

Immunoglobulin M is Required for Protection Against Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice

Short title **IgM Deficiency Accelerates Atherosclerosis**

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Abstract

Background— IgM natural antibodies bind oxidatively modified low-density lipoprotein (LDL) and apoptotic cells and have been implicated as important for protection from atherosclerosis. We have directly investigated the requirement for IgM by studying the effects of IgM deficiency in LDL receptor-deficient mice (*Ldlr*^{-/-}).

Methods and Results— Mice deficient in serum IgM (sIgM) or complement C1q were crossed with *Ldlr*^{-/-} mice and studied on both low (LF) and high fat (HF) semi-synthetic diets. On both diets, *en face* and aortic root atherosclerotic lesions in *sIgM.Ldlr*^{-/-} mice were substantially larger and more complex, with accelerated cholesterol crystal formation and increased smooth muscle cell content in aortic root lesions. Combined C1q and IgM deficiency had the same effect as IgM deficiency alone. Increased apoptosis was observed in aortic root lesions of both *sIgM.Ldlr*^{-/-} and *C1qa.Ldlr*^{-/-} mice. Since lesions were significantly larger in IgM deficient mice than in the absence of C1q, IgM protective mechanisms therefore appear to be partially independent of classical pathway activation and apoptotic cell clearance. Levels of IgG antibodies against copper-oxidised LDL were lower in high fat fed *sIgM.Ldlr*^{-/-} mice, suggesting compensatory consumption of IgG in the absence of IgM.

Conclusions— This study provides direct evidence that IgM antibodies play a central role in protection against atherosclerosis. The mechanism appears to be at least partly independent of classical pathway complement activation by C1q.

Key Words: atherosclerosis, IgM, complement, apoptosis

Introduction

Oxidative modifications of low-density lipoprotein (LDL) induce inflammatory responses that are key initial steps in the pathogenesis of atherosclerosis.¹ Both innate and adaptive immune responses are thought to contribute to lesion development, with involvement of antibodies which recognise epitopes on oxidised LDL (oxLDL) among other targets.² Both IgG and IgM autoantibodies to oxLDL can be readily detected in both humans and animal models of atherosclerosis, and in some studies antibody titres correlated with disease severity.³⁻⁵ IgM monoclonal antibodies reacting with oxLDL have been cloned from apolipoprotein E deficient (*ApoE*^{-/-}) mice and some have been found to bind the phosphorylcholine (PC) headgroup of oxidatively modified phospholipids.⁶⁻⁸ The prototypic and best characterised of these, EO6, is identical in sequence to T15, a classical natural antibody known to recognise PC expressed as a capsular epitope on *Streptococcus pneumoniae*.⁸

While initial studies focused on the detrimental effects of antibodies in atherosclerosis, evidence has accumulated suggesting that they may also have protective roles.⁹ Numerous studies have shown that immunisation with malondialdehyde-modified LDL (MDA-LDL) reduces atherosclerosis and is associated with robust T cell-dependent IgG anti-oxLDL responses.¹⁰⁻¹² Immunisation of LDL receptor deficient (*Ldlr*^{-/-}) or *ApoE*^{-/-} mice with *Streptococcus pneumoniae* or phosphorylcholine (PC)-conjugated keyhole limpet hemocyanin is also atheroprotective, in association with a rise in IgM anti-PC antibody titre.^{13,14} However, the precise roles of antibodies in active immunization experiments is difficult to interpret conclusively in view of the greater antibody titres achieved by active immunisation compared with those obtained spontaneously,¹⁵ as well as the use of adjuvants

and the effects of immunisation on other immunological parameters, such as regulatory T cells.¹⁶

One mechanism by which IgM antibodies may contribute to protection against atherosclerosis is by facilitating the clearance of apoptotic material from developing lesions. Opsonisation of apoptotic cells with IgM is known to accelerate their non-inflammatory clearance by macrophages, and this process requires cooperation with complement C1q.¹⁷ We have recently shown that C1q deficiency in *C1qa.Ldlr*^{-/-} mice accelerates atherosclerosis.¹⁸ Increased numbers of apoptotic cells were observed in atherosclerotic lesions, consistent with the concept that defective waste disposal leads to accumulation of cellular debris within plaques.¹⁹⁻²¹

In the present study we sought to examine directly the contribution of IgM antibodies in atherogenesis by crossing *Ldlr*^{-/-} mice with mice deficient in serum IgM (*sIgM*^{-/-}) through gene-targeted disruption of the secretory tail of immunoglobulin μ chain.²² Although unable to secrete IgM, *sIgM*^{-/-} B cells maintain membrane IgM expression, and have intact class switching and IgG production.²² We compared *sIgM.Ldlr*^{-/-} mice with *C1qa.Ldlr*^{-/-} mice and with triple deficient *C1qa.sIgM.Ldlr*^{-/-} mice to contrast the effect of IgM on atherogenesis with selective disruption of the classical pathway.

Methods

Mice

sIgM^{-/-} mice were generated as described previously,²² and crossed with *Ldlr*^{-/-} mice (both backcrossed 10 times on C57BL/6 background) to generate *sIgM.Ldlr*^{-/-} mice. *sIgM.Ldlr*^{-/-} mice were further crossed with *C1qa.Ldlr*^{-/-} mice¹⁸ to create “triple knockout” *C1qa.sIgM.Ldlr*^{-/-} mice. From 10 to 22 weeks of age, experimental groups of female mice received either a high fat (HF) diet or a low fat (LF) semisynthetic reference diet (see Supplementary Methods for details). The following numbers of mice were used for LF and HF diets respectively: *Ldlr*^{-/-} (n=12, n=12), *C1qa.Ldlr*^{-/-} (n=12, n=15), *sIgM.Ldlr*^{-/-} (n=14, n=15) and *C1qa.sIgM.Ldlr*^{-/-} (n=10, n=9). The aortic root of one LF-fed *C1qa.sIgM.Ldlr*^{-/-} mouse was damaged in processing and was not used for analysis. Animal care and procedures were conducted according to institutional guidelines, and mice were kept under specific pathogen-free conditions. Total serum cholesterol and triglycerides were measured using colorimetric enzymatic assays (Infinity, Alpha Labs, Eastleigh, UK). Lipoprotein profiles were generated by fast performance liquid chromatography (FPLC) on a Superose 6 size-exclusion column.

Atherosclerotic lesion analysis

Mice were killed by CO₂ inhalation and blood removed from the inferior vena cava. Using a cannula inserted in the left ventricle, hearts were perfused sequentially with Krebs-Henseleit buffer at 37°C for 5 minutes, 2% formalin for 5min, and 2mL of Sudan IV solution by direct slow injection over 5 minutes. Each aorta was microdissected to remove adventitial fat, cut open longitudinally, destained briefly in 80% ethanol and photographed. *En face* plaque quantification was performed using ImagePro software (Media Cybernetics, MD) by a single

operator blinded to group allocation. Aortic root cryosection, Oil red O staining and lesion quantification were performed as previously described.¹⁸

Immunohistochemistry

Aortic root frozen sections were stained using standard immunohistochemistry to identify the following cell types: macrophages (MOMA-2 rat mAb, Serotec), VSMC (alkaline phosphatase (AP)-conjugated anti- α -smooth muscle actin Ab, Sigma-Aldrich, Poole, UK), T cells (goat anti-mouse CD3 ϵ Ab, Santa Cruz Biotechnology, Santa Cruz, CA). The presence of lesional deposition of C5b-9 (rabbit anti-human C5b-9 Ab, Calbiochem, Merck Biosciences, Darmstadt, Germany) was identified using the same technique, and quantified as percentage lesion area staining positive using ImagePro. Lesional C3 (FITC-conjugated goat anti-mouse C3 Ab, MP Biomedicals, Cambridge, UK) and IgG (FITC-conjugated goat anti-mouse IgG Ab, Sigma-Aldrich) were identified using immunofluorescence and quantified as mean fluorescence intensity per pixel.

Quantification of lesional apoptosis

Apoptotic cells were detected using TUNEL (Roche, Welwyn Garden City, UK) on aortic root cryosections, following the manufacturer's instructions. Randomised slides were quantified by a single operator blinded to group allocation, and assessed for number of TUNEL positive cells fitting morphological criteria for apoptosis, including cell shrinkage, nuclear condensation or fragmentation and expressed as percentage of lesional cells.

Confocal microscopy

For confocal microscopy,¹⁸ aortic root cryosections were double-immunostained for CD68 (Alexafluor 488-conjugated anti-CD68 Ab, Molecular Probes, Invitrogen, Paisley, UK) and

IgM (biotin-conjugated mouse anti-mouse IgM^b mAb and biotin-conjugated mouse anti-mouse IgM^a, BD Pharmingen, Oxford, UK) secondarily labelled with Alexa 568-conjugated streptavidin (Molecular Probes), counterstained with TOPRO-3.

Lipoprotein isolation and modification

Human LDL (density 1.019-1.063g/mL) was isolated from plasma of healthy donors after overnight fasting by differential density ultracentrifugation,²³ and modified with either freshly synthesized malondialdehyde (MDA) or CuSO₄ to generate MDA-LDL and copper-oxidised LDL (CuOxLDL) (see Supplementary Methods).²⁴

Serum autoantibody measurement and assessment of autoimmunity

For the detection of anti-MDA-LDL²⁴ and anti-CuOxLDL antibodies, non-irradiated microtiter plates (Greiner Bio-One, Stonehouse, UK) were coated overnight at 4°C with either 15µg/mL MDA-LDL, CuOxLDL or native LDL in a coating buffer of 100mM NaHCO₃ and 1mg/mL Na₂EDTA or with coating buffer alone to assess non-specific binding. After washing with PBS with 0.5% Tween-20, plates were blocked with 5% BSA. Sera diluted in PBS-Tween were applied. AP-conjugated anti-mouse IgG, IgM or IgG₁ Abs (Southern Biotech, Birmingham AL) were used for detection and plates were developed with *p*-nitrophenol phosphate. As *Ldlr*^{-/-} mice on the C57BL/6 background produce IgG_{2c} (equivalent to IgG_{2a}^b) antibodies, whereas *sIgM.Ldlr*^{-/-} mice (carrying a 129-derived interval over the Ig gene region) produce IgG_{2a} (equivalent to IgG_{2a}^a) (unpublished, M.Botto), to detect IgG_{2a} and IgG_{2c} anti-MDA-LDL or anti-CuOxLDL antibodies, we used an AP-conjugated polyclonal goat anti-mouse IgG_{2a} (Southern Biotech) with equal specificity for both mouse IgG_{2a} and IgG_{2c} over the detection range used (see Supplementary data). Anti-single-stranded DNA, anti-dsDNA, anti-chromatin and anti-histone antibodies were assayed

by ELISA.²⁵ For anti-cardiolipin and anti- β_2 glycoprotein I antibody ELISA assays and scoring of renal histology for glomerulonephritis see Supplementary Methods.

Statistical analysis

Results were analysed using Graphpad Prism version 3.0 (Graphpad Software, San Diego CA). Where data were not normally distributed non-parametric statistical tests were used and results are expressed as median (interquartile range): to compare two groups Mann-Whitney test was used; to compare three or more groups Kruskal-Wallis test followed by Dunn's post-test was used. Normally distributed data was compared using unpaired two-tailed Student's t-test with Bonferroni correction for multiple comparisons.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Localisation of IgM in plaques of Ldlr^{-/-} mice

Confocal microscopy was used to examine IgM deposition within atherosclerotic plaques and localisation relative to macrophages. IgM was found within lesions following either low fat (LF) or high fat (HF) diets, particularly in the acellular core and with a reciprocal relationship to CD68-positive macrophages (Figure 1A). No IgM was detected within atherosclerotic lesions of *sIgM.Ldlr^{-/-}* mice, indicating detection specificity (Figure 1B).

Accelerated atherosclerosis in serum IgM deficient mice

No significant differences between all four mouse strains on either diet were noted in final body weight, total serum cholesterol or triglycerides (Supplementary Table 1), or lipoprotein FPLC profiles (not shown). Atherosclerotic plaque burden was quantified in *sIgM.Ldlr^{-/-}* mice and compared with *Ldlr^{-/-}* control mice and with *C1qa.Ldlr^{-/-}* mice. On the LF diet, a hierarchy was apparent such that *C1qa.Ldlr^{-/-}* mice showed a moderate increase (2.8-fold) in atherosclerotic lesion area fraction at the aortic root (*C1qa.Ldlr^{-/-}*, median 4.58% (interquartile range 1.42-8.44%), n=12 vs. *Ldlr^{-/-}*, 1.64% (1.11-2.72%), n=12; P<0.05, using Kruskal-Wallis test with Dunn's post-test), whereas both *sIgM.Ldlr^{-/-}* and *C1qa.sIgM.Ldlr^{-/-}* mice demonstrated substantially increased lesion size of 6.1-fold and 4.1-fold respectively (*sIgM.Ldlr^{-/-}* 10.0% (4.96-12.9%), n=14; *C1qa.sIgM.Ldlr^{-/-}* 6.76% (5.11-10.4%), n=9; both P<0.001 vs. *Ldlr^{-/-}*) (Figure 2A and 2B). On the HF diet (Figure 2A and 2C), aortic root lesions in *sIgM.Ldlr^{-/-}* mice were 29% increased compared to *Ldlr^{-/-}* mice (*sIgM.Ldlr^{-/-}* 35.8% (31.1-39.1%), n=15; *Ldlr^{-/-}* 27.8% (24.0-30.5%), n=12; P<0.05) and 33% increased in *C1qa.sIgM.Ldlr^{-/-}* mice (36.9% (33.5-39.9%), n=9; P<0.01). There was no

difference between *C1qa.Ldlr*^{-/-} (26.4% (20.7-31.8%), n=15, P=1.00) and *Ldlr*^{-/-} mice following the HF diet confirming our previously published data.¹⁸

Whole aorta *en face* atherosclerotic lesion area mirrored the aortic root lesion data, showing a comparable hierarchy of effects of C1q and sIgM deficiency. On the LF diet *C1qa.Ldlr*^{-/-} mice showed a moderate (although not statistically significant) increase of approximately 2.1-fold in *en face* atherosclerotic lesion area compared to *Ldlr*^{-/-} mice (*C1qa.Ldlr*^{-/-} 2.87% (1.71-3.56%), n=12 vs. *Ldlr*^{-/-} 1.36% (0.73-1.72%), n=12; P=0.26) (Figure 3A and 3B). A substantially greater increase (~7-fold) in *en face* staining was evident in the *sIgM.Ldlr*^{-/-} group compared to *Ldlr*^{-/-} controls, with an equal increase seen in *C1qa.sIgM.Ldlr*^{-/-} triple knockouts (*sIgM.Ldlr*^{-/-}, 9.48% (5.36-10.9%), n=14; *C1qa.sIgM.Ldlr*^{-/-}, 7.18% (4.79-11.4%), n=10; both P<0.001 vs. *Ldlr*^{-/-}). On the HF diet, a 66% increase in Sudan IV staining was still seen in the *sIgM.Ldlr*^{-/-} group compared to *Ldlr*^{-/-} controls (12.4% (8.48-17.2%), n=15 vs. 7.49% (6.13-8.77%), n=12; P<0.05) (Figure 3C and 3D).

C1qa.sIgM.Ldlr^{-/-} showed a similar level of *en face* atherosclerotic lesions (10.1% (7.88-14.1%), n=9) as *sIgM.Ldlr*^{-/-} mice, while *C1qa.Ldlr*^{-/-} (5.23% (4.77-6.70%), n=15) were not statistically different to *Ldlr*^{-/-} mice (P=0.16).

Increased lesion complexity in the absence of serum IgM

Aortic root lesions in *Ldlr*^{-/-} mice fed the LF diet were largely fatty streaks, composed virtually entirely of macrophages. On both LF (Figure 4A) and HF (Figure 4B) diets, *sIgM.Ldlr*^{-/-} lesions showed a significant increase in population by vascular smooth muscle cells (VSMC), identified by positive staining for α -smooth muscle actin (LF diet: *sIgM.Ldlr*^{-/-} 4.95% (1.80-7.87%), n=14 vs. *Ldlr*^{-/-} 0.0% (0.0-2.29%), n=12; P=0.008; HF diet: *sIgM.Ldlr*^{-/-} 19.7% (11.6-33.9%), n=15 vs. *Ldlr*^{-/-} 6.73% (3.85-10.4%), n=12;

P=0.0004), with a reciprocal drop in macrophage content. On the HF diet, substantial numbers of *sIgM.Ldlr*^{-/-} aortic root sections demonstrated fibrous caps (Figure 4C). Even on the LF diet, cholesterol crystal deposition was noted in the aortic root lesions of *sIgM.Ldlr*^{-/-} mice (Figure 4C), whereas there was no evidence of cholesterol crystal deposition in the LF fed *Ldlr*^{-/-} mice. There was no difference between *sIgM.Ldlr*^{-/-} and *Ldlr*^{-/-} in the proportion of lesional T lymphocytes (assessed with anti-CD3ε) on either diet (Figure 4A & B). Using immunofluorescence for IgG and C3, mean fluorescent intensity per pixel was quantified as an estimate of the density of lesional IgG and C3 deposition. There was no difference in the mean fluorescent intensity for IgG or C3 deposition in aortic root lesions between *Ldlr*^{-/-} and *sIgM.Ldlr*^{-/-} mice on either diet (Figure 4A, B and C), nor any difference in the level of lesional C5b-9 (not shown).

Clearance of apoptotic cell debris in atherosclerotic lesions in the absence of IgM

Lesional apoptosis in *sIgM.Ldlr*^{-/-} mice was at a level similar to that found in *C1qa.Ldlr*^{-/-}, which were previously demonstrated to have impaired lesional apoptotic cell clearance.¹⁸ Thus, using a combination of TUNEL staining and morphological identification, aortic root lesions of *sIgM.Ldlr*^{-/-} mice fed the LF diet contained increased numbers of apoptotic cells compared to *Ldlr*^{-/-} controls, reaching statistical significance when analysed by Mann-Whitney test (P=0.047). However, using more stringent Kruskal-Wallis post-tests only *C1qa.Ldlr*^{-/-} mice showed increased numbers of apoptotic cells (P<0.01), and neither *sIgM.Ldlr*^{-/-} nor *C1qa.sIgM.Ldlr*^{-/-} mice reached significance, compared to *Ldlr*^{-/-} controls (Figure 5A and 5B). More apoptotic cells were detectable in *Ldlr*^{-/-} controls following the HF diet, such that differences between HF-fed groups were not significant (Figure 5C).

Autoantibody production in $sIgM.Ldlr^{-/-}$ mice

$sIgM^{-/-}$ mice on a mixed C57BL/6 \times 129 background have previously been reported to produce anti-double-stranded DNA (dsDNA) and aCL antibodies.²⁶ However $sIgM^{-/-}$ mice backcrossed for 10 generations on the C57BL/6 background do not produce autoantibodies (unpublished data, M. Botto). Serological tests were conducted to check for evidence of autoimmunity in $sIgM.Ldlr^{-/-}$ mice. LF-fed $sIgM.Ldlr^{-/-}$ mice showed low levels of IgG anti-single-stranded DNA (ssDNA) antibodies, which were not statistically different to levels in $Ldlr^{-/-}$ mice (Table 1). However a significant increase in IgG anti-ssDNA antibodies was noted when $sIgM.Ldlr^{-/-}$ mice were fed the HF diet compared to HF-fed $Ldlr^{-/-}$ controls ($P < 0.001$, Kruskal-Wallis post-test). Although raised, levels of anti-ssDNA antibodies in HF-fed $sIgM.Ldlr^{-/-}$ mice were low compared with 6 month old MRL/ lpr mouse serum that was used as a standard (1000 ELISA units). Only HF-fed $Ldlr^{-/-}$ mice showed modest titres of IgG anticardiolipin antibodies (aCL), while all other groups showed non-significant levels. Other autoantibodies, including antinuclear antibodies, anti-dsDNA, antichromatin and anti- β_2 glycoprotein I antibodies were essentially undetectable in all experimental groups. There was no evidence of proteinuria or glomerulonephritis in any group (not shown).

Changes in levels of IgG antibodies against MDA-LDL and CuOxLDL

To examine whether IgM deficiency influences IgG antibody responses against oxidised LDL (oxLDL), titres of anti-MDA-LDL and anti-CuOxLDL antibodies were assayed in $Ldlr^{-/-}$ and $sIgM.Ldlr^{-/-}$ mice at 22 weeks of age by ELISA. There was a significant increase in IgM and IgG antibodies to CuOxLDL, but not to MDA-LDL, in HF-fed versus LF-fed $Ldlr^{-/-}$ mice, and, as expected, IgM antibodies were not detected in $sIgM.Ldlr^{-/-}$ sera (Figure 6). There were no significant differences in IgG anti-MDA-LDL or anti-CuOxLDL antibody titre in $sIgM.Ldlr^{-/-}$ mice compared with $Ldlr^{-/-}$ mice on the LF diet. However, there was a

significant reduction in IgG anti-CuOxLDL antibodies in *sIgM.Ldlr^{-/-}* mice fed a HF diet compared with HF-fed *Ldlr^{-/-}* mice.

Hypercholesterolaemia in *ApoE^{-/-}* mice has been shown to cause a T_h1 to T_h2 switch resulting in increased IgG₁ anti-MDA-LDL antibody formation.²⁴ To investigate whether increased atherosclerosis in *sIgM.Ldlr^{-/-}* mice was associated with an increased T_h2 response, IgG₁ and IgG_{2a/2c} isotypes were also assayed. For both isotypes generally no differences were seen between *sIgM.Ldlr^{-/-}* and *Ldlr^{-/-}* mice, with the exception of slightly lower IgG_{2a/2c} anti-CuOxLDL antibodies observed in LF-fed *sIgM.Ldlr^{-/-}* mice (significant only at the lowest dilution).

Discussion

In this paper, we have shown that *Ldlr*^{-/-} mice deficient in serum IgM display a substantial acceleration of atherosclerosis on both a LF and HF diet, with larger lesions, an increase in lesional cholesterol crystal formation and an increase in VSMC content consistent with fibrous cap formation. Overall, our data provide the first direct evidence that endogenous IgM natural antibodies are necessary for atheroprotection, and build on previous experimental studies on humoral immunity in mice that have shown reduction of atherosclerosis by (i) B cell rescue of splenectomised mice;²⁷ (ii) administration of polyclonal human IgG;^{28,29} (iii) administration of a monoclonal human IgG₁ antibody against MDA-LDL;^{30,31} and (iv) raising IgM anti-PC antibody levels by vaccination with pneumococci¹³ or PC conjugated to keyhole limpet hemocyanin.¹⁴

One of the primary functions of IgM is to activate complement. Previous studies in mouse models have shown increased arterial lipid deposition and macrophage infiltration in mice with deficiency of C1q or C3, suggesting that complement has atheroprotective effects.^{18,32,33} However, whilst C1q deficiency was associated with increased VSMC content in lesions,¹⁸ the opposite was reported in C3 deficient mice,³² raising the possibility that different modes of complement activation may have distinct effects on atherogenesis. The direct comparison of *sIgM.Ldlr*^{-/-} mice with *C1qa.Ldlr*^{-/-} and triple “knockout” *C1qa.sIgM.Ldlr*^{-/-} mice allowed us to gauge how much of the IgM-related atheroprotection was mechanistically linked to downstream classical pathway complement activation via C1q. As previously published, *C1qa.Ldlr*^{-/-} mice on the LF diet showed a modest increase in aortic root lesion size, but C1q deficiency had no detectable effect in mice fed a HF diet.¹⁸ The differences in this paper between *sIgM.Ldlr*^{-/-} and *C1qa.Ldlr*^{-/-} mice now demonstrate a hierarchy, with IgM playing a dominant role. Consistent with this, combined deficiency of C1q and sIgM

showed no difference in the level of atherosclerosis compared to IgM deficiency alone. It is possible that in the absence of C1q, IgM may bypass the classical pathway and instead activate the lectin pathway,³⁴ as has been shown in ischaemia-reperfusion injury.³⁵ However, in the *sIgM.Ldlr^{-/-}* mice we found no difference in the density of lesional C3 or C5b-9 staining compared with the *Ldlr^{-/-}* mice, suggesting that at least part of the mechanism by which IgM exerts its protective effect in atherosclerosis is independent of complement activation in the arterial wall.

Both C1q and IgM have been implicated as important recognition molecules mediating clearance of dying cells.^{17,36} We observed increased levels of apoptotic cells and TUNEL positive cellular debris within aortic root atherosclerotic plaques of *sIgM.Ldlr^{-/-}* mice, similar to those seen in *C1qa.Ldlr^{-/-}*.¹⁸ Increased lesional apoptosis was not observed in *C1qa.sIgM.Ldlr^{-/-}* mice, raising the possibility of compensatory mechanisms occurring within the triple knock-outs. There was no difference in apoptotic cell numbers between the groups on the HF diet, primarily due to increased lesional apoptosis in *Ldlr^{-/-}* controls. It is important to note that although defective clearance of cellular debris within atherosclerotic plaques may be a significant contributing factor to the increase in lesion size noted in *sIgM.Ldlr^{-/-}* and *C1qa.Ldlr^{-/-}* mice on the LF diet, increased numbers of lesional apoptotic cells may also be a function of lesion size. Overall, the data on lesional apoptotic cells do not support a failure of apoptotic cell clearance being the only or even the major mechanism by which IgM deficiency accelerates atherosclerosis, and certainly do not account for the greater lesion size in *sIgM.Ldlr^{-/-}* compared to *C1qa.Ldlr^{-/-}* mice.

Although deficiency of serum IgM has been shown to predispose to the development of anti-dsDNA and aCL antibodies on certain murine genetic backgrounds,^{26,37} *sIgM^{-/-}* mice do not

develop autoantibodies when fully backcrossed onto the C57BL/6 background (unpublished, M.Botto). Unexpectedly we observed that *sIgM.Ldlr^{-/-}* mice on the HF diet developed anti-ssDNA autoantibodies, supporting previous observations that there is an interaction between hypercholesterolaemia and predisposition to lupus-like autoimmunity.¹⁹ Hence IgM deficiency enhances autoimmunity both in a strain-dependent and lipid-dependent manner. It should be noted however that the levels of anti-ssDNA in *sIgM.Ldlr^{-/-}* mice on the HF diet were low compared to those seen in classical lupus models (e.g. MRL/lpr), and no mice developed renal disease. It therefore seems unlikely that lupus-like autoimmunity contributed significantly to the accelerated arterial disease we observed.

The main purpose of measuring levels of IgG antibodies against MDA- and CuOxLDL was to check whether removal of IgM would result in a change in predominant IgG isotype or a compensatory increase in levels of IgG antibodies to these antigens. There was no evidence in *sIgM.Ldlr^{-/-}* mice of a change in IgG isotype reflecting a T_h1 to T_h2 switch.²⁴

Paradoxically, there was a significant reduction in IgG antibodies against CuOxLDL in *sIgM.Ldlr^{-/-}* mice fed a HF diet compared with *Ldlr^{-/-}* mice. Whilst theoretically this may be due to an idiosyncratic reduction in IgG synthesis related to IgM deficiency, class switching is thought to be relatively normal in *sIgM^{-/-}* mice.²² One plausible explanation is that the lower IgG CuOxLDL antibody level in HF-fed *sIgM.Ldlr^{-/-}* mice is due to depletion of IgG by oxLDL particles that would have bound IgM in *Ldlr^{-/-}* mice. This raises the question as to what extent the protective effect of IgM is related to actions such as inhibition of uptake of oxLDL by macrophages within lesions,⁷ and how much is due to facilitating safe clearance of oxLDL from the circulation or other tissues. For example, IgM may well be important for opsonising circulating oxLDL particles and targeting them to the liver, which is known to be the major organ for circulating oxLDL uptake.³⁸

Although mechanistic conclusions cannot be drawn from clinical studies relating antibody levels with disease, it is relevant that there have been reports showing inverse correlations between serum levels of IgM anti-oxLDL or anti-phosphorylcholine antibodies and progression of carotid atherosclerosis,³⁹⁻⁴¹ as well as angiographically determined coronary artery disease.⁴² Taken together with the emerging experimental evidence in this and previous studies that IgM and IgG antibodies can be atheroprotective, there is therefore increasing rationale for therapeutic strategies aimed at boosting humoral immunity. Conversely, B cell depletion therapy using the anti-CD20 monoclonal antibody rituximab is increasingly used to treat autoimmune conditions including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Up to 20% of patients with RA treated with repeated courses of B cell depletion develop abnormally low levels of sIgM, with less effect on serum IgG levels.⁴³ It seems possible that repeated B cell depletion might increase cardiovascular risk due to preferential depletion of serum IgM, in a population already susceptible to cardiovascular complications.⁴⁴

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Disclosures

None.

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Figure Legends

Figure 1. Deposition of IgM in atherosclerotic lesions. Confocal images of aortic root sections double-immunostained for CD68 (green) and IgM (red) and counterstained with TOPRO nuclear dye (blue). (A) Low power view of aortic root lesion from LF-fed *Ldlr*^{-/-} mouse showing IgM deposition in the acellular base of the lesion and its relationship to lesional macrophages. (B) Low power view of similar lesion from LF-fed *sIgM.Ldlr*^{-/-} mouse showing complete absence of IgM.

Figure 2. Accelerated atherosclerotic lesion formation in aortic roots of sIgM deficient mice. (A) Representative sections from *Ldlr*^{-/-}, *C1qa.Ldlr*^{-/-}, *sIgM.Ldlr*^{-/-} and *C1qa.sIgM.Ldlr*^{-/-} mice stained with Oil Red O and haematoxylin. Scale bars 500µm. (B, C) Cross-sectional aortic root lesion area fraction (%) on (B) LF or (C) HF diet. Each point represents the mean of 5 sections per mouse, and bars show overall median. *P<0.05, **P<0.01, ***P<0.001 by Kruskal-Wallis test with Dunn's post-test.

Figure 3. Accelerated lesion formation in *en face* aorta preparations of sIgM deficient mice. (A, C) Representative Sudan IV-stained *en face* aorta preparations from *Ldlr*^{-/-}, *C1qa.Ldlr*^{-/-}, *sIgM.Ldlr*^{-/-} and *C1qa.sIgM.Ldlr*^{-/-} mice following 12 weeks of (A) LF or (C) HF diet. Scale bars 0.5mm. (B, D) Quantification of *en face* aorta lesion area expressed as % lesion area fraction on (B) LF or (D) HF diet, with bars showing the median. *P<0.05, **P<0.01, ***P<0.001 by Kruskal-Wallis test with Dunn's post-test.

Figure 4. Increased complexity of aortic root lesions in sIgM deficient mice. MOMA-2 positive macrophages, α -smooth muscle actin positive VSMC and CD3+ T cells expressed as % of lesional cells on (A) low fat (*Ldlr*^{-/-} n=12, *sIgM.Ldlr*^{-/-} n=14) and (B) high fat diet

(*Ldlr*^{-/-} n=12, *sIgM.Ldlr*^{-/-} n=15). Density of aortic root lesion IgG and C3 deposition measured as mean fluorescence intensity per pixel on (A) low fat and (B) high fat diet (n numbers as before), expressed as relative fluorescence units (RFU) per pixel. Bars represent median, and statistical analysis was by Mann-Whitney test. (C) Representative photomicrographs of aortic root lesions showing cholesterol crystals under polarizing microscopy (age 22 wk, LF diet), smooth muscle fibrous cap formation (blue), lesional IgG and C3 deposition using fluorescence microscopy on HF diet in *sIgM.Ldlr*^{-/-} compared to *Ldlr*^{-/-} mice. Autofluorescence using a FITC-conjugated isotype control is shown below. Scale bars 100µm.

Figure 5. Increased apoptosis in atherosclerotic lesions of LF-fed *C1qa.Ldlr*^{-/-}, *sIgM.Ldlr*^{-/-} and *C1qa.sIgM.Ldlr*^{-/-} mice. (A) Representative photomicrographs of TUNEL staining (brown) from LF-fed mice, counterstained with haematoxylin, showing few apoptotic cells in *Ldlr*^{-/-} mice, but increased lesional apoptotic cells in *C1qa.Ldlr*^{-/-}, *sIgM.Ldlr*^{-/-} and *C1qa.sIgM.Ldlr*^{-/-} mice. Scale bars 50µm. Quantification of lesional apoptotic cells on basis of TUNEL positive staining and morphological nuclear changes consistent with apoptosis on (B) LF diet and (C) HF diet, expressed as % of total lesional nuclei. Bars represent median. **P<0.01 by Kruskal-Wallis post-test.

Figure 6. IgG and IgM anti-oxLDL antibodies in *Ldlr*^{-/-} and *sIgM.Ldlr*^{-/-} mice. Serial dilution curves showing titres of (A) anti-MDA-LDL and (B) anti-CuOxLDL antibodies in *Ldlr*^{-/-} and *sIgM.Ldlr*^{-/-} mice on low and high fat diets at 22 weeks of age. Non-specific binding to native LDL is shown at a single dilution. Error bars show SEM. †P<0.05 *sIgM.Ldlr*^{-/-} (LF) vs. *Ldlr*^{-/-} (LF). **P<0.01 *sIgM.Ldlr*^{-/-} (HF) vs. *Ldlr*^{-/-} (HF). §P<0.05,

§§P<0.01, §§§P<0.001 *Ldlr*^{-/-} (LF) vs. *Ldlr*^{-/-} (HF). Statistical analysis by unpaired t-test with Bonferroni correction for multiple comparisons.

Table 1. Autoantibody profiles in *Ldlr*^{-/-} and *sIgM.Ldlr*^{-/-} mice at 22 weeks of age

	LF diet		HF diet	
	<i>Ldlr</i> ^{-/-} n=12	<i>sIgM.Ldlr</i> ^{-/-} n=14	<i>Ldlr</i> ^{-/-} n=12	<i>sIgM.Ldlr</i> ^{-/-} n=15
ssDNA	14.2 (10.5-24.66)	22.0 (16.2-37.8)	14.3 (4.70-29.3)	49.6 (26.7-87.9)*
dsDNA	2.16 (1.67-4.22)	1.46 (1.10-5.35)	2.50 (1.62-3.44)	5.27 (3.08-9.12)
Chromatin	9.98 (6.67-15.2)	11.5 (8.25-26.5)	17.1 (13.7-25.5)	16.1 (9.82-22.3)
aCL	7.43 (0-43.7)	14.2 (0-44.7)	38.4 (0-75.1)	0 (0-18.3)
β ₂ GPI	4.84 (2.43-7.27)	1.96 (0.26-10.5)	3.74 (2.42-7.43)	4.21 (1.22-8.64)

Serum IgG anti-single-stranded DNA (ssDNA), anti-dsDNA, anti-chromatin, anticardiolipin (aCL) and anti-β₂glycoprotein I (β₂GPI) antibody levels expressed as median (interquartile range) in arbitrary ELISA units, compared against a reference standard of pooled MRL/lpr mouse sera. *P<0.001 vs. HF-fed *Ldlr*^{-/-} (post-test following Kruskal-Wallis test)

Fig. 1

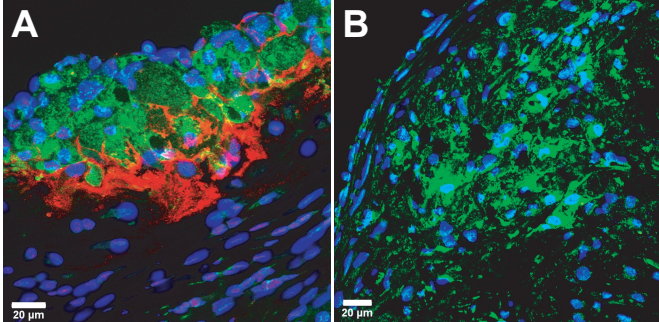


Fig. 2

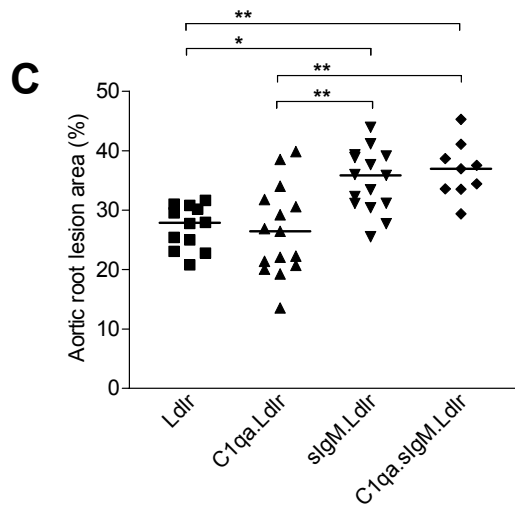
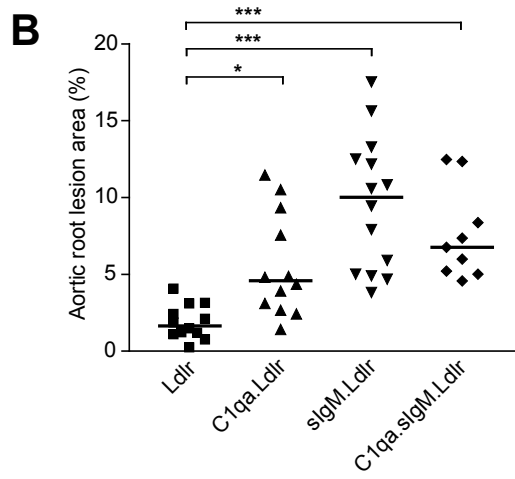
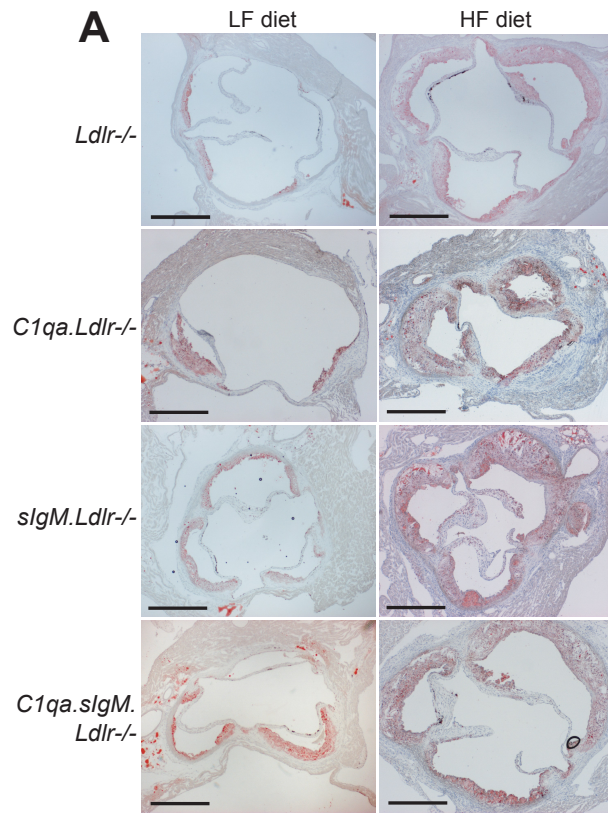


Fig.3

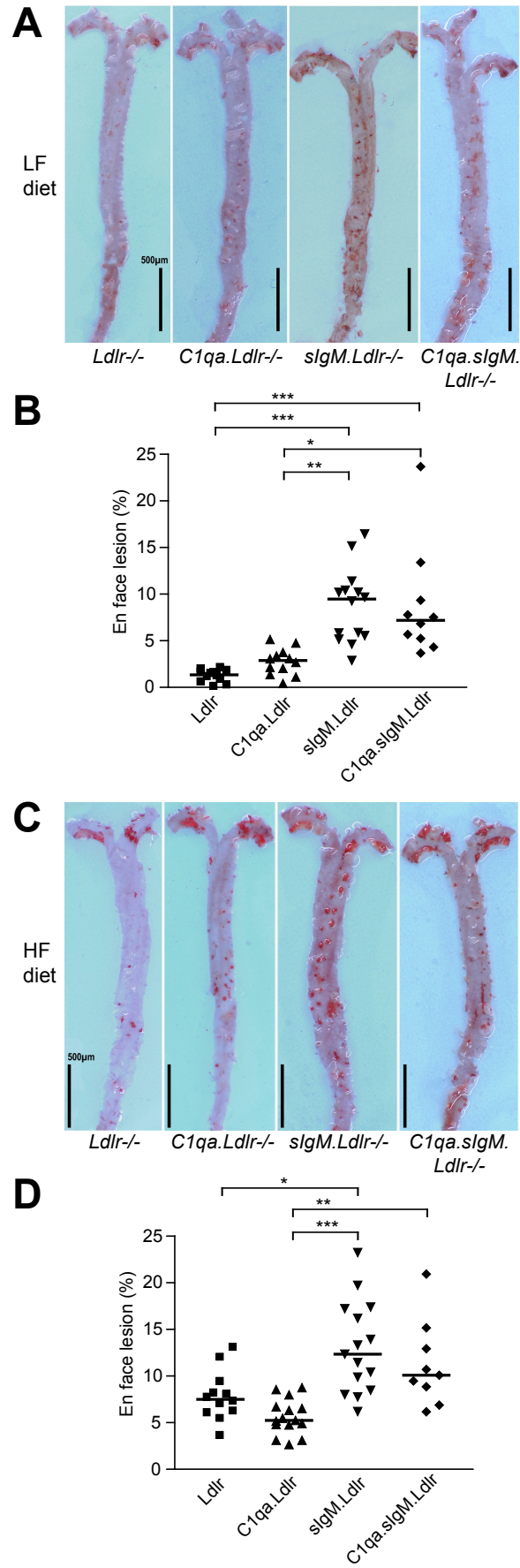


Figure 4

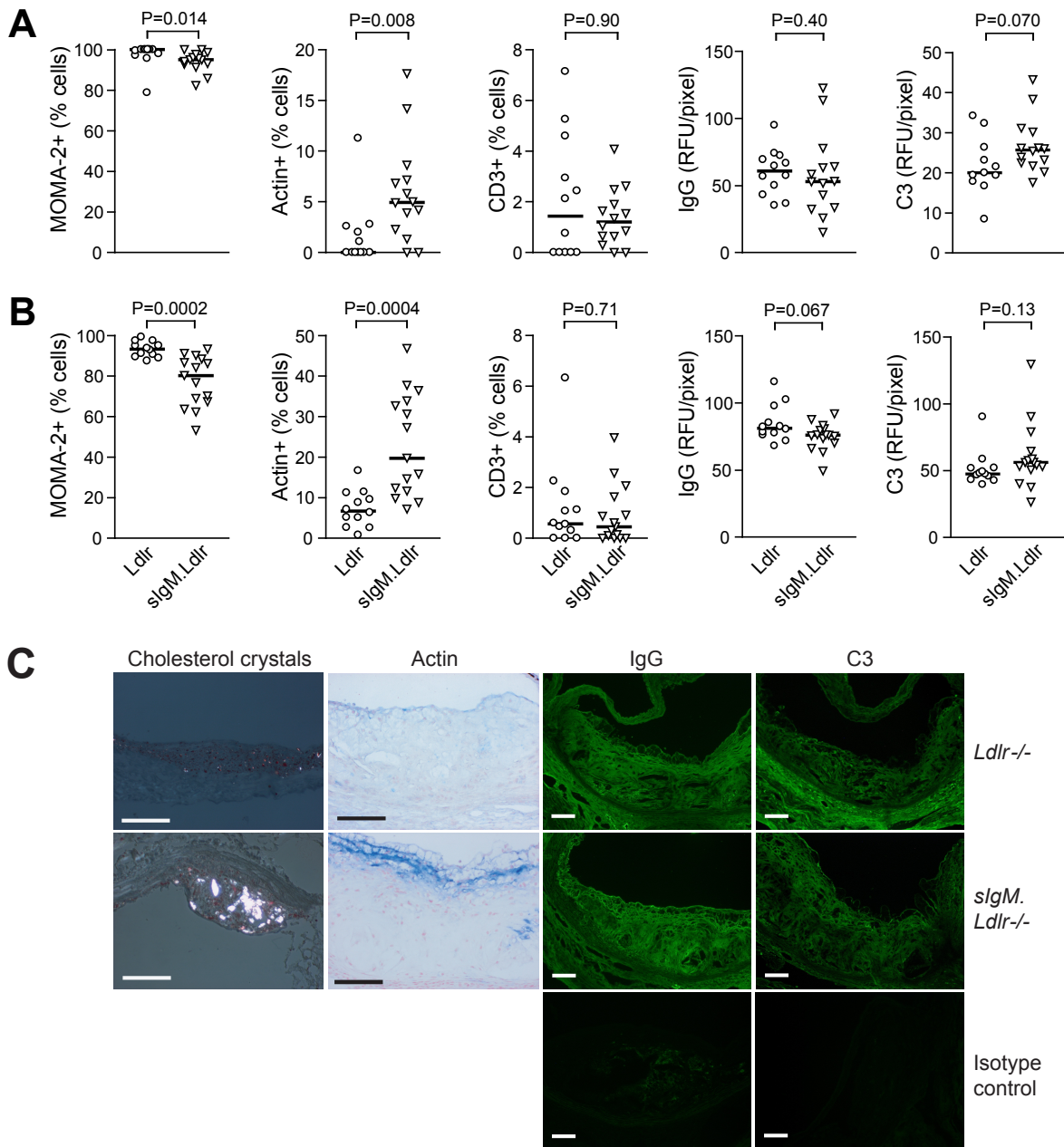


Figure 5

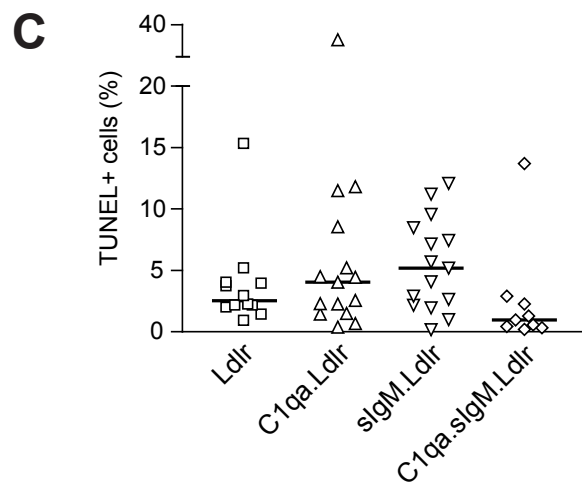
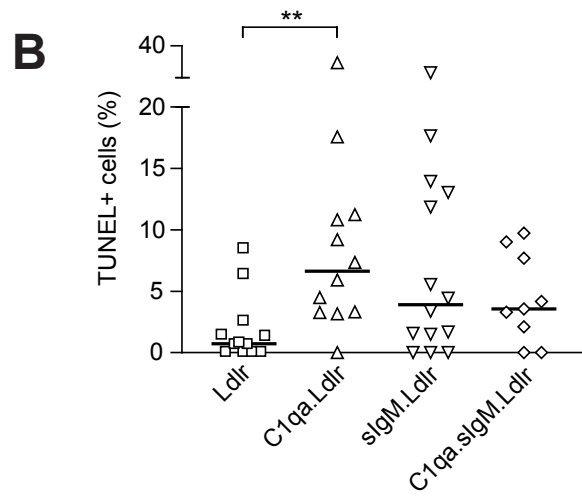
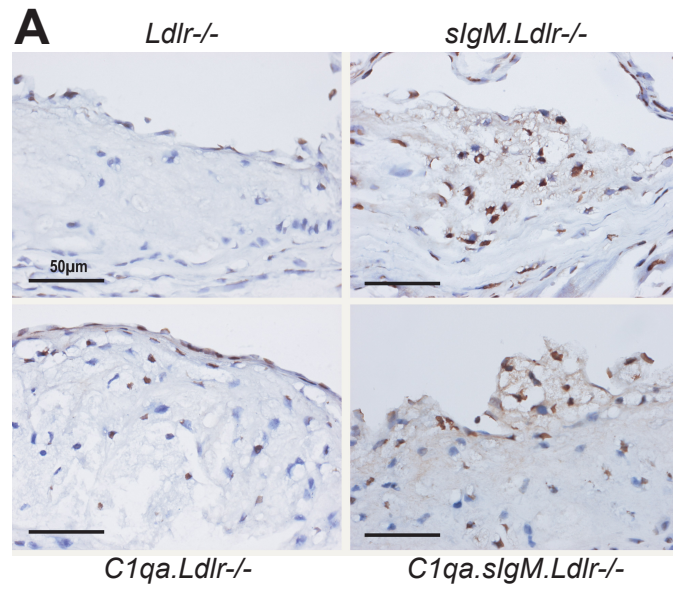
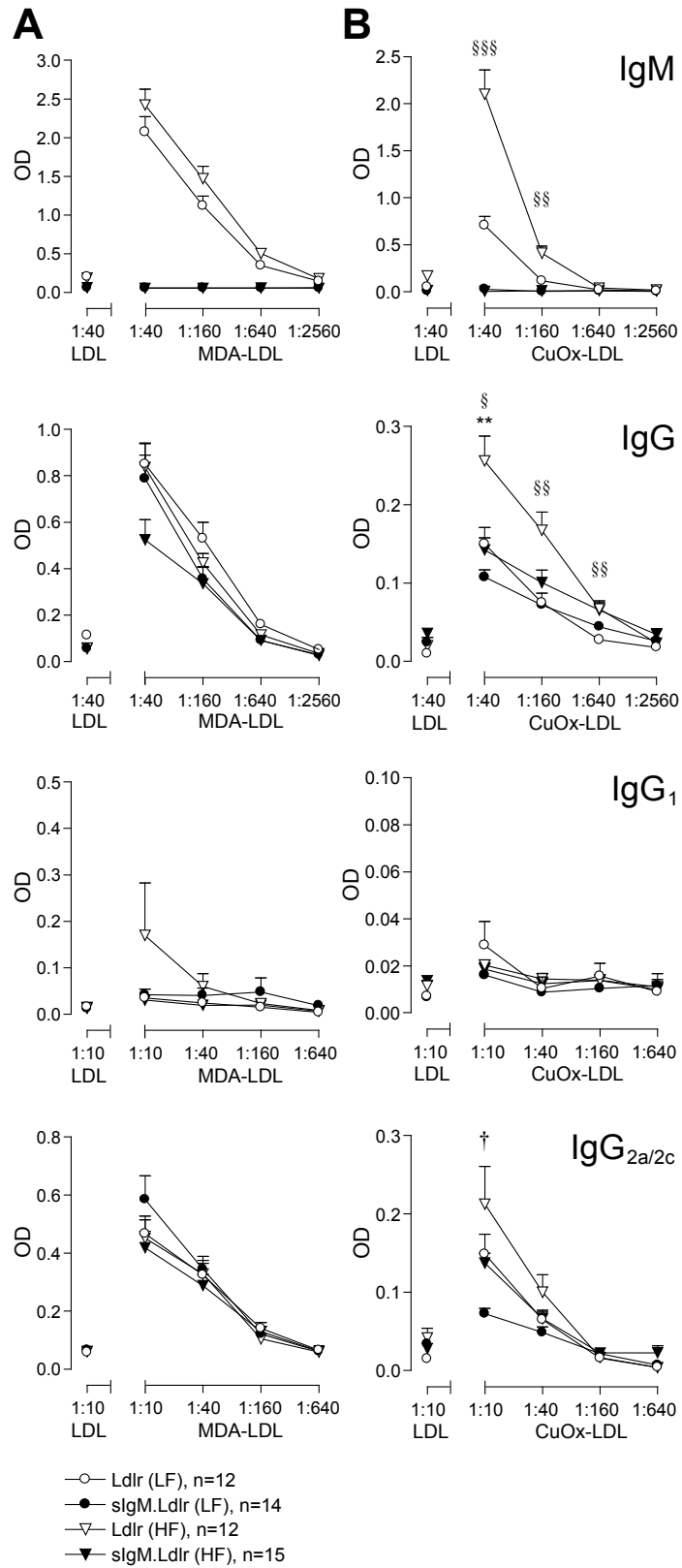


Figure 6



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 Na₂EDTA 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 ddH₂O 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 CuSO₄ for 16 hours at 37°C. After oxidation MDA-LDL or CuOxLDL was gel filtered to remove residual MDA or CuSO₄. To limit further modification, Na₂EDTA 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-β₂glycoprotein I antibodies

To measure anticardiolipin (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-β₂glycoprotein I antibodies, 96 well polystyrene plates (NUNC Maxisorp) were coated overnight at 4°C with purified human β₂glycoprotein 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

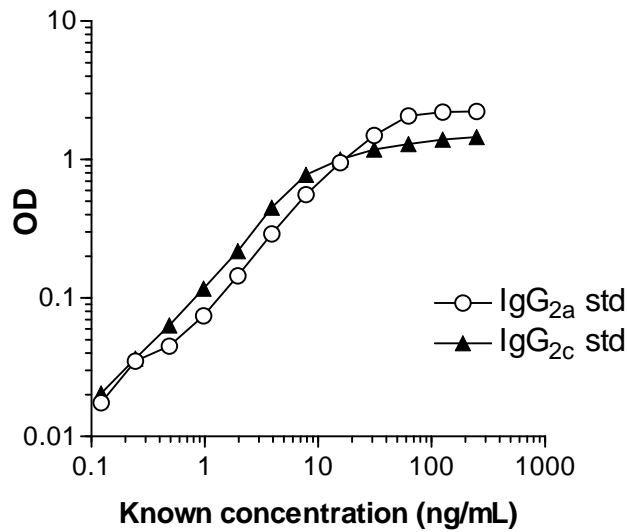
Specificity testing of antibody for both mouse IgG_{2a} and IgG_{2c}

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*^{-/-}, *Clqa.Ldlr*^{-/-}, *sIgM.Ldlr*^{-/-} and *Clqa.sIgM.Ldlr*^{-/-} mice aged 22 weeks.

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

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