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

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

Methods and Results—Mice deficient in serum IgM (sIgM) or complement C1q were crossed with Ldlr–/– mice and studied on both low-fat and high-fat semisynthetic 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 C1q.Ldlr–/– mice. Because lesions were significantly larger in IgM-deficient mice than in the absence of C1q, IgM protective mechanisms appear to be partially independent of classical pathway activation and apoptotic cell clearance. Levels of IgG antibodies against copper-oxidized LDL were lower in sIgM.Ldlr–/– mice fed a high-fat diet, 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 and apoptotic cell clearance. Levels of IgG antibodies against copper-oxidized LDL were lower in sIgM.Ldlr–/– mice fed a high-fat diet, suggesting compensatory consumption of IgG in the absence of IgM.

Key Words: antibodies ■ apoptosis ■ atherosclerosis ■ complement C1q ■ immune system ■ immunoglobulin M ■ pathology

Oxidative modifications of low-density lipoprotein (LDL) induce inflammatory responses that are a 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 that recognize epitopes on oxidized LDL (oLDL) among other targets.2 Both immunoglobulin G (IgG) and IgM autoantibodies to oLDL can be readily detected in both humans and animal models of atherosclerosis, and in some studies, antibody titers correlated with disease severity.3–5 IgM monoclonal antibodies reacting with oLDL have been cloned from apolipoprotein E–deficient (ApoE–/–) mice, and some have been found to bind the phosphorylcholine head group of oxidatively modified phospholipids.6–8 The prototypic and best characterized of these, EO6, is identical in sequence to T15, a classical natural antibody known to recognize phosphorylcholine expressed as a capsular epitope on Streptococcus pneumoniae.8

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Although initial studies focused on the detrimental effects of antibodies in atherosclerosis, evidence has accumulated suggesting that they also may have protective roles.9 Numerous studies have shown that immunization with malondialdehyde-modified LDL (MDA-LDL) reduces atherosclerosis and is associated with robust T-cell–dependent IgG anti-oLDL responses.10–12 Immunization of LDL receptor–deficient (Ldlr–/–) or ApoE–/– mice with S pneumoniae or phosphorylcholine-conjugated keyhole limpet hemocyanin is also atheroprotective, in association with a rise in IgM anti-phosphorylcholine antibody titer.13,14 However, the precise roles of antibodies in active immunization experiments are difficult to interpret conclusively in view of the greater antibody titers achieved by active immunization compared with those obtained spontaneously,15 as well as the use of adjuvants and the effects of immunization 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. Opsonization of apoptotic cells with IgM is known to accelerate their noninflammatory clearance by macrophages, and this process requires cooperation with complement C1q.\textsuperscript{17} We have recently shown that C1q deficiency in C1qa.sIgM.Ldlr\textsuperscript{−/−} mice accelerates atherosclerosis.\textsuperscript{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.\textsuperscript{19–21}

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

**Methods**

**Mice**

sIgM.Ldlr\textsuperscript{−/−} mice were generated as described previously\textsuperscript{22} and crossed with Ldlr\textsuperscript{−/−} mice (both backcrossed 10 times on C57BL/6 background) to generate sIgM.Ldlr\textsuperscript{−/−} mice. sIgM.Ldlr\textsuperscript{−/−} mice were further crossed with C1qa.Ldlr\textsuperscript{−/−} mice\textsuperscript{22} to create “triple-knockout” C1qa.sIgM.Ldlr\textsuperscript{−/−} 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 the Methods section in the online-only Data Supplement for details). The following numbers of mice were used for the LF and HF diets, respectively: Ldlr\textsuperscript{−/−}, \( n = 12 \) and \( n = 11 \); C1qa.Ldlr\textsuperscript{−/−}, \( n = 12 \) and \( n = 15 \); sIgM.Ldlr\textsuperscript{−/−}, \( n = 14 \) and \( n = 15 \); and C1qa.sIgM.Ldlr\textsuperscript{−/−}, \( n = 10 \) and \( n = 9 \). The aortic root of 1 LF-fed C1qa.sIgM.Ldlr\textsuperscript{−/−} 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 with colorimetric enzymatic assays under specific pathogen-free conditions. Total serum cholesterol and triglycerides were measured with colorimetric enzymatic assays. Total serum cholesterol and triglycerides were measured with colorimetric enzymatic assays.

**Atherosclerotic Lesion Analysis**

Mice were killed by CO\textsubscript{2} inhalation, and blood was removed from the inferior vena cava. Using a cannula inserted in the left ventricle, we perfused hearts sequentially with Krebs-Henseleit buffer at 37°C for 5 minutes, 2% formalin for 5 minutes, and 2 mL 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 were performed as previously described.\textsuperscript{18} We perfused hearts sequentially with Krebs-Henseleit buffer at 37°C for 5 minutes, 2% formalin for 5 minutes, and 2 mL 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 were performed as previously described.\textsuperscript{18}

**Quantification of Lesional Apoptosis**

Apoptotic cells were detected with terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL; Roche, Welwyn Garden City, UK) on aortic root cryosections, following the manufacturer’s instructions. Randomized 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,\textsuperscript{18} aortic root cryosections were double immunostained for CD68 (Alexafluor 488–conjugated anti-CD68 antibody, Molecular Probes, Invitrogen, Paisley, UK) and IgM (biotin-conjugated mouse anti-mouse IgM monoclonal antibody and biotin-conjugated mouse anti-mouse IgG, BD Pharmingen, Oxford, UK) secondarily labeled with Alexa 568–conjugated streptavidin (Molecular Probes) and counterstained with TOPRO-3.

**Lipoprotein Isolation and Modification**

Human LDL (density, 1.019 to 1.063 g/mL) was isolated from plasma of healthy donors after an overnight fast by differential density ultracentrifugation\textsuperscript{23} and modified with either freshly synthesized MDA or CuSO\textsubscript{4} to generate MDA-LDL and copper-oxidized LDL (CuOxLDL) (see the Methods section of the online-only Data Supplement).\textsuperscript{24}

**Serum Autoantibody Measurement and Assessment of Autoimmunity**

For the detection of anti–MDA-LDL\textsuperscript{24} and anti-CuOxLDL antibodies, nonirradiated microtiter plates (Greiner Bio-One, Stonehouse, UK) were coated overnight at 4°C with 15 \( \mu \)g/mL MDA-LDL, CuOxLDL, or native LDL in a coating buffer of 100 mmol/L NaHCO\textsubscript{3} and 1 mg/mL Na\textsubscript{2}EDTA or with coating buffer alone to assess nonspecific binding. After washing with PBS with 0.5% Tween-20, plates were blocked with 5% BSA. Sera diluted in PBS-Tween were applied. Alkaline phosphatase–conjugated anti-mouse IgG, IgM, or IgG1 antibodies (Southern Biotech, Birming-

**Immunohistochemistry**

Aortic root frozen sections were stained by use of standard immunohistochemistry to identify the following cell types: macrophages (MOMA-2 rat monoclonal antibody, Serotec, Kidlington, Oxford, UK), vascular smooth muscle cells (alkaline phosphatase–conjugated anti-\( \alpha \)-smooth muscle actin antibody, Sigma-Aldrich, Poole, UK), and T cells (goat anti-mouse CD3\textsubscript{e} antibody, Santa Cruz Biotechnology, Santa Cruz, Calif). The presence of lesional deposition of C5b-9 (rabbit anti-human C5b-9 antibody, Calbiochem, Merck Biosciences, Darmstadt, Germany) was identified through the use of the same technique and quantified as percentage lesional area staining positive with ImagePro. Lesional C3 (FITC-conjugated goat anti-mouse C3 antibody, AbD Serotec, Oxford, UK) and IgG (FITC-conjugated goat anti-mouse IgG antibody, Sigma-Aldrich) were identified with immunofluorescence and quantified as mean fluorescence intensity per pixel.

**Statistical Analysis**

Results were analyzed with Graphpad Prism version 3.0 (Graphpad Software, San Diego, Calif). When data were not normally distributed, nonparametric statistical tests were used, and results are expressed as median (interquartile range). To compare 2 groups,
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1A). No IgM was detected within atherosclerotic lesions of slgM.Ldlr−/− mice, indicating detection specificity (Figure 1B).

Accelerated Atherosclerosis in Serum IgM–Deficient Mice
No significant differences between all 4 mouse strains on either diet were noted in final body weight, total serum cholesterol, triglycerides (Table I of the online-only Data Supplement), or lipoprotein fast-performance liquid chromatography profiles (not shown). Atherosclerotic plaque burden was quantified in slgM.Ldlr−/− mice and compared with Ldlr−/− control mice and with C1qa.Ldlr−/− mice. On the LF diet, a hierarchy was apparent so 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% to 8.44%], n=12; Ldlr−/−: 1.64% [interquartile range, 1.11% to 2.72%], n=12; P<0.05, Kruskal-Wallis test with Dunn post test), whereas both slgM.Ldlr−/− and C1qa.sIgM.Ldlr−/− mice demonstrated substantially increased lesion size of 6.1-fold and 4.1-fold, respectively (slgM.Ldlr−/−: 10.0% [interquartile range, 4.96 to 12.9%], n=14; C1qa.sIgM.Ldlr−/−: 6.76% [interquartile range, 5.11% to 10.4%], n=9; both P<0.001 versus Ldlr−/−; Figure 2A and 2B). On the HF diet (Figure 2A and 2C), aortic root lesions in slgM.Ldlr−/− mice were 29% increased compared with Ldlr−/− mice (slgM.Ldlr−/−: 35.8% [interquartile range, 31.1% to 39.1%], n=15; Ldlr−/−: 27.8% [interquartile range, 24.0% to 30.5%], n=12; P<0.05) and 33% increased in C1qa.sIgM.Ldlr−/− mice (median, 36.9% [interquartile range, 33.5% to 39.9%], n=9; P<0.01). There was no difference between C1qa.Ldlr−/− (26.4% [interquartile range, 20.7% to 31.8%], n=15; P=1.00) and Ldlr−/− mice on 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 ≈2.1-fold in en face atherosclerotic lesion area compared with Ldlr−/− mice (C1qa.Ldlr−/−: 2.87% [interquartile range, 1.71% to 3.56%], n=12; Ldlr−/−: 1.36% [interquartile range, 0.73% to 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 slgM.Ldlr−/− group compared with Ldlr−/− controls, with an equal increase seen in C1qa.sIgM.Ldlr−/− triple-knockout mice (slgM.Ldlr−/−: 9.48% [interquartile range, 5.36% to 10.9%], n=14; C1qa.sIgM.Ldlr−/−: 7.18% [interquartile range, 4.79% to 11.4%], n=10; both P<0.001 versus Ldlr−/−). On the HF diet, a 66% increase in Sudan IV staining was still seen in the slgM.Ldlr−/− group compared with Ldlr−/− controls (12.4% [interquartile range, 8.48% to 17.2%], n=15, versus 7.49% [interquartile range, 6.13% to 8.77%], n=12; P<0.05) (Figure 3C and 3D). C1qa.sIgM.Ldlr−/− mice showed a level of en face atherosclerotic lesions (10.1% [interquartile range, 7.88% to 14.1%], n=9) similar to slgM.Ldlr−/− mice, whereas C1qa.Ldlr−/− mice (5.23% [interquartile range, 4.77% to 6.70%], n=15) were not statistically different from Ldlr−/− mice (P=0.16).

Results
Localization of IgM in Plaques of Ldlr−/− Mice
Confocal microscopy was used to examine IgM deposition within atherosclerotic plaques and localization relative to macrophages. IgM was found within lesions in mice on either an LF or HF diet, particularly in the acellular core and with a reciprocal relationship to CD68-positive macrophages (Figure

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 a similar lesion from LF-fed slgM.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−/−, slgM.Ldlr−/−, and C1qa.slgM.Ldlr−/− mice stained with Oil Red O and hematoxylin. Scale bars = 500 μm. B and C, Cross-sectional aortic root lesion area fraction (%) on an LF (B) or HF (C) diet. Each point represents the mean of 5 sections per mouse; bars show overall median. *P<0.05, **P<0.01, ***P<0.001 by Kruskal-Wallis test with Dunn post test.

Figure 3. Accelerated lesion formation in en face aorta preparations of sIgM-deficient mice. A and C, Representative Sudan IV-stained en face aorta preparations from Ldlr−/−, C1qa.Ldlr−/−, slgM.Ldlr−/−, and C1qa.slgM.Ldlr−/− mice after 12 weeks of an LF (A) or HF (C) diet. Scale bars = 0.5 mm. B and D, Quantification of en face aorta lesion area expressed as percent lesion area fraction on LF (B) or HF (D) diet, with bars showing the median. *P<0.05, **P<0.01, ***P<0.001 by Kruskal-Wallis test with Dunn post test.
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, slgM.Ldlr−/− lesions showed a significant increase in population by vascular smooth muscle cells, identified by positive staining for α-smooth muscle actin—positive vascular smooth muscle cells, and CD3+ T cells expressed as percent of lesional cells on an LF (A; Ldlr−/−, n=12; slgM.Ldlr−/−, n=14) and HF (B; Ldlr−/−, n=12; slgM.Ldlr−/−, n=15) diet. Density of aortic root lesion IgG and C3 deposition measured as mean fluorescence intensity per pixel on an LF (A) and HF (B) diet (n as before) and expressed as relative fluorescence units (RFU) per pixel. Bars represent median. Statistical analysis was by Mann–Whitney test. C, Representative photomicrographs of aortic root lesions showing cholesterol crystals under polarizing microscopy (age, 22 weeks; LF diet), smooth muscle fibrous cap formation (blue), lesional IgG, and C3 deposition with fluorescence microscopy on HF diet in slgM.Ldlr−/− vs Ldlr−/− mice. Autofluorescence with an FITC-conjugated isotype control is shown below. Scale bars=100 μm.

Figure 4. Increased complexity of aortic root lesions in slgM-deficient mice. MOMA-2–positive macrophages, α-smooth muscle actin–positive vascular smooth muscle cells, and CD3+ T cells expressed as percent of lesional cells on an LF (A; Ldlr−/−, n=12; slgM.Ldlr−/−, n=14) and HF (B; Ldlr−/−, n=12; slgM.Ldlr−/−, n=15) diet. Density of aortic root lesion IgG and C3 deposition measured as mean fluorescence intensity per pixel on an LF (A) and HF (B) diet (n as before) and expressed as relative fluorescence units (RFU) per pixel. Bars represent median. Statistical analysis was by Mann–Whitney test. C, Representative photomicrographs of aortic root lesions showing cholesterol crystals under polarizing microscopy (age, 22 weeks; LF diet), smooth muscle fibrous cap formation (blue), lesional IgG, and C3 deposition with fluorescence microscopy on HF diet in slgM.Ldlr−/− vs Ldlr−/− mice. Autofluorescence with an FITC-conjugated isotype control is shown below. Scale bars=100 μm.

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Ldlr−/− mice in the proportion of lesional T lymphocytes (assessed with anti-CD3ε) on either diet (Figure 4A and 4B). Using immunofluorescence for IgG and C3, we quantified mean fluorescent intensity per pixel 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 slgM.Ldlr−/− mice on either diet (Figure 4A through 4C) 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 slgM.Ldlr−/− mice was at a level similar to that found in C1qa.Ldlr−/− mice, which were previously demonstrated to have impaired lesional apoptotic cell clearance.18 Thus, using a combination of TUNEL staining and morphological identification, we found that aortic root lesions of slgM.Ldlr−/− mice fed the LF diet contained increased numbers of apoptotic cells compared with Ldlr−/− controls, reaching statistical significance when analyzed by Mann–Whitney test (P = 0.047). However, when more stringent Kruskal-Wallis post tests only were used, C1qa.Ldlr−/− mice showed increased numbers of apoptotic cells (P < 0.01), and neither slgM.Ldlr−/− nor C1qa.sIgM.Ldlr−/− mice reached significance compared with Ldlr−/− controls (Figure 5A and 5B). More apoptotic cells were detectable in Ldlr−/− controls fed the HF diet; differences between HF-fed groups were not significant (Figure 5C).

Autoantibody Production in slgM.Ldlr−/− Mice
slgM.Ldlr−/− mice on a mixed C57BL/6×129 background have previously been reported to produce anti–double-stranded DNA and anti-cardiolipin antibodies.26 However, slgM.Ldlr−/− mice backcrossed for 10 generations on the C57BL/6 background do not produce these autoantibodies (M. Botto, unpublished). Serological tests were conducted to check for evidence of autoimmunity in slgM.Ldlr−/− mice. LF-fed slgM.Ldlr−/− mice showed low levels of IgG anti–single-stranded DNA antibodies, which were not statistically different from levels in Ldlr−/− mice (the Table). However, a significant increase in IgG anti–single-stranded DNA antibodies was noted when slgM.Ldlr−/− mice were fed the HF diet compared with HF-fed Ldlr−/− controls (P < 0.001, Kruskal-Wallis post test). Although raised, levels of anti–single-stranded DNA antibodies in HF-fed slgM.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 titers of IgG anti-cardiolipin antibodies; all other groups showed nonsignificant levels. Other autoantibodies, including anti-nuclear antibodies, anti-double-stranded DNA, anti-chromatin, and anti-β2-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 oxLDL, titers of anti–MDA-LDL and
anti-CuOxLDL antibodies were assayed in Ldlr<sup>-/-</sup> and slgM, Ldlr<sup>-/-</sup> 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 compared with LF-fed Ldlr<sup>-/-</sup> mice, and as expected, IgM antibodies were not detected in slgM, Ldlr<sup>-/-</sup> sera (Figure 6). There were no significant differences in IgG anti–MDA-LDL or anti–CuOxLDL antibody titer in slgM, Ldlr<sup>-/-</sup> mice compared with Ldlr<sup>-/-</sup> mice on the LF diet. However, there was a significant reduction in IgG anti-CuOxLDL antibodies in slgM, Ldlr<sup>-/-</sup> mice fed an HF diet compared with HF-fed Ldlr<sup>-/-</sup> mice.

Hypercholesterolemia in ApoE<sup>-/-</sup> mice has been shown to cause a T<sub>1</sub>1-to-T<sub>2</sub>2 switch, resulting in increased IgG<sub>1</sub> anti–MDA-LDL antibody formation. To investigate whether increased atherosclerosis in slgM, Ldlr<sup>-/-</sup> mice was associated with an increased T<sub>2</sub>2 response, IgG<sub>1</sub> and IgG<sub>2a,2c</sub> isotypes also were assayed. For both isotypes, generally no differences were seen between slgM, Ldlr<sup>-/-</sup> mice, except for slightly lower IgG<sub>2a,2c</sub> anti–CuOxLDL antibodies observed in LF-fed slgM, Ldlr<sup>-/-</sup> mice (significant only at the lowest dilution).

### Discussion

Here, we have shown that Ldlr<sup>-/-</sup> mice deficient in serum IgM display a substantial acceleration of atherosclerosis on both an LF and HF diet, with larger lesions, an increase in lesional cholesterol crystal formation, and an increase in vascular smooth muscle cell content consistent with fibrous cap formation. Overall, our data provide the first direct evidence that endogenous IgM natural antibodies are necessary for humoral protection and build on previous experimental studies on humoral immunity in mice that have shown a reduction in atherosclerosis by (1) B-cell rescue of splenectomized mice, (2) administration of polyclonal human IgG, (3) administration of a monoclonal human IgG<sub>1</sub> antibody against MDA-LDL, and (4) increase in IgM anti-phosphorylcholine antibody levels by vaccination with pneumococci or phosphorylcholine conjugated to keyhole limpet hemocyanin.

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 C1q or C3 deficiency, suggesting that complement has atheroprotective effects. However, although C1q deficiency was associated with increased vascular smooth muscle cell content in lesions, the opposite was reported in C3-deficient mice, raising the possibility that different modes of complement activation may have distinct effects on atherogenesis. The direct comparison of slgM, Ldlr<sup>-/-</sup> mice with C1qa,Ldlr<sup>-/-</sup> and triple-knockout C1qa,slgM, Ldlr<sup>-/-</sup> 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<sup>-/-</sup> mice on the LF diet showed a modest increase in aortic root lesion size, but C1q deficiency had no detectable effect in mice fed an HF diet. The differences here between slgM, Ldlr<sup>-/-</sup> and C1qa,Ldlr<sup>-/-</sup> mice demonstrate a hierarchy, with IgM playing a dominant role. Consistent with this, the combined deficiency of C1q and slgM showed no difference in the level of atherosclerosis compared with IgM deficiency alone. It is possible that in the absence of C1q, IgM may bypass the classical pathway and instead activate the lectin pathway, as has been shown in ischemia/reperfusion injury. However, in the slgM, Ldlr<sup>-/-</sup> mice, we found no difference in the density of lesional C3 or C5b-9 staining compared with the Ldlr<sup>-/-</sup> 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 the clearance of dying cells. We observed increased levels of apoptotic cells and TUNEL-positive cellular debris within aortic root atherosclerotic plaques of slgM, Ldlr<sup>-/-</sup> mice, similar to those seen in C1qa,Ldlr<sup>-/-</sup> mice. Increased lesional apoptosis was not observed in C1qa,slgM, Ldlr<sup>-/-</sup> mice, raising the possibility of compensatory mechanisms occurring within the triple-knockout mice. There was no difference in apoptotic cell numbers between the groups on the HF diet, primarily because of increased lesional apoptosis in Ldlr<sup>-/-</sup> 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 slgM, Ldlr<sup>-/-</sup> and C1qa,Ldlr<sup>-/-</sup> mice on the LF diet, increased numbers of lesional apoptotic cells also may be a function of lesion size. Overall, the data on lesional apoptotic cells do not support a failure of apoptotic cell clearance as

| Table. Autoantibody Profiles in Ldlr<sup>-/-</sup> and slgM,Ldlr<sup>-/-</sup> Mice at 22 Weeks of Age |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                | LF Diet                        |                                | HF Diet                        |                                |
|                                | n=12                           | n=14                           | n=12                           | 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)               |
| aCL                            | 7.43 (0.43–77)                 | 14.2 (0–44.7)                  | 38.4 (0.75–1)                 | 0 (0–18.3)                     |
| β<sub>2</sub>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-β<sub>2</sub>GPI antibody levels expressed as median (interquartile range) in arbitrary ELISA units, compared against a reference standard of pooled MRL/pr mouse sera. *P<0.001 vs HF-fed Ldlr<sup>-/-</sup> (post-test following Kruskal-Wallis test).
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ple comparisons. †

tistical analysis by unpaired
mice on LF and HF diets at 22 weeks of age. Nonspecific binding
Figure 6. IgG and IgM anti-oxLDL antibodies in Ldlr−/− and slgM-
LDlr−/− mice. Serial dilution curves showing titers of anti-MDA-LDL
(A) and anti-CuOxLDL (B) antibodies in Ldlr−/− and slgM-LDlr−/−
mice on LF and HF diets at 22 weeks of age. Nonspecific binding
to native LDL is shown at a single dilution. Error bars show SEM. Sta-
tistical analysis by unpaired t test with Bonferroni correction for mul-
tiple comparisons: *P<0.05, slgM-LDlr−/− (LF) vs Ldlr−/− (LF). **P<0.01,
IgM-LDlr−/− (HF) vs Ldlr−/− (HF). §§P<0.05, §§§P<0.001,
LDlr−/− (LF) versus Ldlr−/− (HF). OD indicates optical density.

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 slgM-LDlr−/− compared with C1qa-LDlr−/− mice.

Although deficiency of serum IgM has been shown to
predispose to the development of anti–double-stranded DNA
and anti-cardiolipin antibodies on certain murine genetic
backgrounds,26,27 slgM-LDlr−/− mice do not develop autoan-
tibodies when fully backcrossed onto the C57BL/6 back-
ground (M. Botto, unpublished). Unexpectedly, we observed that slgM-LDlr−/− mice on the HF diet developed anti–single-
stranded DNA autoantibodies, supporting previous observa-
tions that there is an interaction between hypercholesterol-
emia and predisposition to lupus-like autoimmunity.19 Hence,
IgM deficiency enhances autoimmunity in both a strain-
dependent and lipid-dependent manner. It should be noted,
however, that the levels of anti–single-stranded DNA in slgM-LDlr−/− mice on the HF diet were low compared with
those seen in classical lupus models (eg, MRL/lpr), and no mice developed renal disease. It therefore seems unlikely that
lupus-like autoimmunity contributed significantly to the ac-
celerated arterial disease we observed.

The main purpose of measuring levels of IgG antibodies
against MDA-LDL 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 antibod-
ies to these antigens. There was no evidence in slgM-LDlr−/−
mice of a change in IgG isotype reflecting a T h1-to-Th2
switch.24 Paradoxically, there was a significant reduction in
IgG antibodies against CuOxLDL in slgM-LDlr−/− mice fed
an HF diet compared with Ldlr−/− mice. Although theoreti-
cally this may be due to an idiosyncratic reduction in IgG
synthesis related to IgM deficiency, class switching is thought
to be relatively normal in slgM-LDlr−/− mice.22 One plausible
explanation is that the lower IgG CuOxLDL antibody level in
HF-fed slgM-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? 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
opsonizing 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 corre-
lations between serum levels of IgM anti-oxLDL or anti-
phosphorylcholine antibodies and progression of carotid ather-
therosclerosis,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 increas-
ing rationale for therapeutic strategies aimed at boosting
humoral immunity. Conversely, B-cell depletion therapy with
the anti-CD20 monoclonal antibody rituximab is increasingly
used to treat autoimmune conditions, including systemic
lupus erythematosus and rheumatoid arthritis. Up to 20% of
patients with rheumatoid arthritis treated with repeated
courses of B-cell depletion develop abnormally low levels of
slgM, with less effect on serum IgG levels.43 It seems possible that repeated B-cell depletion might increase cardio-
vascular risk as a result of preferential depletion of serum IgM in a population already susceptible to cardiovascular complications.44

Acknowledgments

We are grateful for assistance from H.T. Cook (renal pathology), D.P. Patel (LDL purification), N. Navaratnam (fast-performance liquid chromatography), D. Carassiti (immunofluorescence), M. Lewis (histology processing), and the Biological Services Unit staff for the welfare of the animals.

Sources of Funding

This study was funded by program grants from the British Heart Foundation and the Wellcome Trust. Dr Lewis is the recipient of a Wellcome Trust Clinical Research Fellowship.

Disclosures

None.

References

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CLINICAL PERSPECTIVE

Previous immunization experiments in animals and clinical association studies have suggested that IgM antibodies may have protective effects against the development of atherosclerosis. In this study, we have addressed the role of IgM in atherogenesis by crossing the low-density lipoprotein (LDL) receptor–deficient (Ldlr−/−) mouse model of atherosclerosis with mice that have no IgM in their serum as a result of defective release of IgM from B lymphocytes (sIgM−/−). sIgM.Ldlr−/− mice showed marked acceleration of atherosclerosis compared with Ldlr−/− mice with larger lesions, early lesional cholesterol crystal formation, and an increase in vascular smooth muscle cell content consistent with fibrous cap formation. Although IgM is a potent activator of complement and thus has an important function in disposing of apoptotic cells, atheroprotection by IgM, in part, is not mediated through complement activation and consequently is at least partially independent of apoptotic cell clearance. Overall, our data provide the first direct evidence that endogenous IgM natural antibodies are necessary for arterial homeostasis and corroborate clinical studies that have observed an inverse correlation between IgM anti–oxidized LDL antibodies and progression of carotid atherosclerosis or angiographically determined coronary artery disease. This work lends support for developing immunization strategies to boost natural antibody protection and potentially limit atherosclerosis.
Immunoglobulin M Is Required for Protection Against Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient Mice
Myles J. Lewis, Talat H. Malik, Michael R. Ehrenstein, Joseph J. Boyle, Marina Botto and Dorian O. Haskard

Circulation. 2009;120:417-426; originally published online July 20, 2009;
doi: 10.1161/CIRCULATIONAHA.109.868158

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/120/5/417

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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. 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). 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 IgG2a and IgG2c**

A standard ELISA was set up to measure levels of IgG2a and IgG2c, using a goat anti-mouse Ig antibody (Southern Biotech) as the capture antibody. Serial dilutions of commercial mouse IgG2a standard (HOPC-1 clone, Southern Biotech) or IgG2c 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 IgG2a, 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.

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

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


