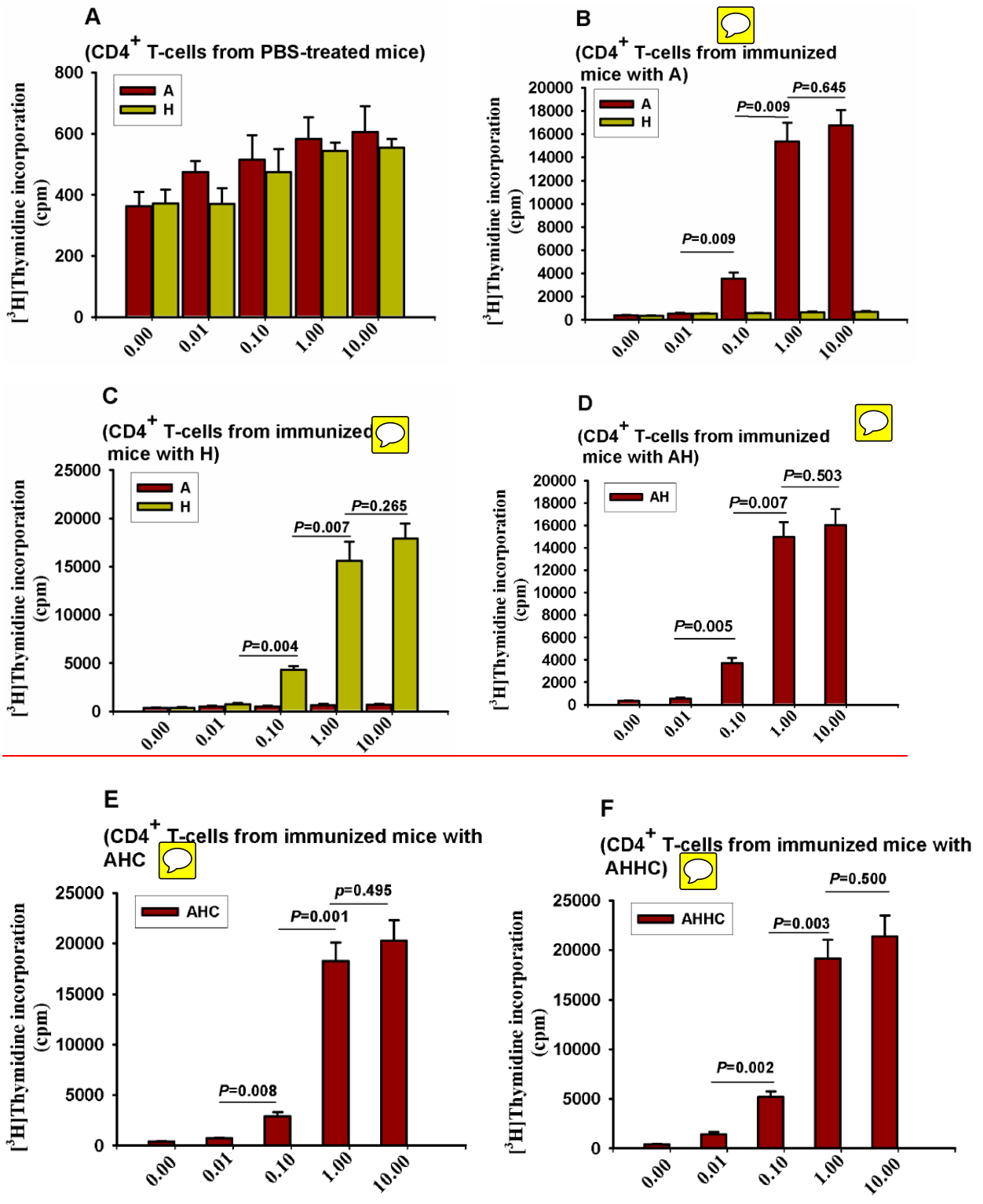

A
(CD4+ T-cells from PBS-treated mice)
[3H]Thymidine incorporation (cpm)
A
H
0.00
0.01
0.10
1.00
10.00
B
(CD4+ T-cells from immunized mice with A)
P=0.009
P=0.009
P=0.645
C
(CD4+ T-cells from immunized mice with H)
P=0.004
P=0.007
P=0.265
D
(CD4+ T-cells from immunized mice with AH)
AH
P=0.005
P=0.007
P=0.503
E
(CD4+ T-cells from immunized mice with AHC
AHC
P=0.008
P=0.001
p=0.495
F
(CD4+ T-cells from immunized mice with AHHC)
AHHC
P=0.002
P=0.003
P=0.500

Figure S5: Analysis of the proliferative response of $CD4^+$ T-cells isolated from the spleen of ApoB100 only, $LDLr^{-/-}$ mice fed a high-fat diet after immunization with constructs A, H, AH, AHC and AHHC after stimulation with appropriate construct. [^{3}H]-thymidine incorporation was detected in triplicate cultures and counts (cpm) and SEM are shown.

A: $CD4^+$ T-cells from PBS-treated mice were tested with both constructs.

B: $CD4^+$ T-cells from construct A-immunized mice were stimulated with both constructs A and H showing significant response to construct A and no response to construct H.

C: $CD4^+$ T-cells from construct H-immunized mice were stimulated with both constructs A and H showing significant response to construct H and no response to construct A.

D: $CD4^+$ T-cells from construct AH-immunized mice were stimulated with AH showing significant response to AH

E: $CD4^+$ T-cells from construct AHC-immunized mice were stimulated with AHC showing significant response to AHC.

F: $CD4^+$ T-cells from construct AHHC-immunized mice were stimulated with AHHC showing significant response to AHHC.