SUPPLEMENT

Additional Methods:

Conversion of IK17-Fab into IK17-scFv: The human Fab monoclonal antibody IK17 was cloned from a phage display library as previously described(1). To convert the IK17-Fab into an scFv fragment, two rounds of PCR were used to introduce a seven amino acid linker connecting the VL and VH regions and restriction sites for cloning. Briefly, according to the germline genes identified previously, in first round PCR, we used forward primer 5'-GGGCCCAGGCGGCCGAGCTCGTGWTGACRCAGTCTCC (targets human κ3 gene) and backward primer 5'-GGAAGATCTAGAGGAACCACCTTTGATCTCCAGCTTGGTCCC (targets human Jκ2) for VL; and forward primer 5'-GGTGGTTCCTCTAGATCTTCCGAGGTGCAGCTGCTCGAGTCGGG (targets human VH3 gene) and backward primer 5'-CCTGGCCGGCCTGGCCACTAGTGACCGATGGGCCCTTGGTGGARGC (targets human γ gene) for VH. The separately amplified VL and VH products were then 1:1 mixed and used for overlap PCR with VL forward and VH backward primers to assemble the IK17-scFv of VL-linker-VH structure. The IK17-scFv was sequenced to confirm the gene structure and inserted into a prokaryotic vector pARA for expression. The purified IK17-scFv was used to evaluate its binding properties and biological functions using the methods described below. The coding region of IK17-scFv was amplified with primers: 5'-ACGAAGCTTGCTCGTGTTGACGCAGTCTCC (forward) and 5'-CGAGCGGCCGCTGAGGAGACGGTGACCCGG (backward); then subcloned into HindIII and NotI sites of the eukaryotic expression vector pSectTag2A (Invitrogen, Carlsbad, CA), which contain a mouse kappa signal sequence for expression and secretion. This vector also has a c-Myc tag, allowing detection with an anti-myc antibody.

Generation of recombinant adenovirus vector expressing the IK17-scFv gene: The expression and secretion cassette of IK17-scFv gene was isolated from the pSecTag-IK17-scFv plasmid by a PCR reaction using primers: 5'-TGGAATTCATACGACTCACTATAGGGAGAC (forward) and 5'-AGTGTCGACAACTAGAAGGCACAGTCGAGG (backward); and then inserted into the EcoRV (blunt) site of the adenovirus shuttle plasmid pDelatE1Z, which expresses the transgene from the

hCMV promoter. The resulting adeno-shuttle plasmid pDeltaE1Z-IK17 was cotransfected with E1-defective adenovirus backbone genome JM17 DNA into the HEK293 cells. Two to three weeks after co-transfection, plaques were isolated and amplified to examine the IK17-scFv expression. Adv-vectors were then purified through two-rounds of CsCl centrifugation and titers were measured in two ways: plaque formation on 293 cells (pfu/ml) and OD_{260} reading (particles/ml). As a control of a non-relevant gene, the green florescent protein (GFP) gene was also inserted into the same adenovirus vector to generate adenovirus expression GFP (Adv-EGFP).

Western blotting: The supernatants from the transfected 293 cells or plasma samples were run on 10-20% gradient NuPage SDS-gel (Invitrogen, Carlsbad, CA), transferred onto a nitrocellulose membrane and detected using alkaline phosphatase (AP) labeled anti-c-myc monoclonal antibody (Sigma, St. Louis, MO).

Assessment of gene expression by real-time PCR: RNA was extracted from heart, liver, kidney, lung and spleen using RNeasy kit (Qiagen; Valencia, CA), reverse transcribed into cDNA using Superscript III (Invitrogen, Carlsbad, CA), and used as the template for real-time PCR to examine the expression of the transgenes. Real-time PCR was performed using the PerkinElmer ABI Prism 7700 and Sequence Detection System software (PerkinElmer Life Sciences) using sets of TaqMan PCR primers and probes for IK17 and GAPDH previously described(2). All samples were run in duplicate.

Chemiluminescent immunoassays: The ability of IK17-Fab or IK17-scFv to bind to MDA-LDL and Cu-OxLDL was determined using chemiluminescent immunoassays as previously described(1). In brief, varying dilutions of culture supernatants or plasma (containing IK17-Fab or IK17-scFv) were added to microtiter wells containing MDA-LDL or Cu-OxLDL (or various controls) and after incubations and appropriate washings, the extent of binding determined using an alkaline phosphatase (AP) labeled goat anti-human IgG Fab (Sigma) to detect IK17-Fab or AP labeled monoclonal anti-myc (9E10, Sigma) to detect myc-labeled IK17-scFv. The amount

of binding was determined using LumiPhos 530 solution (Lumigen) and the resulting light emission was measured as relative light units (RLU) over 100 milliseconds using a Dynex Luminometer (Dynex Technologies). To determine the specificity of binding, competition immunoassays were performed as described(3,4). In brief, a fixed and limiting dilution of IK17-Fab or scFv (in culture or plasma) were added to microtiter wells containing MDA-LDL or Cu-OxLDL in the absence or presence of indicated competitors and then the extent of binding determined and expressed as a ratio of binding in the presence or absence of competitor (B/B_0).

For Study 1, measurements of IgG and IgM autoantibodies to MDA-LDL and Cu-OxLDL, and oxidized phospholipids on mouse apoB-100 particles (OxPL/apoB) were determined as previously described(5). Anti-phosphocholine T15/E06 IgM antibody levels were measured using the T15 anti-idiotype antibody AB1-2 as described(3). The binding of murine anti-IK17 antibodies to IK17 Fab was performed by adding serial dilutions of plasma to purified IK17-Fab (5 μg/ml) plated in microtiter wells, and the extent of antibody binding determined using anti-murine IgG or IgM (Sigma) as described above. IK17-Fab plasma concentrations were measured at selected time points in plasma obtained just prior to the next IK17 infusion by sandwich ELISA by plating goat anti-human Fab as capture antibody, adding plasma at 1:100 dilution and detecting IK17 Fab with a goat anti-kappa chain human IgG antibody (Sigma). A standard curve was generated with purified IK17 Fab. IgG and IgM IK17-immune complexes (IC), representing mouse anti-IK17 antibodies bound to human IK17-Fab, were measured by sandwich ELISA by plating the goat anti-human Fab, adding plasma at 1:100 dilution and detecting IK17 ICs with IgG or IgM goat anti-mouse antibodies.

Atherosclerosis Quantification: Atherosclerosis was quantified by computer-assisted image analysis in Sudan-stained *en face* preparations of the entire aorta as previously described(1). In addition, aortic root cross-sectional atherosclerosis was measured by cutting 9 μm paraffin sections from the origin of the aortic valve where the first leaflet was seen until the last leaflet, resulting in ~40-60 sections. Modified van Gieson elastic stain was used to enhance the contrast between the intima and surrounding tissue. Quantitative analysis of lesion area was

performed on every sixth section starting from the time each of the three aortic valve leaflets was visible until a total of 7 sections were analyzed (i.e. spanning ~42 total sections or 378µm from the origin of the first visible leaflet). The results are presented as total lesion area in mm^2 of all aortic cross-sections analyzed. Quantification was performed by investigators blinded to treatment assignment using computer-assisted image analysis(2).

Immunohistochemistry: Atherosclerotic lesions from human and mouse were paraffin-embedded, and sectioned. Immunostaining for oxidation-specific epitopes recognized by expressed IK17-scFv from the plasma of Adv-IK17-scFv injected animals was performed as described(3). Briefly, the tissue sections were deparaffinized and incubated in 3% hydroperoxide to block the endogenous peroxidase activity. After blocking non-specific binding with non-immune serum from the species, in which the secondary antibody was made, the sections were incubated with serial dilutions of plasma of an AdvIK17 injected mouse, and IK17-scFv bound detected using biotinylated anti-myc antibodies followed by an avidin-biotin-peroxidase complex and the DAB substrate (Vector labs). As a control, adjacent sections were stained with the plasma from Adv-EGFP injected mouse. The sections were then counterstained with Hematoxylin.

Immunostaining of murine tissues with rat anti-macrophage (RM0029-11H3, Santa Cruz Biotechnology) and guinea pig antisera against MDA epitopes (MDA3) was performed using the VectorLabs ABC-AP system (Vector Laboratories, Burlingham, CA). Serial 9 µm-thick sections of the paraffin-embedded aortic sinus were deparaffinized in xylene. Slides were incubated consecutively with 5% normal serum to block non-specific binding, primary antibody for 1 hour and alkaline phosphatase labeled secondary anti-rat or anti-guinea pig antibody for 30 minutes and Vectastain ABC-AP reagent for 30 minutes at room temperature. The reaction products were identified by immersing the slides in alkaline phosphatase substrate solution to give a red reaction product. The slides were then counterstained with VectoLabs hematoxylin QS and mounted. Negative controls included replacement of the primary antibody by irrelevant isotype-matched antibodies or non-immune serum.

Smooth muscle cells (SMCs) were analyzed by Movat`s pentachrome staining. Collagen content was analyzed by picrosirius red and polarized light microscopic imaging. Percent-positive area for immunohistochemical or picrosirius red staining was quantified by Photoshop-based image analysis as described(6). Briefly, pixels with similar chromogen characteristics were selected with the “magic wand” tool and the “select similar” command, and the ratio of the positively stained area to the total lesion area studied was calculated with the “histogram” command in Photoshop. All quantitative morphometric and immunohistochemical data were collected independently by three experienced operators blinded to the mice genotypes.

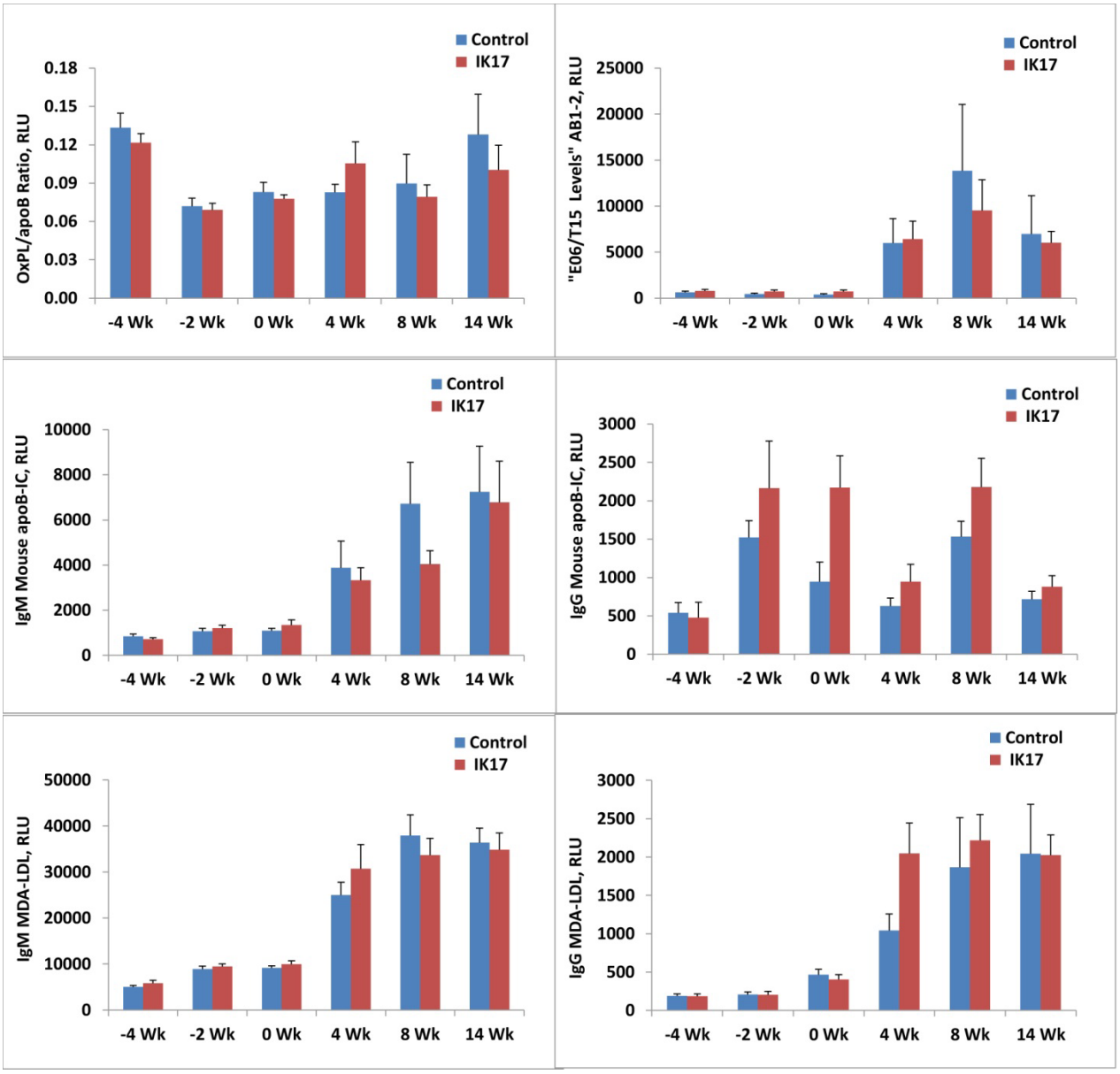
Macrophage binding and competition assay: The assay was conducted as previously described with modifications(4). Briefly, isolated human LDL was biotinylated according to manufacturer’s protocol (Cat# 21326; Pierce Biotechnology) prior to modification or oxidation to prepare various biotinylated OxLDL ligands. The biotinylated OxLDL (1.5 μg/ml) was incubated with serially diluted competitors and controls in 1% BSA-PBS. The ligand-competitor solutions were incubated overnight at 4°C.

J774 murine macrophages were cultured in 10% fetal bovine serum in DMEM and plated in 100 □L L929-fibroblast conditioned media at 25,000 cells/well in sterile 96-well flat-bottom white plates (Greiner Bio-One). The plating media consisted of 20% L929-fibroblast conditioned DMEM-10 and 80% fresh DMEM-10 and served as a source of growth factors, including macrophage colony-stimulating factor (M-CSF).

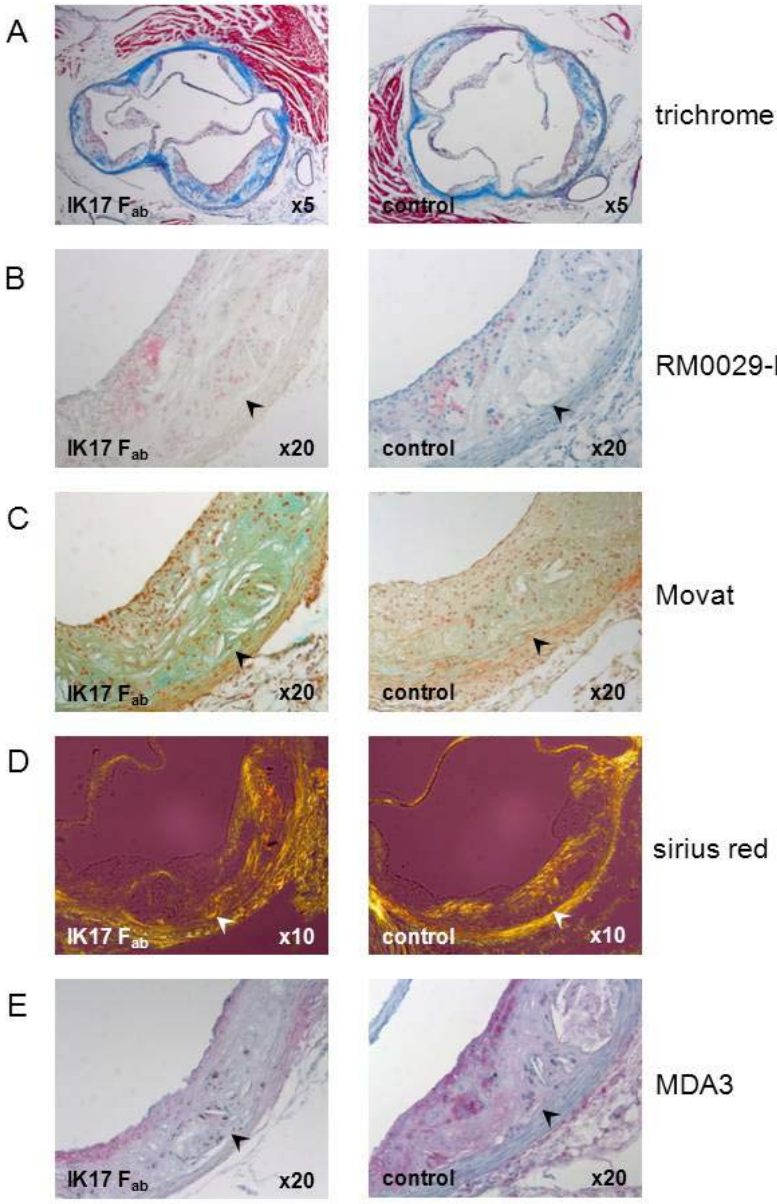
After 72 hours, plates were washed gently 5 times with PBS using a microtiter plate washer (Dynex Technologies, Chantilly, VA), and wells were blocked with ice-cold 200 μL 1% BSA-PBS for 30 min, while plates were kept on ice. After washing, macrophages were incubated with ice-cold ligand-competitor solutions (100 μL/well) for 2 hours on ice, washed again, and fixed with ice-cold 3.7% formaldehyde in PBS for 30 min in the dark. Macrophage-bound biotinylated OxLDL was detected with NeutrAvidin-conjugated alkaline phosphatase (Pierce Biotechnology), LumiPhos 530 (Lumigen, Southfield, MI), and a Dynex Luminometer (Dynex Technologies). Data were recorded as relative light units counted per 100 milliseconds (RLU/100ms).

RESULTS:

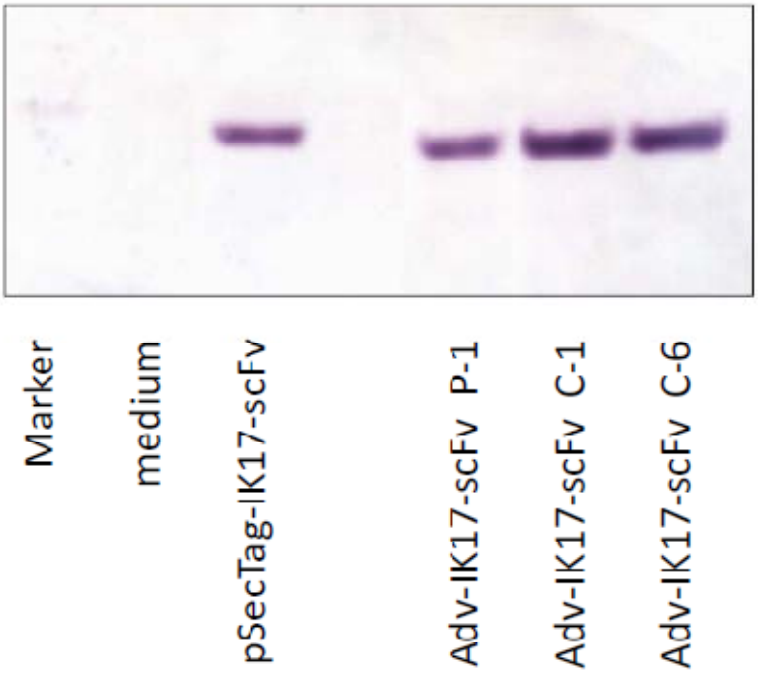
Supplemental Fig.1. Immunologic effects in LDLR$^{-/-}$ mice following infusion of IK17-Fab. There were no differences between groups, but significant increases were present in "E06/T15" levels and apoB-immune complexes and autoantibodies to MDA-LDL in response to the high cholesterol diet.



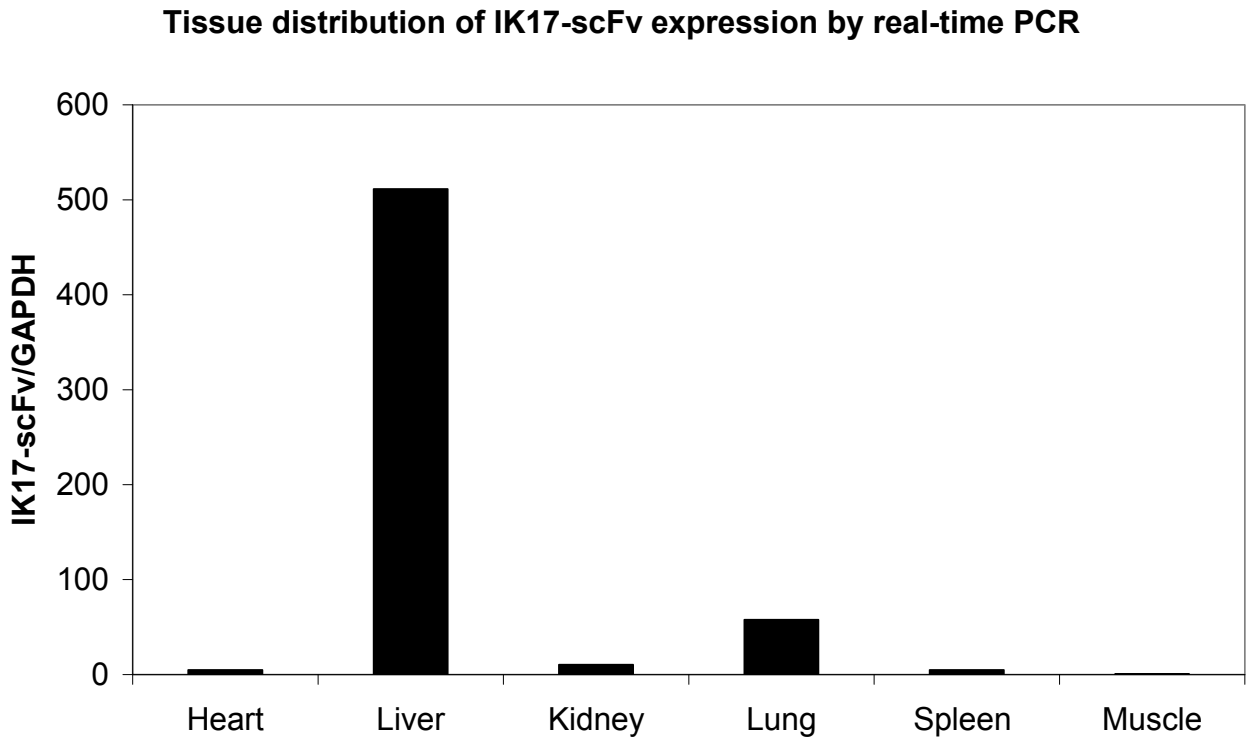
Supplemental Fig. 2. Representative examples of atherosclerotic lesions located in the aortic sinus inner aortic arch intima (lesser curvature) of IK17 Fab treated and control mice. Slides were stained with trichrome (A) for quantification of total lesion area, the rat antibody RM0029-H3 for quantification of macrophages (B), Movat´s pentachrome for quantification of SMCs (C), sirius red with subsequent polarization for quantification of collagen (D) and the guinea pig antiserum MDA3 for quantification of MDA (E). Percent-positive area for macrophages (B, red-stained areas), smooth muscle cells (C, red-stained areas), collagen (D, areas with yellow, green, orange, or red polarized colour) and MD-LDL (E, red-stained areas) were quantified by Photoshop-based image analysis. In B to E, the lumen is to the upper left corner and the demarcation between intima and media is indicated by arrowheads.



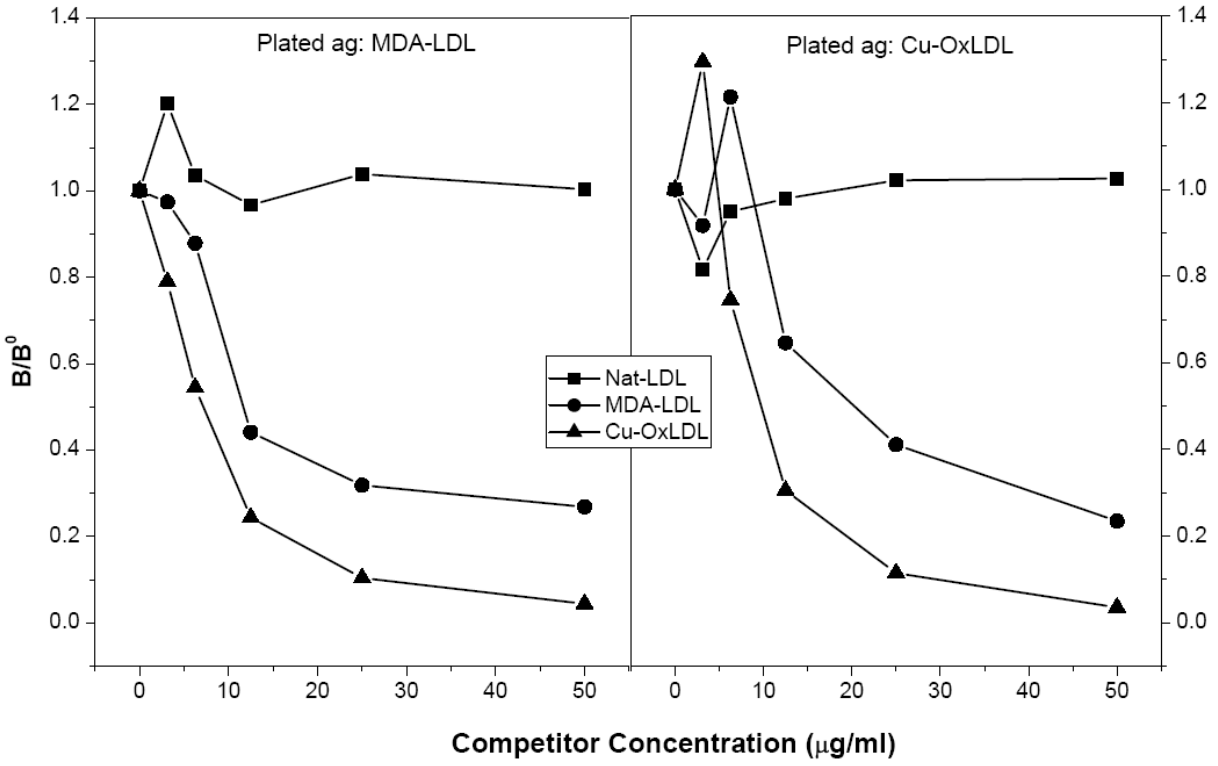
Supplemental Fig. 3. Supernatants collected from 293 cells were run on a 10-20% gradient SDS gel and transferred onto a nitrocellulose membrane. After blocking with 5% fat free milk, the expressed IK17-scFv protein was detected by anti-c-myc-AP mouse monoclonal antibody. This experiment demonstrated that a single peptide of IK17-scFv was secreted into the media by transfected pSecTag-IK17-scFv vector, as well as by the infection of adenovirus vector. P1 was the initial plaque of recombinant adenovirus; C1 and C6 were two stains of adenovirus selected from single colonies.



Supplement Fig. 4. RNA was extracted from indicated tissues from three mice sacrificed one week post-injection of $1.0X10^{11}$ v.p. of Adv-IK17-scFv. RNA was converted to cDNA, which was used as the template for Taqman real-time PCR reaction using specific primers for the IK17-scFv gene to detect the presence of the virus mediated transgene. The transgene was primarily located in liver, with little distribution to other tissues. The results are expressed as the average of organs from three mice. For each reaction GAPDH DNA was used as housekeeping gene to normalize the genomic DNA.



Supplement Fig 5. Specificity of the plasma IK17-scFv expressed from Adv-IK17-scFv. Pooled mouse plasma (1:100 dilution) was added to Cu-OxLDL or MDA-LDL coated wells in the absence or presence of indicated competitors, and the amount of IK17-scFv bound was detected by anti-myc antibody. Data expressed as the ratio of IK17-scFv bound in the presence (B)/absence (B_0) of competitors.



References

1. Shaw PX, Horkko S, Tsimikas S, et al. Human-derived anti-oxidized LDL autoantibody blocks uptake of oxidized LDL by macrophages and localizes to atherosclerotic lesions in vivo. Arterioscler Thromb Vasc Biol 2001;21:1333-9.
2. Li AC, Brown KK, Silvestre MJ, Willson TM, Palinski W, Glass CK. Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. Journal of Clinical Investigation 2000;106:523-531.
3. Binder CJ, Horkko S, Dewan A, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nature medicine 2003;9:736-43.
4. Binder CJ, Hartvigsen K, Chang MK, et al. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. Journal of Clinical Investigation 2004;114:427-437.
5. Merki E, Graham MJ, Mullick AE, et al. Antisense oligonucleotide directed to human apolipoprotein B-100 reduces lipoprotein(a) levels and oxidized phospholipids on human apolipoprotein B-100 particles in lipoprotein(a) transgenic mice. Circulation 2008;118:743-53.
6. Lehr HA, Mankoff DA, Corwin D, Santeusanio G, Gown AM. Application of photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. The journal of histochemistry and cytochemistry 1997;45:1559-65.