Electronic Supplementary Information (ESI)

S100A9-targeted tobacco mosaic virus nanoparticles exhibit high specificity toward atherosclerotic lesions in ApoE$^{-/-}$ mice

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1. Experimental details

1.1. Sample characterization and measurements

a. **Transmission electron microscopy (TEM):** TEM samples were prepared by dropcasting nanoparticles solutions onto a 3-mm lacey carbon grid, followed by negative staining with 2% (w/v) uranyl acetate for 2 min. TEM images were obtained using FEI Tecnai F30.

b. **Ultraviolet-visible (UV-vis) spectroscopy:** UV-vis spectra were obtained to determine the concentration of the particles and the number of fluorescent molecules conjugated to a particle using a Nanodrop^{TM} 2000 spectrometer. TMV samples were dispersed at 1 mg/mL in 10 mM KP. The extinction coefficients for TMV and Cy5 are 3 mL mg^{-1} cm^{-1} at 260 nm and 271000 M^{-1} cm^{-1} at 649 nm, respectively.

c. **Hydrodynamic size measurement via dynamic light scattering (DLS):** A DynaPro Nanostar (Wyatt Technology) with a disposable cuvette was used to measure hydrodynamic sizes of TMV samples. All TMV nanoparticles were suspended in 10 mM KP (pH 7.4) at the concentration of 0.1 mg/mL.

d. **Fast protein liquid chromatography (FPLC):** The structural integrity of particle samples was characterized using ÄKTAFPLC chromatography system (GE Healthcare). The absorbance at 260 and 280 nm was measured with at a flow rate of 0.5 mL/min.

e. **Confocal fluorescence microscopy:** After immunofluorescence staining, the cryosectioned aortas were imaged using Leica TCS SPE confocal microscope (Leica Mircosystems). The microscope was equipped with four lasers with different wavelengths (488, 530, 588, and 630 nm). The imaging was performed in sequence with multiple channels for different fluorophores.

f. **SDS-PAGE:** Particle samples in NuPAGE LDS sample buffer (1:4 dilution) were denatured at 95℃ for 5 min. Denatured particle samples were then separated in a 4−12% NuPAGE gel in 1×MOPS buffer at 200 V for 40 min. The gels were stained with Coomassie Blue and imaged with an FluorChem R (Protein simple). Note that fluorescence image of SDS gel was captured prior to Coomassie Blue staining.

1.2. Procedures

a. Peptide Synthesis

The sequences of S100A9-targeting peptides were obtained from the published article.[1] To facilitate peptide conjugation, the spacer sequence of GGGSC was inserted to C-terminus of the peptides (please see the complete peptide sequences in Table S1). The S100A9-specific (H6 and G3) and scrambled peptides (ScH6 and ScG3) were ordered from Genscript.

b. Preparation of fluorescent S100A9-targeted TMV

Conjugation of fluorescent molecules to TMV-lys

As illustrated Fig. S1, the preparation of fluorescent TMV-lys consists of two steps: (1) attachment of alkyne functional groups using carbodiimide chemistry and (2) attachment of Cy5 molecules via Cu(I) catalyzed azide-alkyne cycloaddition. Alkyne functional groups were attached to carboxylic acid groups at the interior of TMV-lys using carbodiimide linking chemistry. First, TMV-lys solution (10 mL at 1 mg/mL of TMV-lys) was prepared in 10 mM HEPES (pH 7.4) and mixed with 100 molar equivalents (eq) of propargylamine (P50900; Sigma Aldrich) per a coat protein. Then, 50 molar eq of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, E6383; Sigma Aldrich) was added twice (0 and 18 h) to activate carboxyl groups in TMV-lys for spontaneous reaction with propargylamine. The reaction was allowed to undergo for 24 h at room temperature. TMV-lys modified with alkynes (ialk-TMV-lys) was centrifuged at 160,000 ×*g* for 3 h to remove unreacted propargylamine and resuspended in 2 mL of 10 mM phosphate buffer (KP). Next, 2 e.q. of sulfo Cy5-azide molecules (B3330; Lumiprobe) were added to ialk-TMV-lys solution in the presence of 2 mM aminoguanidine (103760250; Acros chemical), 2 mM ascorbic acid (A92902; Sigma Aldrich), and 1 mM $CuSO_4$ (AC197720000; Fisher Scientific) and reacted on

ice for 30 min. After the removal of excess Cy5 via centrifugation, Cy5-labeld TMV-lys was resuspended in 10 mM KP at a concentration of 2 mg/mL.

Conjugation of peptides to TMV-lys

For peptide conjugation, Cy5-labeled TMV-lys was modified with a sulfurhydryl-reactive crosslinker (*i.e.*, NHS-PEG_8-Mal, 22108; Thermo Scientific), followed by spontaneous reaction between maleimide and sulfurhydryl groups. Briefly, 1 molar eq of NHS-PEG_8-Mal was added to iCy5-TMV-lys and reacted at room temperature for 2 h. After the excess of the crosslinker was removed by a spin desalting column, the intermediate nanoparticles (iCy5-TMV-Mal) were conjugated with the peptide by the addition of peptides (0.5 eq). The peptide-conjugated TMV nanoparticles were purified again using a spin desalting column. For the preparation of non-targeting TMV, TMV-lys was modified with 10 e.q. of methoxyl PEG succinimidyl ester (NHS-PEG MW2000, PG1-SC-2k; Nanocs) for 2 h at room temperature and subsequently purified with a spin desalting column.

c. *In vitro* assay to assess specific targeting function of S100A9-targeted TMV

In vitro assays were performed to assess specific targeting to S100A9 protein using TMV-H6 and -G3 and compared to the control TMV. A 100 μL of hS100A9 or mS100A9 (2 μg/mL, R&D Systems) in 10 mM phosphate-buffered saline (PBS, pH 7.4) was applied to each well in a 96 well plate, which was then stored at 4℃. After overnight incubation at 4℃, the well plate was rinsed with three times using PBS. Then, the well plates were blocked with 1% (w/v) bovine serum albumin (BSA, 10735078001; Sigma Aldrich) solution in PBS for 1 h.

A dilution series (0 to 5 μg/mL) of S100A9-targeted and control TMV was prepared by spiking in 10 mM KP with 1.0% BSA. These solutions were applied to the wells, incubated for 2 h, and rinsed three times with 10 mM KP. In the next step, 100 μL of polyclonal rabbit α-TMV antibody (0.2 μg/mL in 10 mM PBS containing 1.0% BSA) was added to each well, incubated for 2 h, and rinsed with 10 mM PBS four times.

Then, 100 μL of α-rabbit IgG conjugated with HRP (1:4000 dilution in PBS with 1.0% BSA, Thermo Fisher) was pipetted in each well and incubated for 1 h. Unbound antibody were subsequently removed by rinsing four times with 10 mM KP. A 50 μL of stock TMB substrate solution (34028; Thermo Fisher) was added for color development, which was stopped after 30 min by applying 50 μL of 2.0 M H_2SO_4 solution. Then, the absorbance at 450 nm was measured using Infinite® 200 PRO (Tecan). The binding affinity (*i.e.*, dissociation constant, K_D) of the S100A9-targeted TMV nanoparticles was determined by nonlinear regression analysis using Sigmaplot software and a single-site binding model.

d. Basic Local Alignment Search Tool (BLAST) analysis

BLAST analysis is an algorithm for finding similarity between biological sequences, such as amino acid sequences of nucleotides or proteins.[2] Therefore, we used BLAST analysis to examine the similarities in the amino acid sequences of human and mouse S100A9 to theoretically explain cross reactivity of S100A9-targting TMV to both S100A9. The information on amino acid sequences was obtained from the National Center for Biotechnology Information (NCBI) database by searching gene ID numbers of the proteins (P06702 and P31725 for human and mouse S100A9, respectively) from the vendor.

e. *Ex vivo* fluorescent imaging of atherosclerosis in ApoE$^{-/-}$ mice

All experiments were carried out by following Case Western Reserve University IACUC approved procedures. For atherosclerosis disease model, ApoE$^{-/-}$ mice were purchased from Jackson Laboratory. ApoE$^{-/-}$ mice were fed a high fat/high cholesterol (D12108C, Research Diets, Inc.) for 16-19 weeks prior to imaging. For imaging experiments, mice were intravenously injected with various particle formulations (PBS, TMV-PEG, TMV-H6, and TMV-G3) at a dosage of 400 μg TMV per 20 g mouse. After 3 h *in vivo* circulation of TMV, mice were euthanized and the aortas were perfused with PBS before the collection. The collected aortas were fixed in 4% (v/v) paraformaldehyde/PBS solution overnight at 4℃. After fixation, fatty connective

tissues around aortas were removed. Next, the clean aortas were imaged using a Spectrum imaging system to detect Cy5 fluorescence signal accumulated in the aortas. The obtained images were quantitatively analyzed after background signal subtraction.

f. Immunofluorescence microscopy and hematoxylin and eosin (H&E) of cyrosectioned aortas

After *ex vivo* fluorescence imaging, the aortas were sliced into 6 sections. The sectioned aortas were embedded in OCT medium (23-730-571; Thermo Scientific) and frozen in liquid nitrogen. The frozen tissue samples were cut into 10 μm thick sections, which were subsequently mounted on microscope glass slides for immunofluorescence and H&E staining.

For immunofluorescence staining, freshly sectioned aortas were blocked with 10% (v/v) goat serum in PBS for 1 h and rinsed with PBS three times. For antibody staining, a cocktail of antibodies was prepared in 1% (v/v) goat serum: fluorescent α-CD68 for macrophage (1:100 dilution) staining and α-S100A9 for S100A9 staining (1:100 dilution). A droplet (~100 μL) of the antibody cocktail was applied to the sectioned aorta, incubated for 1 h at room temperature, and rinsed with PBS three times. For S100A9 staining, a fluorescently-labeled secondary antibody of α-goat IgG (1:20 dilution) in 1% (v/v) rabbit serum was applied to the slide, incubated for 1 h, and rinsed with PBS three times. Finally, the stained aorta was mounted in a droplet of Fluoroshield™ with DAPI (F6057; Sigma Aldrich) for confocal fluorescence imaging. The detailed information on antibodies for immunofluorescence staining can be found in Table S2.

For H&E staining, the mounted aorta sections were rinsed with deionized water (DI H_2O) to remove OCT residues. Next, the sections were immersed in hematoxylin solution (72804; Thermo Scientific) for 5 min to stain nuclei, followed by rinsing with DI H_2O. The sections were differentiated with 0.3% (v/v) HCl solution for 2 sec and rinsed with Scott's water substitute (1 g sodium bicarbonate and 10 g magnesium sulphate dissolved in 500 mL). Finally, the sections were stained with eosin solution (71225; Thermo Scientific) for 2 min. After rising with DI H_2O and dehydration, the aortas sections were mounted using Permount mounting medium (SP15-500; Fisher Scientific) for imaging.

g. Sudan IV staining of aortas

Sudan IV staining solution was prepared by dissolving 5 g Sudan IV (S4261; Sigma Aldrich) in 1000 mL of the mixture of 70% ethanol and absolute acetone (1:1 v/v). The staining solution was filtered before use. The fixed aortas were briefly rinsed using 70% ethanol, immersed in the staining solution, and stained at room temperature for 20 min. The stained aortas were transferred into 80% ethanol. After 20 min differentiation, the stained aortas were rinsed in pure ethanol to remove excess dyes. The stained aortas were stored in PBS at 4℃ for imaging.

2. Figures

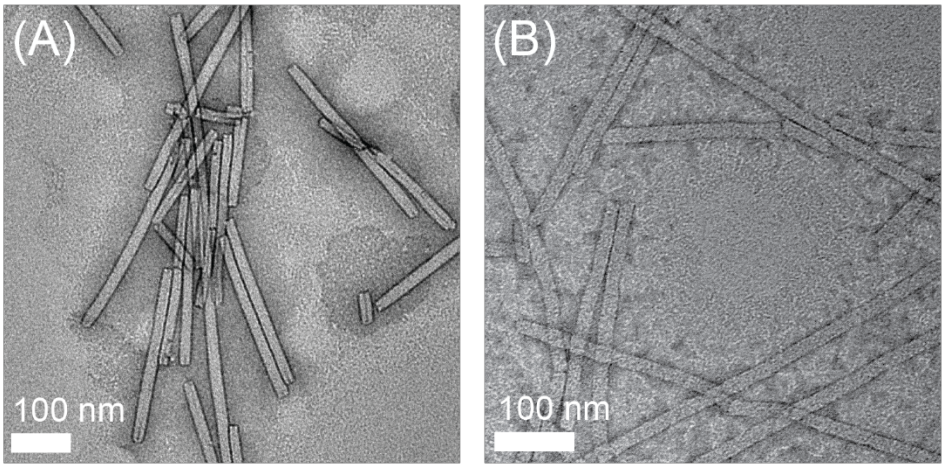


Fig. S1 TEM image of (A) TMV-lys and (B) TMV-PEG nanoparticles.

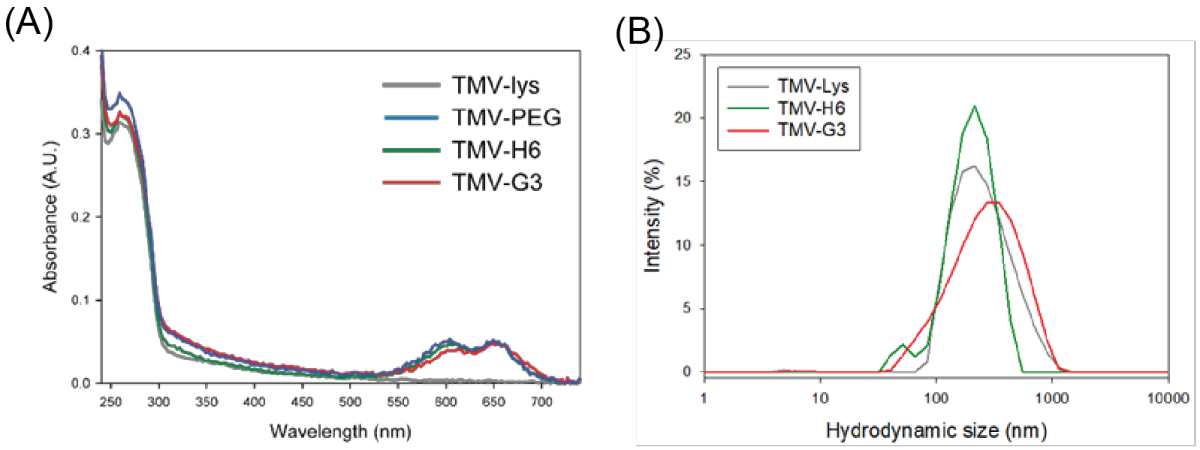
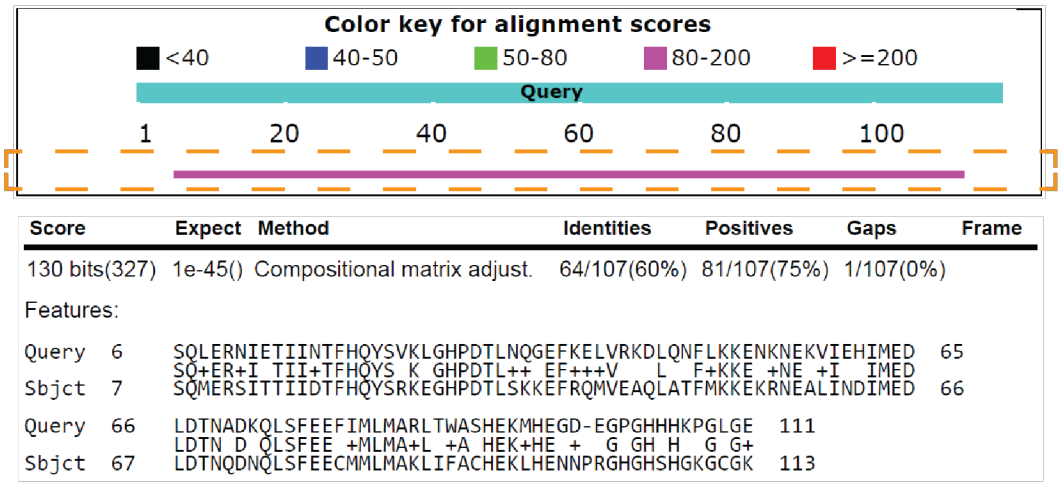


Fig. S2 (A) UV-vis spectra of TMV nanoparticles suspended in 10 mM KP (pH 7.4). The number of Cy5 molecules conjugated with TMV was determined by the absorbances at 260 and 649 nm and Beer-Lambert law. The absorbance ratios (260:280 nm) of TMV-lys, TMV-PEG, TMV-H6, and TMV-G3 are 1.22, 1.22, 1.22, and 1.18, respectively. (B) hydrodynamic sizes of TMV nanoparticles suspended in 10 mM KP (pH 7.4).



Score	Expect	Method	Identities	Positives	Gaps	Frame
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Features:

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Sbjct  7    SQMERSITTIIDTFHQYSRKEGHPDTLSKKEFRQMVEAQLATFMKKEKRNEALINDIMED  66

Query  66   LDTNADKQLSFEEFIMLMARLTWASHEKMHEGD-EGPGHHHKPGLGE  111
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Sbjct  67   LDTNQDNQLSFEECMMLMAKLIFACHEKLHENNPRGHGHSHGKGCGK  113
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Fig. S3 BLAST analysis of human and mouse S100A9 proteins. Query and subject sequences are human and mouse S100A9, respectively. One blast hit of hS100A9 was found in mS100A9 with a high alignment score (highlighted in the dashed box). More importantly, e-value of the hit (10^{-45}) is less than the cut-off value of 10^{-6}, implying that the number of BLAST hit you expect to see by chance is less than 10^{-6}. This indicates that two proteins are very similar even from different species.

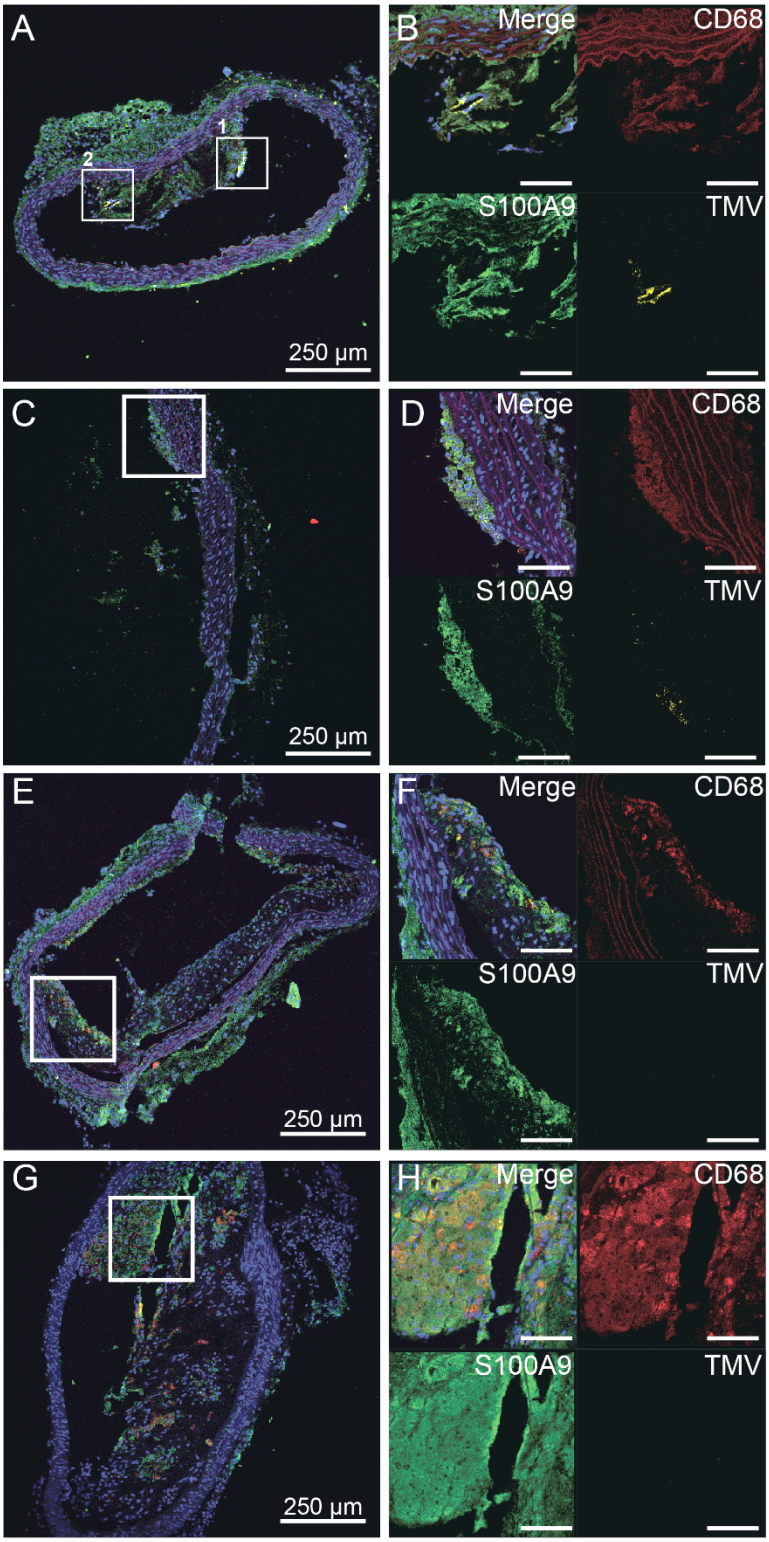

Fig. S4 Confocal immunofluorescence imaging of the sectioned aortas from mice injected with (A and B) TMV-H6, (C and D) TMV-G3, (E and F) PBS, and (G and H) TMV-PEG. The sectioned aortas were stained with DAPI (nucleus), CD68 (macrophage), and S100A9 (mS100A9). Scale bars in (B, D, F, and H) are 75 μm.

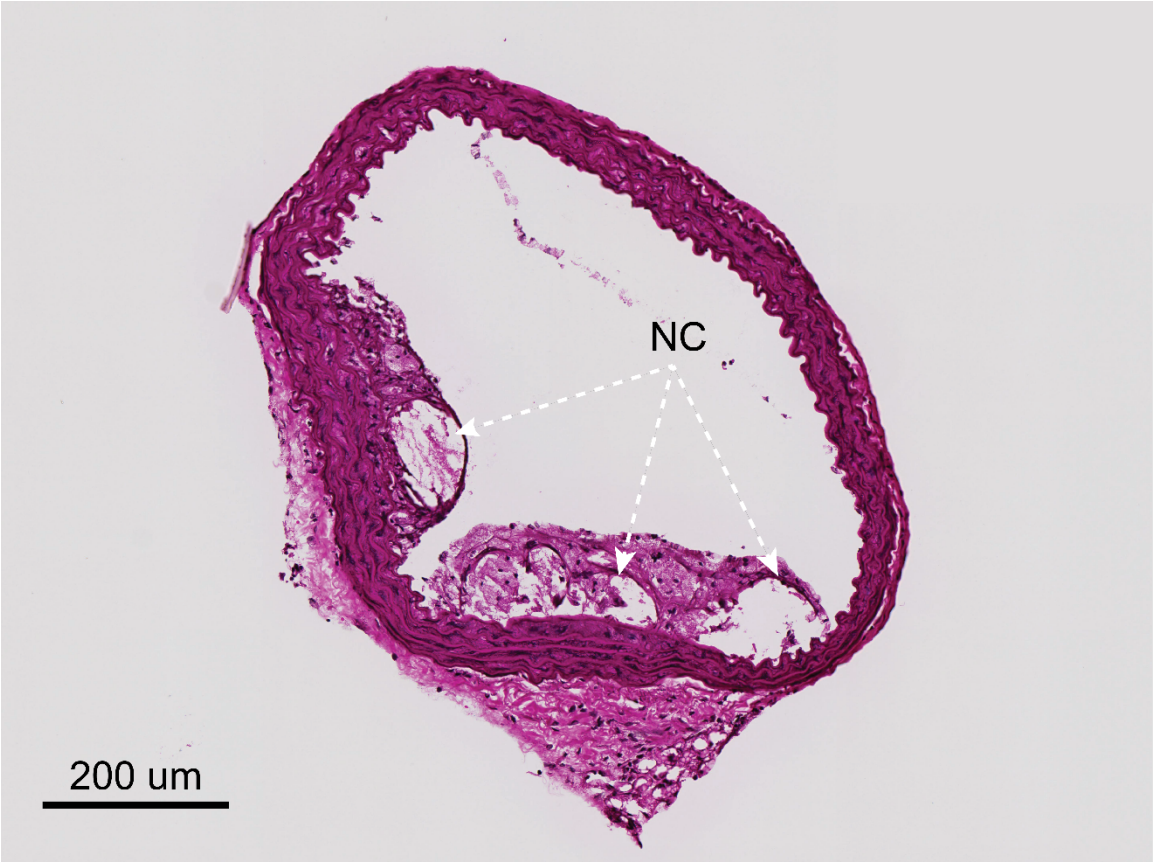

Fig. S5 Representative image of atherosclerotic plaques present in the murine aortic arch after ApoE$^{-/-}$ mice were fed with high fat and high cholesterol diet for more than 20 weeks. Arrows indicate necrotic cores (NC). All aorta sections from ApoE$^{-/-}$ mice (n=3) exhibited necrotic cores.

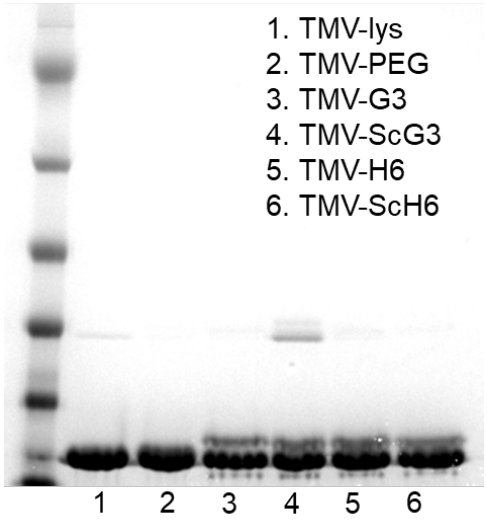

Fig. S6 SDS-PAGE of TMV nanoparticles conjugated with S100A9-targeting (G3 and H6) and its scrambled (ScG3 and ScH6) peptides after Coomassie staining. The gel image showed that coat proteins conjugated with scrambled peptides appeared at the similar molecular weight of coat proteins with S100A9-targeted peptides. Densiometric analysis of the gel image indicated that ~30 % of total CPs were conjugated with peptides.

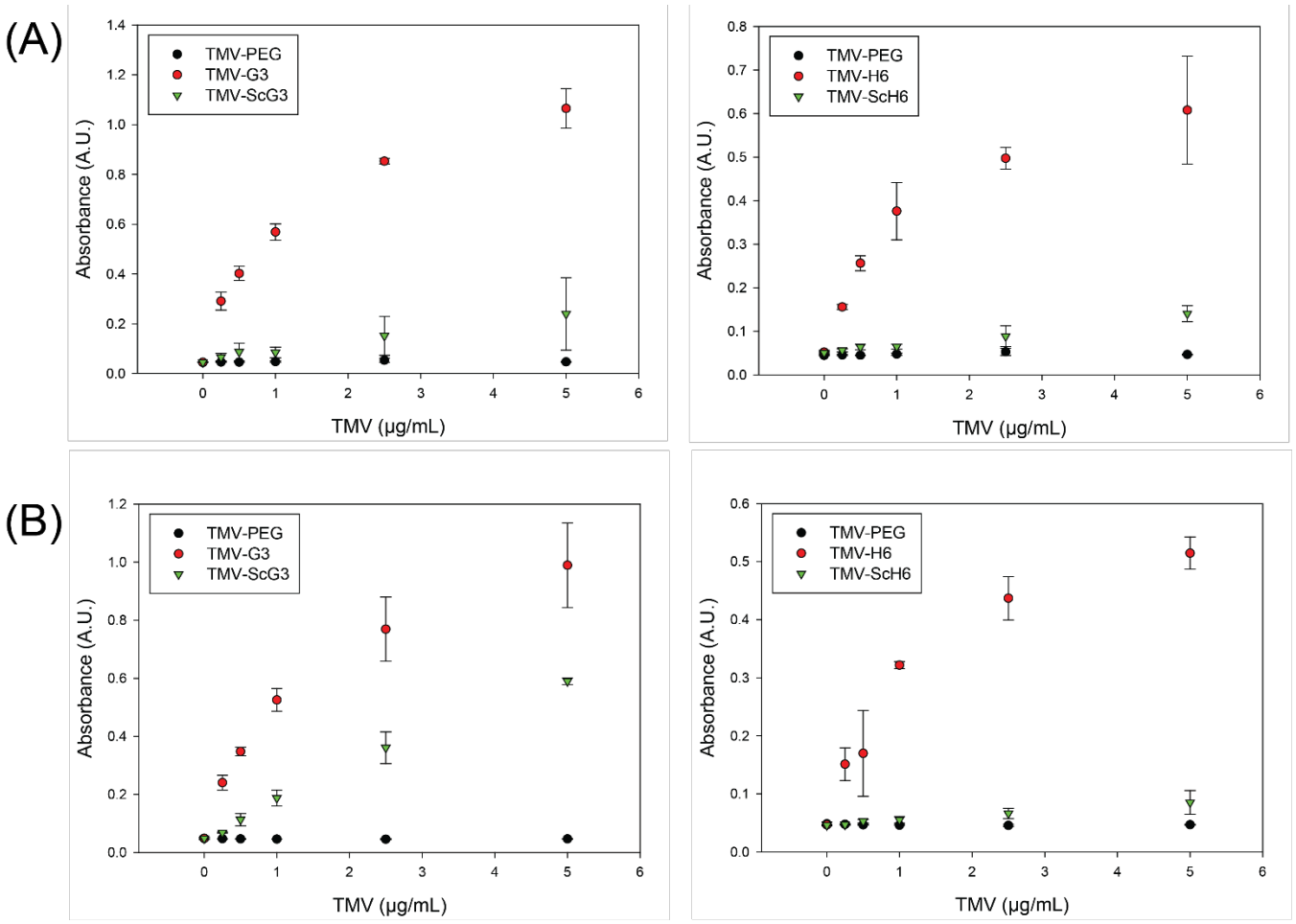

Fig. S7 Comparison of *in vitro* specificity of TMV nanoparticles (TMV-PEG, TMV-G3, TMV-ScG3, TMV-H6, and TMV-ScH6) to (A) mS100A9 and (B) hS100A9. The TMV nanoparticles conjugated with scrambled peptides (TMV-ScG3 and TMV-ScH6) exhibited lower binding affinities to S100A9 proteins when compared to their counterparts (TMV-G3 and TMV-H6).

3. Tables

Table S1. S100A9-targeting and scrambled (non-specific) peptides.

Peptide	Sequence	Approx. molecular weight (Da)
H6	MEWSLEKGYTIK-GGGSC	1846
ScH6	YSELTEIGKMKW-GGGSC	
G3	WGWSLSHGYQVK-GGGSC	1809
ScG3	YSQGWHLKVGWS-GGGSC	

Table S2. Dissociation constant (K_D) of S100A9-targeted TMV nanoparticles.

Particle formulation	K_D to mS100A9 (pM)	K_D to hS100A9 (pM)
TMV-H6	27	44
TMV-G3	25	51

Table S3. List of antibodies used in immunofluorescence study.

Antibody	Vendor	Fluorophore	Clone number	Catalogue number	Stock concentration
α-CD68	Abcam	Alexa Fluor F488	FA-11	ab201844	0.5 mg/mL
α-S100A9	R&D Systems	-	-	AF2065	0.1 mg/mL
α-goat IgG	R&D Systems	PE	-	IC108P	NA

4. References

1. H. Qin, B. Lerman, I. Sakamaki, G. Wei, S. C. Cha, S. S. Rao, J. Qian, Y. Hailemichael, R. Nurieva and K. C. Dwyer, *Nat. Med.*, 2014, **20**, 676.
2. S. F. Altschul, W. Gish, W. Miller, E. W. Myers and D. J. Lipman, *J. Mol. Biol.*, 1990, **215**, 403-410.