SUPPLEMENTAL MATERIAL

Supplemental Methods

Diets

In more detail the constituents of the high fat (HF) diet used were: 15% cocoa butter, 1% corn oil, 0.25% cholesterol, 40.5% sucrose, no cholic acid, total fat content 16% (Arieblok Diet W). The low fat (LF) semisynthetic reference diet contained: 54.3% glucose, 5% soya oil, no added cholesterol, total fat content 5.2% (Arieblok Reference Diet 4068.02). Both diets were purchased from Hope Farms, Woerden, Netherlands.

Lipoprotein isolation

Human LDL (density 1.019-1.063g/mL) was isolated from plasma of healthy donors after overnight fasting by differential density ultracentrifugation.[1] Plasma was centrifuged at a density of 1.019g/mL at 53 000rpm for 16 hours at 5°C in a 70Ti fixed-angle rotor (Beckman Instruments, Inc.). The lower layer was adjusted to density 1.30g/mL, layered under KBr (density 1.006g/mL) and centrifuged in a 50Ti vertical rotor at 50 000rpm for 2½ hours at 8°C. The LDL band was removed, adjusted to density 1.063g/mL with KBr and centrifuged at 43 000rpm in a SW50.1 rotor at 8°C. The purified LDL was gel filtered on PD-10 columns (Pharmacia Biotech AB, Uppsala, Sweden) to remove KBr and sterile filtered. Protein content was determined by nanospectrophotometry. The purified LDL was stored at 4°C in the presence of 1mmol/L Na_2EDTA and used within 2 weeks.

Modification of LDL

Malondialdehyde (MDA) was synthesised by acid hydrolysis of malondialdehyde bis dimethylacetal (Sigma-Aldrich, Poole, UK).[2,3] In summary, 96µL 4M HCl, 704µL

malondialdehyde bis dimethylacetal was mixed with 3.2mL ddH_2O and incubated for 10 minutes at 37°C. The reaction was neutralised by adjusting the pH to 7.4 with 1M NaOH. LDL was gel filtered to remove EDTA and incubated with 0.5M MDA at a ratio of 100µL MDA/mg LDL for 3 hours at 37°C. To generate copper oxidised-LDL (CuOxLDL), LDL diluted in PBS to concentration of 1mg/mL was mixed with 10µM $CuSO_4$ for 16 hours at 37°C. After oxidation MDA-LDL or CuOxLDL was gel filtered to remove residual MDA or $CuSO_4$. To limit further modification, Na_2EDTA was added to a concentration of 2.7mmol/L. Both MDA-LDL and CuOxLDL were used immediately. Agarose gel electrophoresis (Paragon Lipokit, Beckman) confirmed consistent oxidative modification of LDL.

Measurement of anticardiolipin and anti-$\beta_2$glycoprotein I antibodies

To measure anticardiolin (aCL) antibodies, non-irradiated plates were coated overnight at 4°C with bovine cardiolipin in ethanol (1µg/well) and blocked with 10% adult bovine serum (ABS, Sigma-Aldrich). Serum samples were diluted 1:100 in 10% ABS. AP-conjugated anti-mouse IgG antibody (Southern Biotech) diluted 1:1000 in 10% ABS was applied and plates were developed with *p*-nitrophenol phosphate. For the measurement of anti-$\beta_2$glycoprotein I antibodies, 96 well polystyrene plates (NUNC Maxisorp) were coated overnight at 4°C with purified human $\beta_2$glycoprotein I (Crystal Chem, IL) in borate buffered saline, then blocked with 2% BSA. After incubation with serum samples diluted in 2% BSA, AP-conjugated goat anti-mouse IgG antibody was applied and plates developed with *p*-nitrophenol phosphate.

Renal analysis

Urinalysis dipstick (Bayer) was used to screen for proteinuria and haematuria. Kidney portions were harvested at sacrifice prior to perfusion, processed in Bouin's solution for 2

hours, transferred to 70% ethanol, embedded in paraffin, stained with periodic acid-Schiff and scored for glomerulonephritis as previously described.[4]

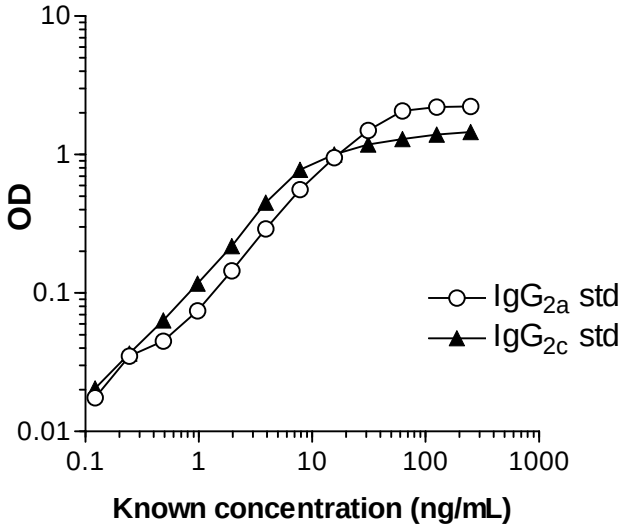
Specificity testing of antibody for both mouse IgG_{2a} and IgG_{2c}

A standard ELISA was set up to measure levels of IgG_{2a} and IgG_{2c}, using a goat anti-mouse Ig antibody (Southern Biotech) as the capture antibody. Serial dilutions of commercial mouse IgG_{2a} standard (HOPC-1 clone, Southern Biotech) or IgG_{2c} reference serum (serum pool from C57BL/6 mice, Bethyl Laboratories) of known concentrations were added. The secondary antibody (alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG_{2a}, Southern Biotech cat. 1080-40) being tested was added. The substrate pNPP was used and plates read at 405nm after 30mins.

Supplemental Table 1. Baseline characteristics in *Ldlr*$^{-/-}$, *C1qa.Ldlr*$^{-/-}$, *sIgM.Ldlr*$^{-/-}$ and *C1qa.sIgM.Ldlr*$^{-/-}$ mice aged 22 weeks.

Mouse genotype	Diet	n	Final body weight (g)	Total cholesterol (mmol/L)	Total triglycerides (mmol/L)
Ldlr$^{-/-}$	LF	12	22.4±0.43	7.48±0.44	1.58±0.22
C1qa.Ldlr$^{-/-}$	LF	12	24.0±0.48	8.61±0.56	1.40±0.15
sIgM.Ldlr$^{-/-}$	LF	14	24.0±0.62	8.61±0.65	1.67±0.16
C1qa.sIgM.Ldlr$^{-/-}$	LF	10	24.4±0.40	7.80±0.77	1.17±0.15
Ldlr$^{-/-}$	HF	12	25.7±1.05	39.6±1.34	4.63±0.45
C1qa.Ldlr$^{-/-}$	HF	15	25.3±0.48	46.4±2.60	6.02±0.62
sIgM.Ldlr$^{-/-}$	HF	15	23.7±0.55	40.7±2.05	4.61±0.34
C1qa.sIgM.Ldlr$^{-/-}$	HF	9	23.8±0.55	44.8±2.18	4.74±0.61

LF= low fat diet, HF= high fat diet. Values are expressed as mean±SEM. Differences between groups were not significant on either diet (statistical analysis by one-way ANOVA).



Supplemental Figure 1. Testing of the specificity of secondary antibody (alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG_{2a}) for mouse IgG_{2a} and IgG_{2c}

Graph shows the testing of the secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG_{2a}, Southern biotech cat. 1080-40) and its specificity for mouse IgG_{2a} and IgG_{2c}. The binding of 1080-40 antibody to both commercial IgG_{2a} standard and IgG_{2c} reference serum of known concentrations is demonstrated, showing that this particular batch of goat anti-mouse IgG_{2a} antibody has similar specificity for both IgG_{2a} and IgG_{2c} below optical densities of ~1.0 for this ELISA setup. According to the manufacturer Southern Biotech, some batches of the 1080-40 antibody recognise both mouse IgG_{2a} and IgG_{2c}.

Supplemental References

1. Wade DP, Knight BL, Soutar AK. Detection of the low-density-lipoprotein receptor with biotin-low-density lipoprotein. A rapid new method for ligand blotting. *Biochem J.* 1985;229:785-790.

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3. Zhou X, Paulsson G, Stemme S, Hansson GK. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J Clin Invest.* 1998;101:1717-1725.

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