

A novel autoimmune IgM antibody attenuates atherosclerosis in IgM deficient low fat diet-fed, but not Western diet-fed *ApoE*^{-/-} mice

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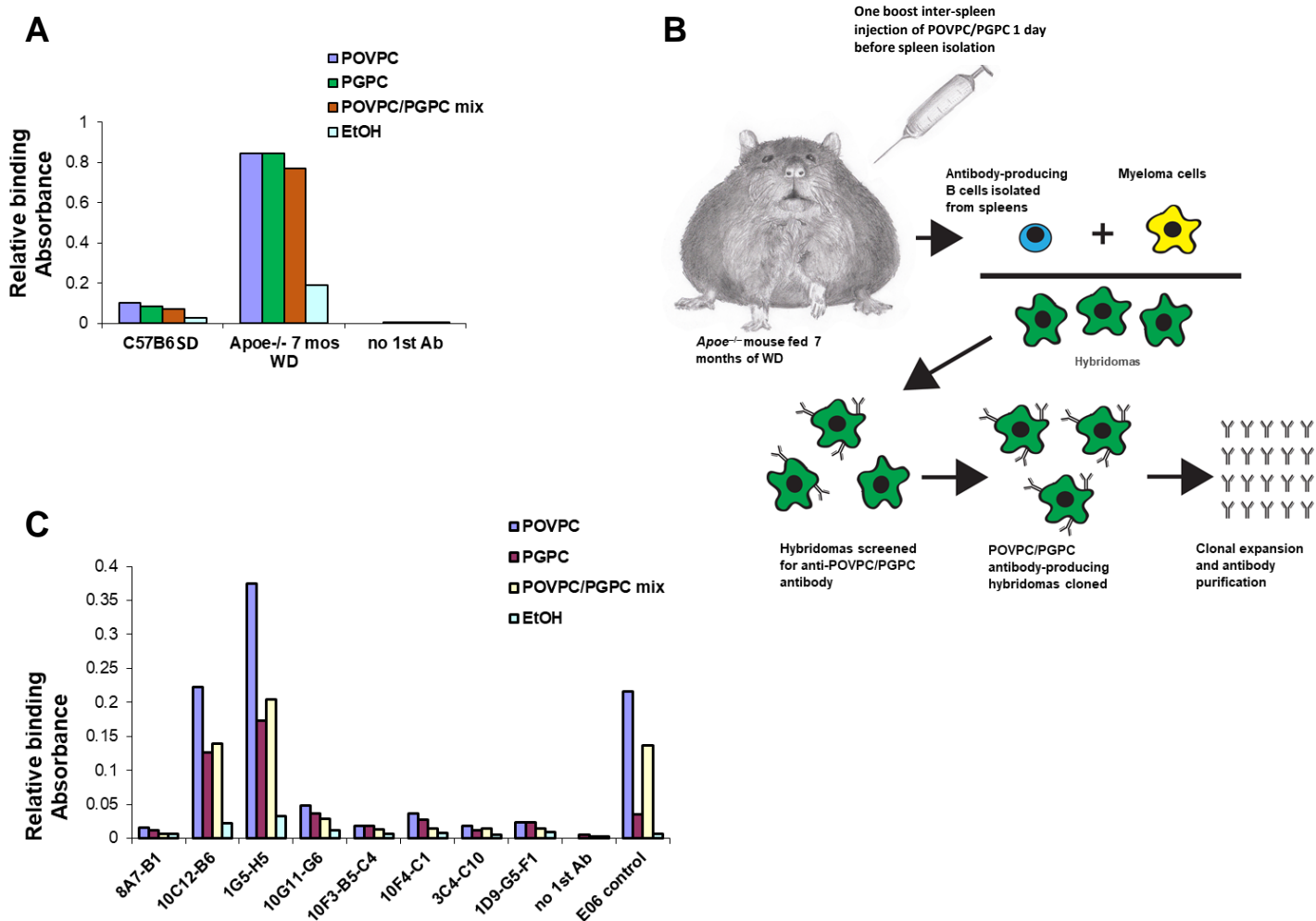
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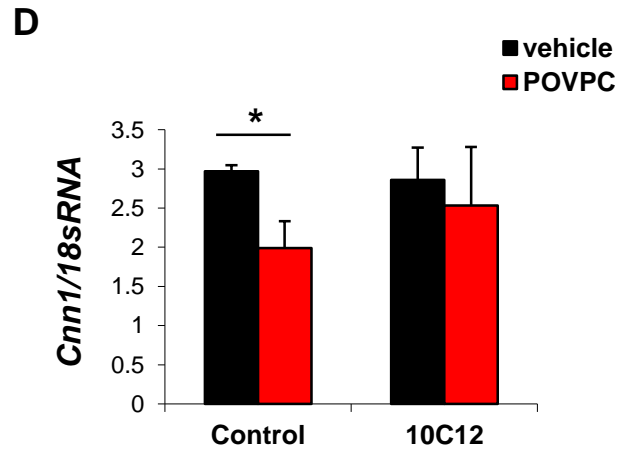
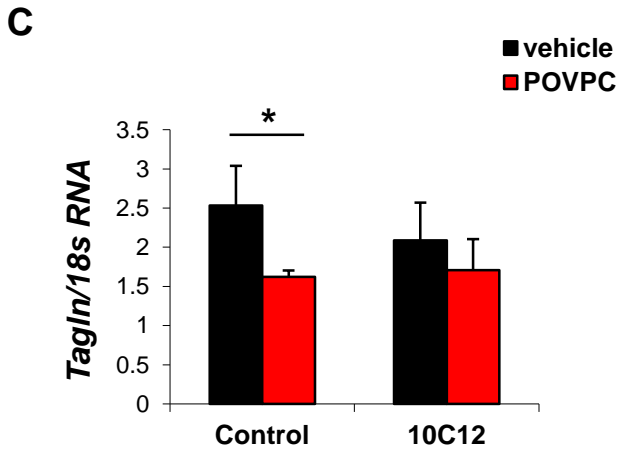
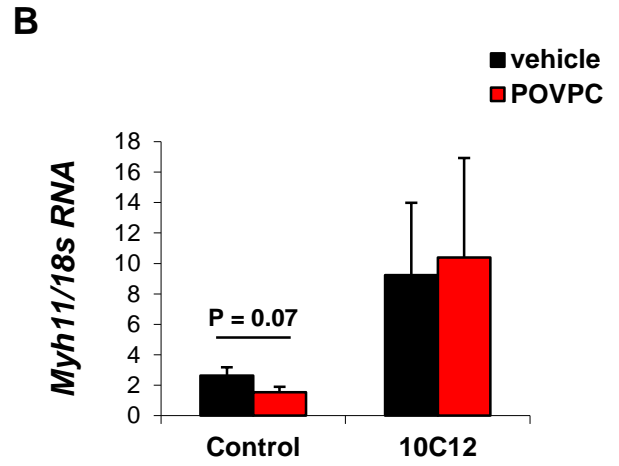
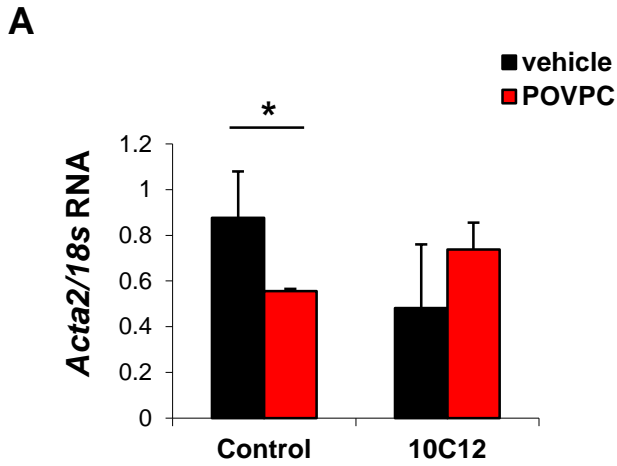
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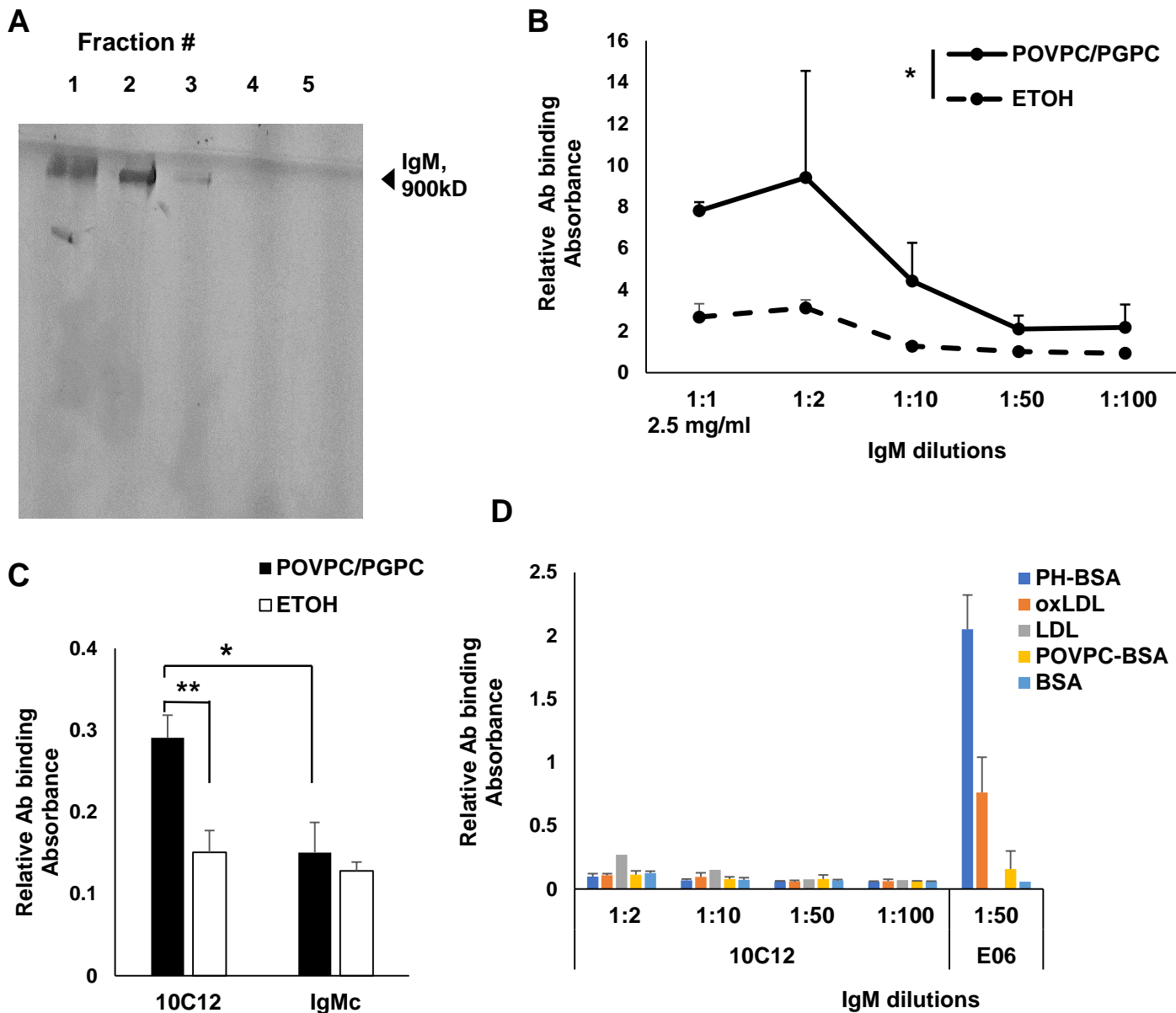
Supplemental Materials



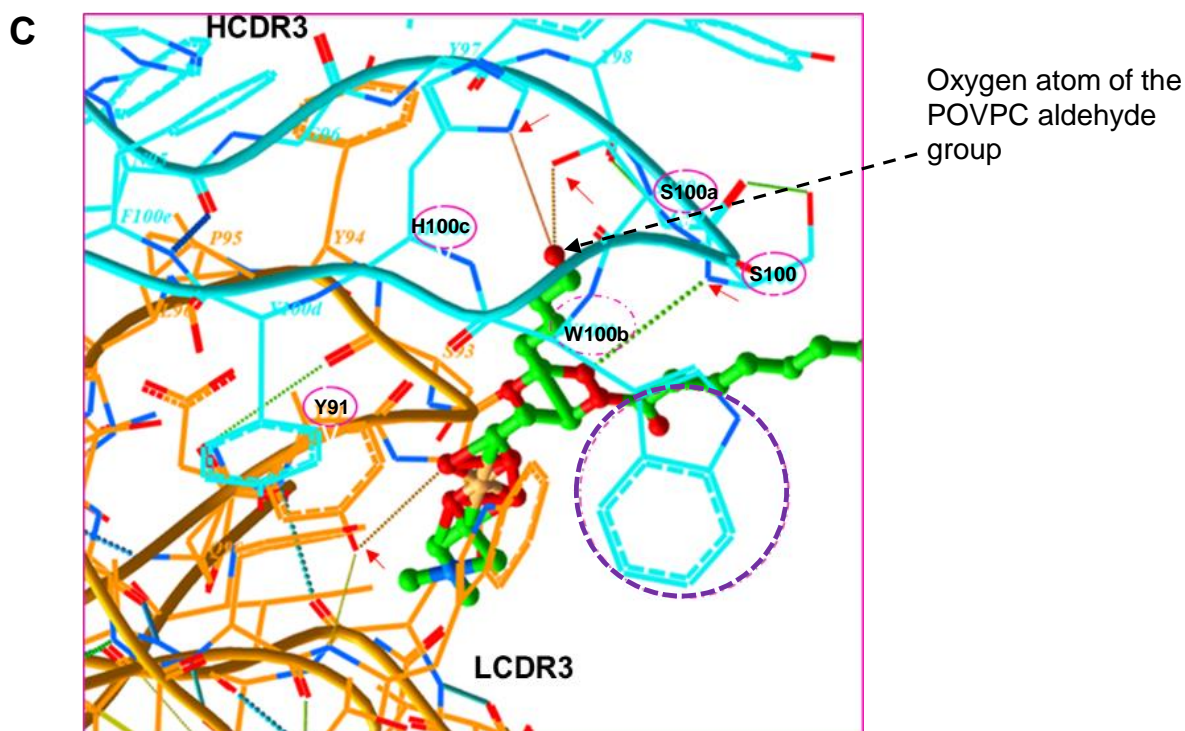
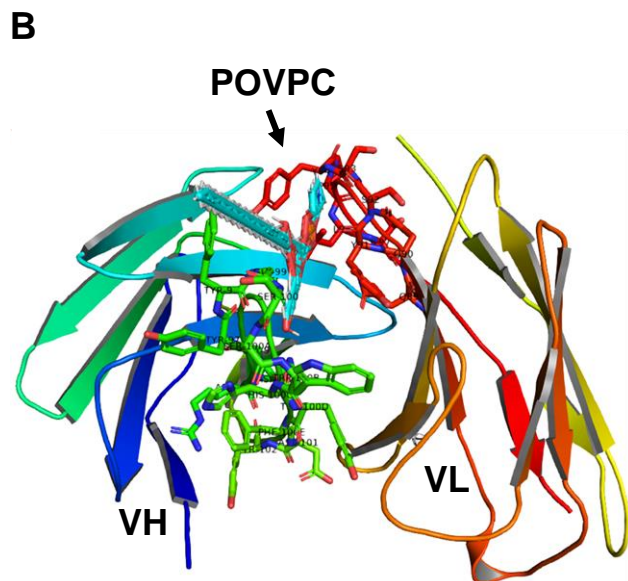
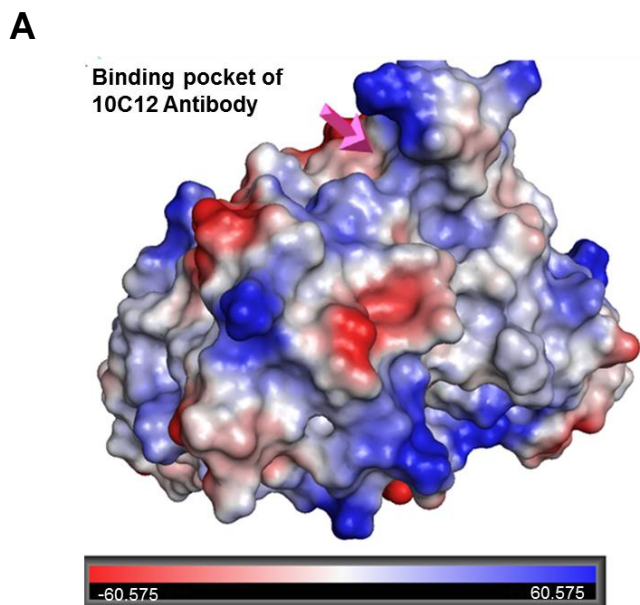
Supplementary Figure I. Generation of a novel 10C12 monoclonal antibody. *Apoe^{-/-}* mice were fed a high-fat Western diet for seven months and given one injection of POVPC/PGPC one day prior to sacrifice to boost immune response. **(A)** Anti-POPVC/PGPC titers from blood serum (1:100 whole serum dilution) of *Apoe^{-/-}* mice fed a high-fat Western diet (WD) for seven months versus C57B6 age-matched mice fed a standard laboratory diet (SD) as determined using an OxPL ELISA. Ethanol (ETOH) was used as a negative vehicle control. **(B)** Schematic for B-cell hybridoma generation. **(C)** A panel of B-cell hybridomas was screened for reactivity to POVPC/PGPC using an OxPL ELISA. Conditioned media from hybridoma cells were collected and used for ELISA. An E06 antibody was used as a positive control. Ethanol (ETOH) was used as a negative vehicle control.



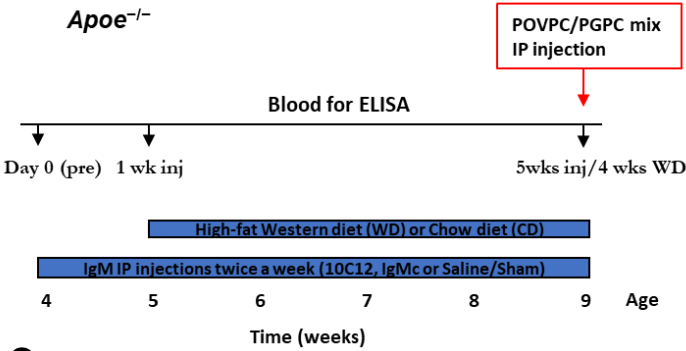
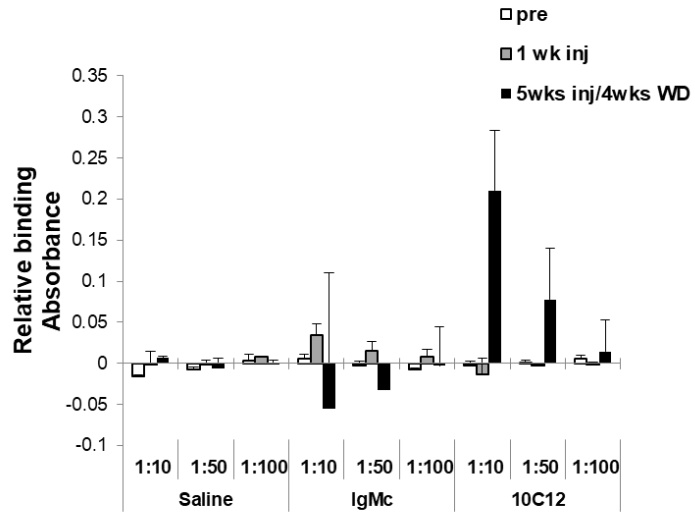
Supplementary Figure II. Cultured media from 10C12 hybridoma cells inhibited POVPC-induced phenotypic switching in cultured SMC. Rat aortic SMC were treated with 10 μ g/ml POVPC or DMSO-vehicle with (10C12) or without (Control) serum-free cultured media from 10C12 hybridoma cells to induce suppression of SMC marker genes, *Acta2*, *Myh11*, *Tagln*, and *Cnn1*. Gene expression was assessed by qRT-PCR and normalized to 18s RNA. Representative experiment, values = mean \pm StDev, * $P < 0.05$ by a Student's *t*-test. The experiment was repeated 3 times.



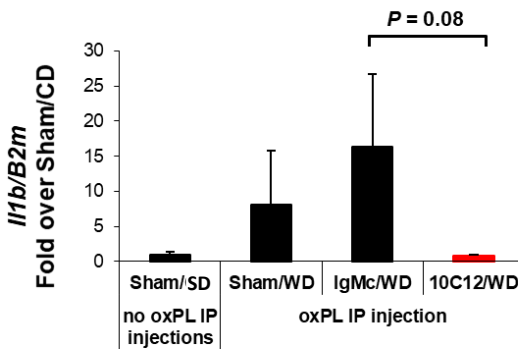
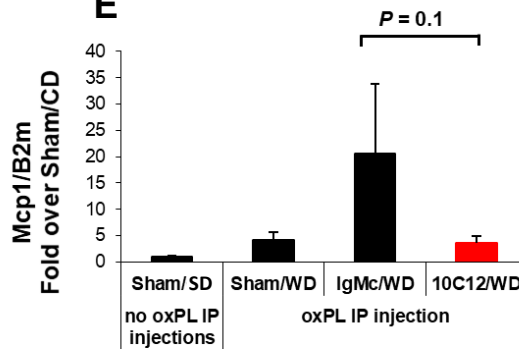
Supplementary Figure III. The 10C12 IgM antibody was purified from hybridoma culture media using HiTrap™ IgM purification HP columns. (A) SDS-PAGE gel stained with Coomassie Brilliant Blue. Lanes 1 – 5 correspond to column fractions containing the maximum protein content. **(B)** Binding of serial dilutions of the 10C12 antibody to POVPC/PGPC based on ELISA assays. Ethanol (ETOH) was used as a negative vehicle control. Data represented as normalization to no 1st antibody POVPC/PGPC control, mean \pm SEM. * $P < 0.05$ POVPC/PGPC vs. ETOH-vehicle control by 2-way ANOVA, $n = 3$ experiments. **(C)** Binding of the 10C12 IgM antibody (250 μ g/ml) or isotype-control IgM (250 μ g/ml) to POVPC/PGPC based on ELISA assays. Values = mean \pm SEM. * $P < 0.05$, * $P < 0.01$ by Student t -test, $n = 3$ experiments. **(D)** Binding of serial dilutions of the 10C12 antibody to various antigens. 1:1 = 2.5 mg/ml. Data represented as normalization to ETOH-vehicle control, $n = 2$ experiments.



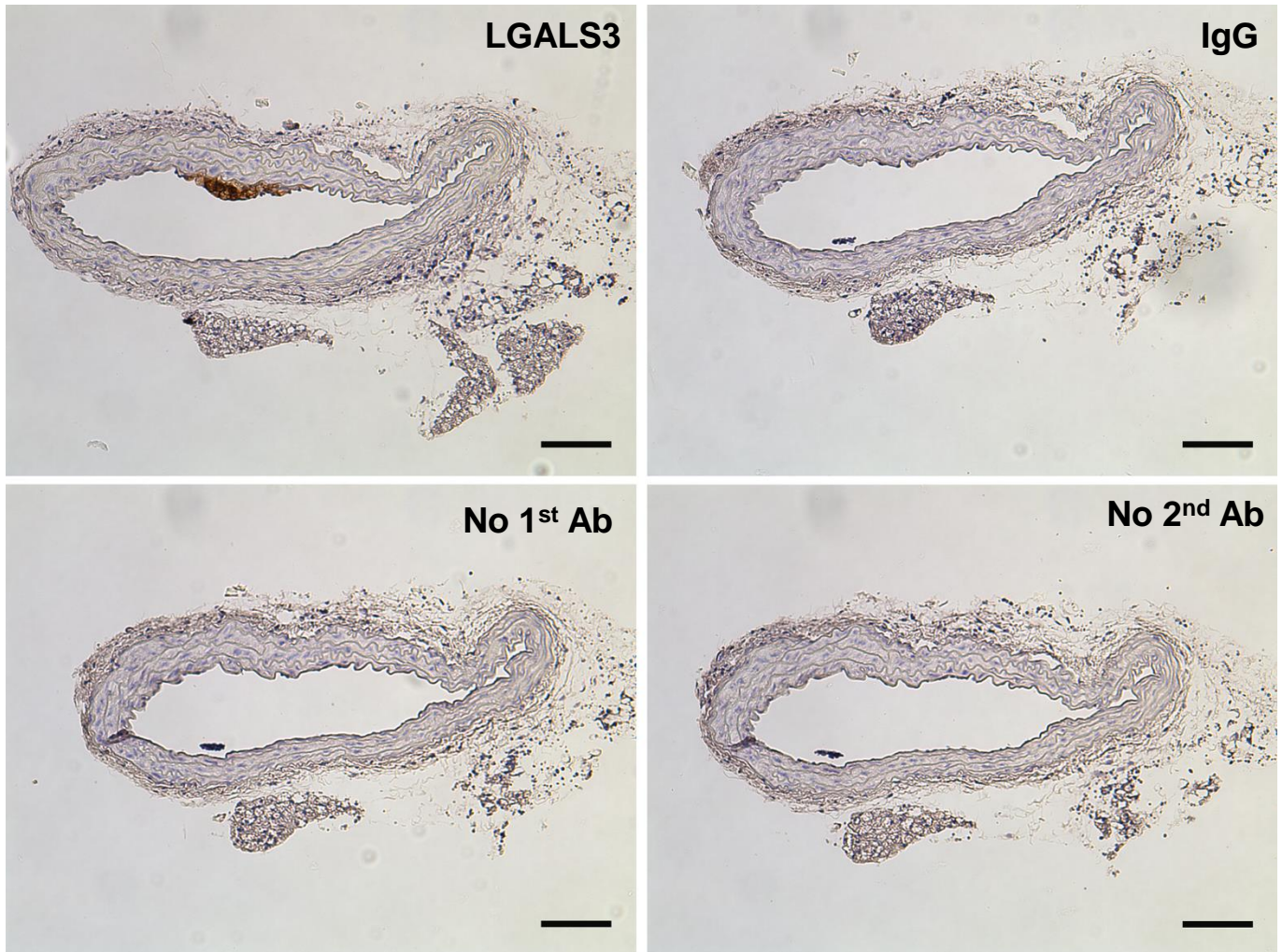
Supplementary Figure IV. 3D structural model of the 10C12 antibody including the predicted POVPC molecular recognition domain. (A) The 10C12 antibody surface is highlighted using a colored molecular surface as a charge-smoothed potential of the surface area in vacuum electrostatics. The antibody binding pocket area appears as a blue patch. The figure was generated using PyMol. **(B)** represents docking models of a single chain variable fragment (scFv) binding pocket. The complementary-determining regions (CDRs) of V_H and V_L of scFv of 10C12 are labeled. **(C)** The close-up view of POVPC interaction with antigen-binding sites indicates that POVPC was surrounded by CDR3 from the V_H domain. Red arrows indicate the key residues of the heavy chain, H^{S100} , H^{S100a} , H^{H100c} , and light chain L^{Y91} , providing non-covalent bonds with the polar oxygens of POVPC. The aromatic circle of the heavy chain tryptophan residue, H^{W100b} (purple dotted circle), forms the π -interaction with an oxygen of POVPC. **(B)** and **(C)** were generated using the Internal Coordinate Mechanics (ICM) software.

A**B****C**

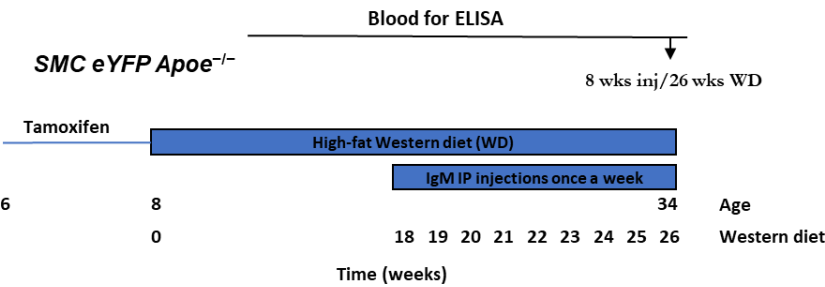
	Cholesterol, mg/dL	Triglycerides, mg/dL
Sham/SD	455 ± 24.9	167 ± 26.4
Sham/WD	1313 ± 99.0	172 ± 24.6
IgMc/WD	1375 ± 73.4	224 ± 25.3
10C12/WD	1326 ± 76.1	223 ± 25.5

D**E**

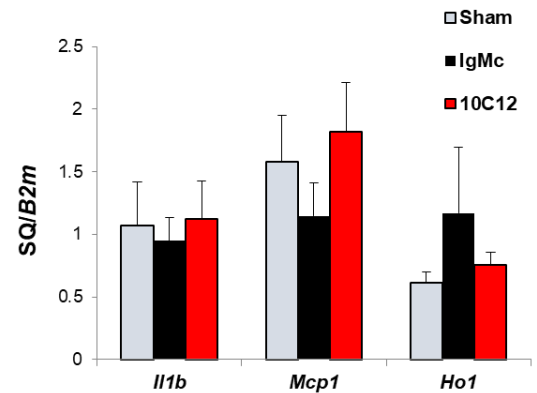
Supplementary Figure V. 10C12 treated *Apoe*^{-/-} mice exhibited increased titers of POVPC/PGPC reactive antibodies and showed impaired cytokine activation within peritoneal lavage cells following acute exposure to POVPC/PGPC intraperitoneal (IP) injections. (A) Experimental design. Four experimental groups were analyzed: Western diet plus IP injections with 10C12 IgM (10C12/WD, $n = 8$), IgM control (IgMc/WD, $n = 6$) or Saline/Sham (Sham/WD, $n = 5$); and standard laboratory low fat diet with Saline/Sham injections (Sham/SD, $n = 7$). A POVPC/PGPC mixture (25 μ g/25 μ g) was injected via IP six hours before mice were euthanized as described in Figure 3. (B) 10C12 antibody injections increased the titer of antibodies to POVPC/PGPC in blood plasma of *Apoe*^{-/-} mice fed a Western diet. The X-axis shows serial dilutions of plasma from randomly choose mice treated with Saline/Sham ($n = 3$), control IgM (IgMc, $n = 3$) or 10C12 ($n = 3$). Blood was collected at the time points indicated in (A). (C) 10C12 antibody treatment did not significantly change metabolic parameters in *Apoe*^{-/-} mice fed a Western diet for 4 weeks. Values = means \pm SEM. (D, E) Effects of 10C12 antibody treatment on expression of *Il1b* ($P = 0.08$) and *Mcp1* ($P = 0.1$) in abdominal lavage cells after POVPC/PGPC IP injections as compared to IgMc/WD group. Results of qRT-PCR normalized to beta2-microglobulin (*B2m*) gene, means \pm SEM, P values were calculated by Student's *t*-test.



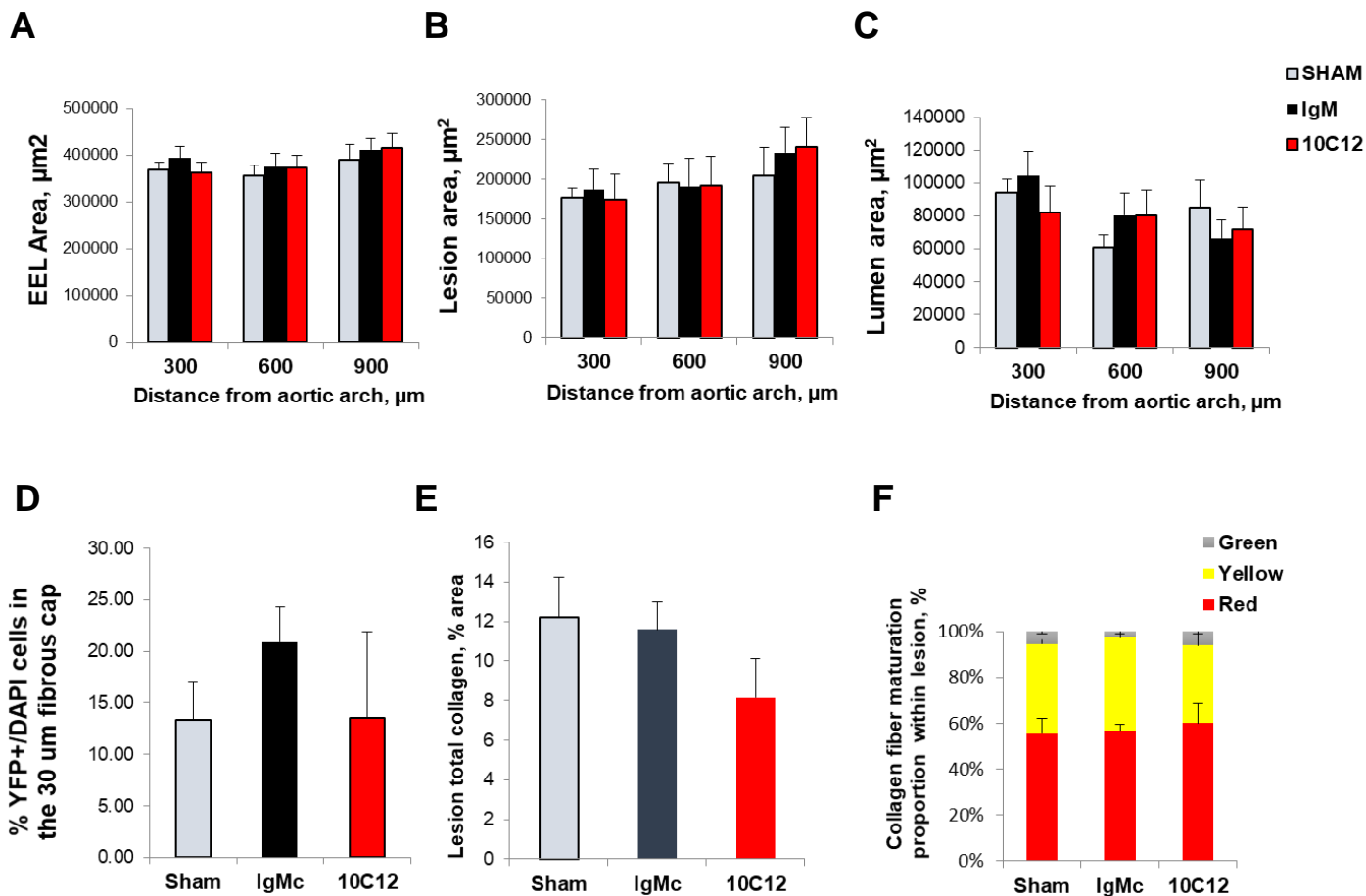
Supplementary Figure VI. Controls for determine the specificity of LGALS3 immunostaining in Figure 3B. Serial sections of the brachiocephalic artery from IgMc/WD treated *ApoE*^{-/-} mouse were stained for LGALS3, or incubated with an isotype rat non-immune IgG at the same concentration as for the anti-LGALS3 antibody, or without first (No 1st Ab), or second (No 2nd Ab). Scale bar = 100 μ m.

A**B**

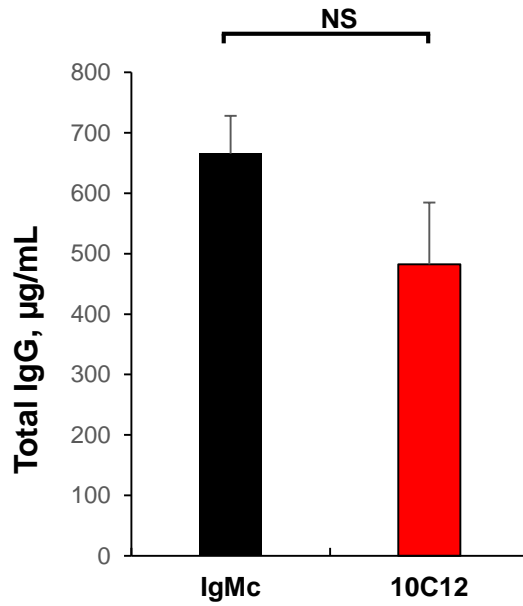
	Sham	IgMc	10C12
Cholesterol, mg/dL	968 ± 138.9	968 ± 146.5	844 ± 107.2
Triglycerides, mg/dL	174 ± 29.9	217 ± 49.1	288 ± 109.6
HDL-Cholesterol, mg/dL	26.7 ± 3.3	30.4 ± 4.5	27.4 ± 3
LDL-Cholesterol, mg/dL	919 ± 137	1073 ± 123.3 [#]	736 ± 126.5[#]
LDL/HDL	39.7 ± 6.7*	44.5 ± 5.0*	28.6 ± 4.8*
Cholesterol/HDL	41.8 ± 6.8*	46.8 ± 4.8*	33.2 ± 3.0*
Body weights, g	40.7 ± 10.4	37.4 ± 8.7	40.4 ± 9.2

C

Supplementary Figure VII. 10C12 antibody treatment of *ApoE*^{-/-} mice between 18 – 26 weeks of a Western diet resulted in improved LDL/HDL and total cholesterol/HDL ratios. (A) Experimental design. SMC-lineage tracing SMC *YFP*^{+/+}*ApoE*^{-/-} mice were treated with tamoxifen between 6 – 8 weeks of age to label MYH11⁺ differentiated SMC with YFP followed by eighteen-weeks of Western diet feeding. Between 18 – 26 weeks of Western diet feeding, mice were injected IP with 10C12 (*n* = 9), or IgM control (IgMc, *n* = 10) antibodies or saline (indicated as “Sham”) (*n* = 10) once a week for eight weeks. Arrows indicate when blood was collected for analyses. (B) 10C12 antibody treatment was not associated with significant decreases in cholesterol, LDL ([#]*P* = 0.09), HDL, triglycerides, or body weights between treatment groups. However, it did induce modest reductions in LDL/HDL and Cholesterol/HDL ratios. Values = means ± SEM, **P* < 0.05 10C12 versus IgMc by Student’s *t*-test. (C) Abdominal fat was collected from SMC *YFP*^{+/+}*ApoE*^{-/-} mice treated with Sham, IgM control or 10C12 and analyzed for inflammatory gene expression. Results of qRT-PCR normalized to beta2-microglobulin (*B2m*) gene, means ± SEM. No differences were observed in *Ho1*, *Il1b* or *Mcp1* gene expression between groups.



Supplementary Figure VIII. The 10C12 antibody treatment of *Apoe*^{-/-} mice between 18 – 26 weeks of Western diet feeding did not alter indices of outward remodeling of the brachiocephalic artery (BCA), lesion size or cell composition and collagen content. (A-C) Quantitative analysis of outward remodeling based on determination of the area within the external elastic lamina (EEL) (A), atherosclerotic lesion area (B), and lumen area (C) based on the Movat staining of the cross-sections of atherosclerotic lesions within BCAs of 10C12 treated mice ($n = 9$) compared to IgMc- ($n = 10$) or Sham-treated ($n = 10$) mice. Values represent mean \pm SEM. No significant differences were found for 10C12 treated mice versus IgMc or Sham-treated mice across multiple locations along the artery. Data were analyzed by linear mixed model ANOVA. (D) Quantification of the percentage of YFP⁺ SMC-derived cells within a 30 μm fibrous cap area of BCA lesions. Values represent the percent of YFP⁺ cells within the total cell population within the fibrous cap area as determined by DAPI staining. (E) Quantification of the total collagen content based on PicroSirius Red staining within the lesion. Values = mean \pm SEM. (F) Evaluation of collagen maturation based on PicroSirius Red staining followed by polarized light microscopic evaluations. No difference was observed in ratios between the amount of green (thin/immature), yellow (intermediate) and red (thick/mature) collagen fibrils within the lesion between experimental groups. Data in E and F were analyzed by Student's *t*-test.



Supplementary Figure IX. Mice treated with the 10C12 antibody and IgMc demonstrated equal IgG levels after eight weeks of injections based on IgG ELISA. NS – non significant by Student *t*-test

Major Resources Tables

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex
mice	The Jackson Laboratory, #002052	C57B6J x 129 <i>Apoe</i> ^{-/-}	males
mice	Previously generated in Dr. Gary Owens' laboratory, Ref. 9, 30, 31	C57B6J <i>ROSA26-STOP^{Flox}eYFP^{+/+};Myh11-CreER^{T2};Apoe</i> ^{-/-} (SMC <i>YFP^{+/+}ApoE</i> ^{-/-})	males
mice	Previously generated in Dr. Coleen McNamara's laboratory, Ref. 41	C57B6J x 129 <i>IgM</i> ^{-/-} <i>Apoe</i> ^{-/-}	males

Animal breeding

	Species	Vendor or Source	Background Strain	Other Information
Parent - Male	mice	Ref. 9, 30, 31	C57B6J	<i>ROSA26-STOP^{Flox}eYFP^{+/+};Myh11-CreER^{T2};Apoe</i> ^{-/-}
Parent - Female	mice	Ref. 9, 30, 31	C57B6J	<i>ROSA26-STOP^{Flox}eYFP^{+/+};Apoe</i> ^{-/-}
Parent - Male	mice	Ref. 41	C57B6J x 129	<i>IgM</i> ^{-/-} <i>Apoe</i> ^{-/-}
Parent - Female	mice	Ref. 41	C57B6J x 129	<i>IgM</i> ^{-/-} <i>Apoe</i> ^{-/-}

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
LGALS3	Cedarlane	CL8942AP	0.1 µg/mL	
Mouse IgG/IgM	Amersham	NXA931	Not available Used as 1:2000 dilution	16810268
Mouse IgM-AP	Southern Biotech	1021-04	Not available Used as 1:4000 dilution	
Monoclonal IgM kappa antibody generated from spleens of <i>Apoe</i> ^{-/-} mice fed a high-fat	Generated in house	10C12	Various (as indicated in manuscript)	

Western diet for seven months				
Cockroach antigen	Lymphocyte Culture Center, University of Virginia	IND-8D6	Various (as indicated in manuscript)	
E06 Antibody	Avanti Polar Lipids	330001	Various (as indicated in manuscript)	

Phospholipids

Phospholipid	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
POVPC	Cayman Chemical	10044	Various (as indicated in manuscript); 10 µg/well for ELISA	
PGPC	Cayman Chemical	10044	Various (as indicated in manuscript); 10 µg/well for ELISA	
DMPC	Cayman Chemical	15097	Various (as indicated in manuscript); 10 µg/well for ELISA	
LDL	Alfa Aesar,	BT-903	1 µg/well for ELISA	903H18A
oxLDL	Alfa Aesar	BT-910	1 µg/well for ELISA	910H18B
PH-BSA	Biosearch Tech	PC-1011-10	1 µg/well for ELISA	110267-04

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)
518 Rat aortic smooth muscle cells	Generated in Dr. Gary Owens laboratory, ref. 42	males