Increased Plasma IgE Accelerate Atherosclerosis in Secreted IgM Deficiency

Dimitrios Tsiantoulas, Ilze Bot, Maria Oszvar-Kozma, Laura Göderle, Thomas Perkmann, Karsten Hartvigsen, Daniel H. Conrad, Johan Kuiper, Ziad Mallat, Christoph J. Binder

Rationale: Deficiency of secreted IgM (sIgM−/−) accelerates atherosclerosis in Ldlr−/− mice. Several atheroprotective effects of increased levels of IgM antibodies have been suggested, including preventing inflammation induced by oxidized low-density lipoprotein and promoting apoptotic cell clearance. However, the mechanisms by which the lack of sIgM promotes lesion formation remain unknown.

Objective: To identify the mechanisms by which sIgM deficiency accelerates atherosclerosis in mice.

Methods and Results: We here show that both sIgM−/− and Ldlr−/−sIgM−/− mice develop increased plasma IgE titers because of impaired generation of B cells expressing the low-affinity IgE receptor CD23, which mediates the clearance of IgE antibodies. We further report that Ldlr−/−sIgM−/− mice exhibit increased numbers of activated mast cells and neutrophils in the perivascular area of atherosclerotic plaques. Treatment with an anti-IgE–neutralizing antibody fully reversed vascular inflammation and accelerated atherosclerotic lesion formation in cholesterol-fed Ldlr−/−sIgM−/− mice.

Conclusions: Thus, our data identify a previously unsuspected mechanism by which sIgM deficiency aggravates atherosclerosis. (Circ Res. 2017;120:78-84. DOI: 10.1161/CIRCRESAHA.116.309606.)

Key Words: arteriosclerosis ■ atherosclerosis ■ B-lymphocytes ■ immunoglobulin E ■ immunoglobulin M ■ immune system ■ inflammation

Secreted IgM (sIgM) antibodies are produced very early in life. In unchallenged conditions, a major portion of sIgM consists of natural IgM, which are mainly derived from innate B-1 B cells. B-1 B cells predominantly localize in the peritoneal cavity, where they are subdivided into B-1a and B-1b B cells, and in contrast to conventional B cells, they produce antibodies in the absence of cognate T-cell help.1,2

A large part of natural IgM recognize oxidation-specific epitopes (OSE),3 which are present on OxLDL, dying cells, and microparticles.3–5 Both epidemiological and experimental studies have suggested a protective role for anti-OxLDL sIgM in atherosclerosis and cardiovascular disease.6 These effects are thought to be mediated by the ability of OSE-specific IgM to block and neutralize the proatherogenic effects of OxLDL and promote the clearance of dying cells and cellular debris.6

However, OSE-specific IgM constitute only ≈30% of total IgM,3 which also possess important homeostatic functions such as regulation of conventional B-2 cell development.7,8 This is of particular interest as B-2 cells have been shown to aggravate atherosclerosis.6 Therefore, sIgM may also have an important modulatory function in atherosclerotic plaque formation that is independent of directly limiting plaque inflammation.

Notably, Ldlr−/− mice deficient in sIgM (sIgM−/−) exhibit accelerated lesion formation,9 but there is no evidence that this effect is a direct result of defective apoptotic cell clearance in the plaque.5 This indicates that sIgM harbor currently unknown atheroprotective properties, which are responsible for the robust proatherogenic effect of sIgM deficiency.

Methods

Mice, Treatments, and Diets

Ldlr−/− mice (on C57BL/6 background), sIgM−/− (on 129 background), and Rag1−/− mice were originally bought from The Jackson Laboratories (USA). S IgM−/− mice were backcrossed onto C57BL/6 background for at least 10 generations. Ldlr−/−sIgM−/− mice were generated by intercrossing Ldlr−/− and sIgM−/− mice. To induce atherosclerosis, mice were fed an atherogenic diet (0.2% cholesterol, 21% fat; E15721-347 bought from Ssniff, Germany) and treated with either an anti-IgE-neutralizing antibody (clone R1E4), which binds free IgE only or a control IgG (Jackson ImmunoResearch Inc.) where indicated.

Original received July 20, 2016; revision received November 23, 2016; accepted November 30, 2016. In October 2016, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15.7 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.116.309606/-/DC1.

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Plasma or Serum Cholesterol and Triglyceride Quantification

Total cholesterol and triglycerides in plasma or serum were measured under standardized conditions in an ISO 15189 accredited medical laboratory on Beckman Coulter AU5400 (Beckman Coulter) or Roche Cobas 8000 (Roche) instruments.

Quantification of Size and Macrophage Content of Atherosclerotic Lesions

Size and macrophage content of atherosclerotic lesions were evaluated by computer-assisted image analysis using Adobe Photoshop Elements 6.0 and ImageJ software as described previously.10,11

Mast Cell and Neutrophil Staining

Mast cells (MCs) and neutrophils were visualized in the perivascular area of cross sections as described previously.12,13

Total and Free IgE Antibody Quantification by ELISA

Total and free IgE serum titers were quantified by ELISA with the Mouse IgE ELISA MAX kit (Biologend). To determine free IgE serum levels, plates were coated with the anti-IgE antibody R1E4 at 5 μg/mL.

Bone Marrow–Derived MC Stimulation

Bone marrow–derived MCs were grown by culturing bone marrow cells at a density of 0.25x10^6 cells in RPMI 1640 (Gibco) containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from PAA), and 10% murine interleukin (IL)-3 containing supernatant (supernatant from WEHI cells over-expressing murine IL-3) for 4 weeks. Bone marrow–derived MCs (5x10^6) were incubated with sterile plasma (1:12 dilution in medium) from Ldlr−/−sIgM−/− mice of study 3 (Online Table I) for 2 hours at 37°C, after which supernatant was collected. IL-6 was determined by ELISA according to the manufacturer’s protocol (BD Biosciences).

Peritoneal Macrophage Stimulation

Macrophages were isolated from the peritoneal cavity of Rag1−/− mice by peritoneal lavage with RPMI 1640 (Gibco) containing 1% fetal bovine serum (Gibco) and 1% penicillin and streptomycin. Cells were plated in a 96-well plate (flat bottom) for at least 2 hours before stimulation to allow adherence. Then, cells were stimulated with 10% peritoneal lavage with RPMI 1640 (Gibco) containing 1% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from PAA), and 10% murine interleukin (IL)-3 containing supernatant (supernatant from WEHI cells over-expressing murine IL-3) for 4 weeks. Bone marrow–derived MCs (5x10^6) were incubated with sterile plasma (1:12 dilution in medium) from Ldlr−/−sIgM−/− mice of study 3 (Online Table I) for 2 hours at 37°C, after which supernatant was collected. IL-6 was determined by ELISA according to the manufacturer’s protocol (BD Biosciences).

Soluble CD23 Quantification by ELISA

Serum soluble CD23 was determined by ELISA using a rabbit anti-CD23 antibody and anti-rabbit IgG conjugated to alkaline phosphatase (Sigma; A3687) on a Synergy 2 luminometer (BIO-TEK).

Flow Cytometry

Flow cytometry analysis in splenic, bone marrow, and peritoneal cells was performed as described previously.14

Total RNA Extraction, cDNA Synthesis, and Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted with thepeqGold total RNA kit (Peqlab), and cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time polymerase chain reaction analysis was performed with the KAPA SYBR green FAST BioRad iCycler kit (Peqlab). For il-6 and germine IgE mRNA quantification, 36B4 or CD19 were used as reference genes, respectively, and the data are expressed as fold change over sIgM+/+ or Ldlr−/−sIgM−/− mice.

Statistical Analyses

Statistical analyses were performed using Graph Pad Prism 5 for Windows (Graph Pad Software). Experimental groups were compared using 2-tailed Student unpaired t or Mann–Whitney U test as appropriate. To analyze multiple group data, either 1-way ANOVA test followed by Newman–Keuls or Dunn or unpaired t test were performed as indicated. Data are presented as mean±SEM or as mean±SD where indicated. P<0.05 was considered significant.

Results

sIgM Deficiency Results in Strongly Increased Plasma IgE

Patients with selective IgM deficiency exhibit increased levels of plasma IgE.14 Although sIgM+/+ mice have been reported to display disturbed basal humoral immunity,15 IgE responses in these mice have not been investigated. We discovered that plasma IgE levels were >8-fold higher in both young and old sIgM−/− mice than in sIgM+/+ controls (Figure 1A and 1B). Similar to sIgM−/− mice, Ldlr−/−sIgM−/− mice fed a regular chow also display robustly increased plasma IgE (>9-fold) (Figure 1C). Although athogenic diet feeding itself has been shown to increase plasma IgE levels,16 Ldlr−/−sIgM−/− mice that were fed an athogenic diet for 16 weeks display much higher levels of IgE in their plasma (>5-fold) compared with the moderately increased levels in control Ldlr−/− mice (Figure 1D). Ldlr−/−sIgM−/− mice that were fed an athogenic diet for 16 weeks developed increased atherosclerosis in the aortic arch, thoracic, and abdominal aorta compared with control Ldlr−/− mice (Figure 1E) despite similar serum cholesterol and triglyceride levels (Online Table I, study 1), which is consistent with Lewis et al.8 Because a proatherogenic role for IgE is supported by both experimental16 and epidemiological studies,17 we hypothesized that increased IgE may mediate the aggravated atherosclerosis in sIgM−/− mice.

IgE Neutralization Reverses the Accelerated Atherosclerosis in Ldlr−/−sIgM−/− Mice

To investigate whether increased IgE titers contribute to accelerated atherosclerosis in these mice, Ldlr−/−sIgM−/− or Ldlr−/− mice were fed an athogenic diet for 6 weeks and at the same time were treated with either a neutralizing anti-IgE antibody (R1E4) or a control antibody (Ctrl), respectively. Anti-IgE treatment resulted in complete neutralization of free IgE antibodies (Figure 2A). Consistent with the study by Lewis et al., Ld lr−/−sIgM−/− mice treated with the control antibody developed 30% increased atherosclerosis in the aortic root compared with Ldlr−/− mice. Importantly, enhanced lesion formation in Ldlr−/−sIgM−/− mice was fully reversed by anti-IgE treatment (Figure 2B). Moreover, anti-IgE treatment did not change plasma cholesterol and triglyceride levels (Online Table I, study 2). Furthermore, similar data were obtained when Ldlr−/−sIgM−/− mice were fed an athogenic diet for 8 weeks (Online Table I, study 3; Figure 2C and 2D) and treated with a control antibody or a neutralizing anti-IgE as described above. Anti-IgE treatment resulted in complete neutralization of free IgE antibodies.
Figure 1. Increased plasma IgE titers in slgM−/− mice. Total plasma IgE antibody levels in (A) 12- to 14-wk-old (n=9–11 mice per group) and (B) 28-wk-old (n=10 mice per group) slgM−/− (light blue bars) and slgM+/+ (dark blue bars) mice measured by ELISA. C, Total plasma IgE of Ldlr−/−slgM−/− (light blue bar) or Ldlr−/−slgM+/+ mice (dark blue bar) fed a regular chow diet (n=6 mice per group). D, Total plasma IgE and (E) representative photomicrographs (left) and dot plot (right) showing the quantification of en face atherosclerotic lesion size expressed as percentage of total aortic area of Sudan VI stained aortas of female Ldlr−/−slgM−/− (light blue bar) or Ldlr−/−slgM+/+ (dark blue bar) mice that were fed an atherogenic diet for 16 wk (Online Table I, study 1; n=13–15 mice per group). Results are represented as mean±SEM. **P<0.01, ***P<0.001, ****P<0.0001 (Mann-Whitney U or unpaired t test).

Figure IIA and IIB). Moreover, we have recently shown that neutrophils, which promote atherosclerosis, are recruited in the vessel wall on MC activation.23 In line with this, we found that the perivascular area of atherosclerotic lesions of Ldlr−/− mice had significantly higher levels of neutrophils compared with Ldlr+/+ mice, which was fully reversed by anti-IgE treatment (Figure 3B; Online Figure IIA and IIB). To assess the proatherogenic properties of pooled plasma IgE from Ldlr−/− slgM−/− mice in vitro, we performed cell-based stimulation assays using plasma from Ctrl and anti-IgE–treated Ldlr−/−slgM−/− mice (study 3) that was collected in a sterile manner. Stimulation of bone marrow–derived MCs with plasma collected from control antibody-treated Ldlr−/−slgM−/− mice (study 3) at the end of the experiment resulted in robust IL-6 secretion (which was >9-fold higher than stimulation with Ldlr−/− plasma; data not shown), whereas stimulation with plasma from anti-IgE–treated Ldlr−/−slgM−/− mice failed to do so (Figure 3C). Moreover, stimulation of peritoneal macrophages with plasma from control antibody-treated Ldlr−/−slgM−/− mice (study 3) resulted in higher IL-6 mRNA and decreased cell survival than stimulation with plasma from anti-IgE–treated Ldlr−/−slgM−/− mice (Figure 3D and 3E). Interestingly, we found no difference in lesional macrophage content between Ldlr−/−slgM−/− and Ldlr−/− mice (Online Figure III). These data indicate that slgM deficiency increases the levels of proinflammatory IgE, which in turn promote vascular inflammation and accelerate the development of atherosclerosis.

B Cells Expressing the Low-Affinity IgE Receptor CD23 Are Reduced in slgM−/− Mice

To investigate whether the increased IgE levels were because of increased IgE production, we quantified IgE producing B cells as defined in Online Figure IVA. We found that slgM−/− and slgM+/+ mice had equivalent numbers of class switched B cells expressing IgE in the spleen (Online Figure VA). In addition, we found no differences in IgE secreting plasma cells (CD138+/ B220+) and plasmablasts (CD138+ B220+) in the spleen (Online Figure VB) and the bone marrow (Online Figure VC) between slgM−/− and slgM+/+ mice. Similar data were obtained for naive B cells (Online Figure VB and VC). In line with these data, germline IgE mRNA in the spleen was not different between slgM−/− and slgM+/+ or between Ldlr−/−slgM−/− and Ldlr−/−slgM+/+ mice (Online Figure VD and VE).

B cells that express CD23, which is the low-affinity receptor for IgE, have previously been shown to mediate clearance of IgE antibodies.20–22 Interestingly, we found that Ldlr−/−slgM−/− mice had reduced CD23-expressing B cells in the spleen (Figure 4A) and the peritoneal cavity (Figure 4B) compared with Ldlr−/−slgM+/+ controls. Similar data were obtained in the spleen and the peritoneal cavity of slgM−/− and slgM+/+ mice (data not shown). In addition, we show that the reduction in splenic CD23-expressing B cells (consisting of follicular and CD23+ transitional stage 2 B cells; FO/T2) in Ldlr−/−slgM−/− mice is because of altered splenic B-cell maturation, which results in increased MZ and CD21+ CD23− B cells and concomitantly reduced FO B cells (Figure 4C). Interestingly, immature B cells that have newly escaped from the bone marrow (newly formed and transitional stage 1 [T1] B cells) are not altered in Ldlr−/−slgM−/− mice (Figure 4C), which suggests that slgM regulate MZ and FO B-cell differentiation by directly...
modulating the splenic microenvironment. Moreover, we found decreased levels of soluble CD23 in the plasma of Ldlr+/−sIgM−/− mice (Figure 4D), which further supports an impaired generation of CD23 expressing B cells. Taken together, the strongly reduced numbers of CD23 expressing B cells in the spleen and peritoneal cavity of Ldlr+/−sIgM−/− mice offer a mechanistic explanation for the accumulation of IgE in these mice.

**Discussion**

IgM Abs that display reactivity toward OxLDL and apoptotic cells have been suggested to confer an atheroprotective effect by blocking OxLDL-induced foam cell formation and promoting apoptotic cell clearance. Lewis et al reported that mice lacking sIgM developed enhanced atherosclerosis. However, Ldlr+/−sIgM−/− mice did not show increased accumulation of apoptotic cells within the lesions, compared with their controls.

Kyaw et al demonstrated that splenectomy of atherosclerotic apolipoprotein E−deficient (ApoE−/−) mice reduced plasma IgM levels by ≈50% and resulted in increased atherosclerosis, which was reversed after the administration of B-1a cells that are able to secrete IgM. However, despite the fact that transfer of B-1a cells from IgM competent mice reversed the proatherogenic effect of splenectomy compared with transfer of B-1a cells from sIgM knockout mice, the deposition of malondialdehyde (MDA)-LDL and apoptotic cells was not different between the 2 groups. Thus, reduction of total IgM in the context of splenectomy may activate unknown proatherogenic mechanisms that are controlled by the pool of total IgM.

In fact, the studies described above do not support the notion that the protective effect of the entire pool of sIgM antibodies is mediated via reduced foam cell formation or improved apoptotic cell clearance. Of note, the difference in plaque size between atherosclerosis-prone sIgM−/− and control mice is larger when fed a low-fat semisynthetic diet compared with atherogenic diet. These data suggest that the accelerated atherosclerosis in Ldlr+/−sIgM−/− mice is predominately mediated via mechanisms that are already present in unchallenged sIgM−/− mice and do not depend on excessive hypercholesterolemia. Here, we report that sIgM−/− mice display strongly elevated IgE levels, which are responsible for accelerated atherosclerosis in these mice. Notably, splenectomy in humans, which also causes a strong reduction in IgM titers, has been reported to result in elevated plasma IgE titers. Thus, even though splenectomy affects multiple immune cell types, it is tempting to hypothesize that IgE may also in part be responsible for accelerated atherosclerosis on splenectomy in humans.

Our data argue against elevated plasma IgE as a consequence of increased IgE production. We propose that impaired generation of B cells expressing the low-affinity receptor CD23, which mediates the clearance of circulating IgE, results in plasma IgE accumulation in sIgM−/− mice. Considering that plasma IgE are increased on hypercholesterolemia in mice and correlate with cardiovascular disease risk in humans, it is tempting to hypothesize that CD23+B cells may be critically

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**Figure 2. IgE neutralization reverses accelerated atherosclerosis in Ldlr−/−sIgM−/− mice.** A. Levels of free plasma IgE antibodies and B) representative photomicrographs of hematoxylin-eosin (H&E)−stained aortic root lesions (×50) and dot plot of the average lesion size in the aortic origin expressed as µm²/section (400 µm) of female Ldlr−/−) (light blue) or Ldlr−/−sIgM−/− (dark blue) mice fed an atherogenic diet for 6 wk and injected intraperitoneally once every week with an anti-IgE−neutralizing antibody (R1E4) or a control IgG. Ldlr−/−+Ctrl IgG, n=14; Ldlr−/−sIgM−/−+Ctrl IgG, n=16; Ldlr−/−sIgM−/−+anti-IgE, n=15 (Online Table I, study 2). C. Levels of free plasma IgE antibodies and D) representative photomicrographs of H&E-stained aortic root lesions (×50) and dot plot of the average lesion size in the aortic origin expressed as µm²/section (400 µm) of female Ldlr−/−sIgM−/− mice fed an atherogenic diet for 8 wk and injected intraperitoneally once every week with an anti-IgE−neutralizing antibody (R1E4) or a control IgG. Ldlr−/−+Ctrl IgG, n=10; Ldlr−/−sIgM−/−+anti-IgE, n=8 (Online Table I, study 3). Data are represented as mean±SEM, *P<0.05, ****P<0.0001 (1-way ANOVA followed by [A] Dunn, [B] Newman–Keuls test, and [C and D] unpaired t test), scale bar: 200 µm.
involved in preventing atherosclerosis progression by promoting the removal of IgE from the circulation.

Moreover, we demonstrate an increased portion of activated MCs and neutrophils in the perivascular area of atherosclerotic lesions of female \( \text{Ldlr}^{-/-} \) (light blue) or \( \text{Ldlr}^{-/-}\text{sIgM}^{-/-} \) (dark blue) mice fed an atherogenic diet for 6 wk and injected intraperitoneally once every week with an anti-IgE-neutralizing antibody (R1E4) or a control IgG. \( \text{Ldlr}^{-/-}+\text{Ctrl IgG}, n=13; \text{Ldlr}^{-/-}\text{sIgM}^{-/-}+\text{Ctrl IgG}, n=15; \text{Ldlr}^{-/-}\text{sIgM}^{-/-}+\text{anti-IgE}, n=14 \) (Online Table I, study 2). C, IL-6 secretion by bone marrow–derived MCs, (D) \( \text{il}-6 \) mRNA production and (E) cell survival of peritoneal resident macrophages stimulated with pooled plasma of \( \text{Ldlr}^{-/-}\text{sIgM}^{-/-} \) mice (Online Table I, study 3), which were fed an atherogenic diet for 8 wk and treated with a control antibody or a neutralizing anti-IgE measured by (C) ELISA, (D) real-time-polymerase chain reaction, or (E) a luminescent cell viability assay. Data represent measurements of triplicate or quadruplicate stimulations and are represented as mean±SEM. *\( P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 \) (1-way ANOVA followed by [A] Newman–Keuls, [B] unpaired \( t \) test, and [C–E] unpaired \( t \) test).

Figure 3. Elevated plasma IgE promote mast cell activation, neutrophil recruitment, and macrophage cell death in \( \text{Ldlr}^{-/-}\text{sIgM}^{-/-} \) mice. A, Representative photomicrographs of chloroacetate esterase stained mast cells (MC) (top left, \( \times 10 \); bottom left, \( \times 40 \)) and dot plot of the average percentages of perivascular activated MC, and (B) dot plot of average numbers of neutrophils in the perivascular area of atherosclerotic lesions of female \( \text{Ldlr}^{-/-} \) mice fed an atherogenic diet for 6 wk and injected intraperitoneally once every week with an anti-IgE-neutralizing antibody (R1E4) or a control IgG. \( \text{Ldlr}^{-/-}+\text{Ctrl IgG}, n=13; \text{Ldlr}^{-/-}\text{sIgM}^{-/-}+\text{Ctrl IgG}, n=15; \text{Ldlr}^{-/-}\text{sIgM}^{-/-}+\text{anti-IgE}, n=14 \) (Online Table I, study 2). C, IL-6 secretion by bone marrow–derived MCs, (D) \( \text{il}-6 \) mRNA production and (E) cell survival of peritoneal resident macrophages stimulated with pooled plasma of \( \text{Ldlr}^{-/-}\text{sIgM}^{-/-} \) mice (Online Table I, study 3), which were fed an atherogenic diet for 8 wk and treated with a control antibody or a neutralizing anti-IgE measured by (C) ELISA, (D) real-time-polymerase chain reaction, or (E) a luminescent cell viability assay. Data represent measurements of triplicate or quadruplicate stimulations and are represented as mean±SEM. *\( P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 \) (1-way ANOVA followed by [A] Newman–Keuls, [B] unpaired \( t \) test, and [C–E] unpaired \( t \) test).
antibody specificities, which thereby contributes largely to the diversity of the total IgM pool that mediates recognition of various types of self-antigens. The latter is particularly important as self-antigen–mediated B-cell receptor signaling dictates proper mature B-2 cell (follicular and marginal zone B cells) development in the spleen.28 Consistent with this, we show that splenic B-2 cell development is disturbed in both sIgM−/− and Ldlr−/− mice and results in strongly impaired CD23-expressing B-cell generation, which as mentioned above are responsible for the clearance of IgE antibodies. Thus, sIgM antibodies influence atherosclerosis in multiple ways. Indeed, both total IgM and MDA-LDL–specific IgM are inversely associated with car- dBulence atherosclerosis in multiple ways. Indeed, both total IgM and MDA-LDL–specific IgM are inversely associated with car-

Sources of Funding

We acknowledge the support from the Netherlands CardioVascular Research Initiative (the Dutch Heart Foundation, Dutch Federation of University Medical Centres, the Netherlands Organisation for Health Research and Development and the Royal Netherlands Academy of Sciences) for the GENIUS project Generating the best evidence-based pharmaceutical targets for atherosclerosis (CVON2011-19). This work was supported by grants of the Austrian Science Fund (SFB F54), the European Union (FP7 VIA), the European Research Council (ERC), and the Dutch Heart Foundation (grant 2012T083).

Disclosures

None.

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Novelty and Significance

**What Is Known?**

- Deficiency of secreted IgM (sIgM) Abs results in accelerated atherosclerosis.
- sIgM Abs protect from atherosclerosis by regulating splenic B-cell development.
- IgE Abs protect from atherosclerosis in mice lacking sIgM Abs. Total and oxidized-low-density lipoprotein-specific IgM Abs have been shown to inversely associate with cardiovascular disease risk. Consistent with this observation, mice lacking sIgM Abs develop accelerated atherosclerosis. However, the underlying mechanism remains unknown. We found that sIgM-deficient mice display strongly increased IgE levels in plasma, which are responsible for the accelerated atherosclerosis in these mice. These results suggest that sIgM impacts atherosclerosis via regulation of B-cell functions and humoral immunity.
Increased Plasma IgE Accelerate Atherosclerosis in Secreted IgM Deficiency
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Circ Res. 2017;120:78-84; originally published online November 30, 2016;
doi: 10.1161/CIRCRESAHA.116.309606

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/120/1/78

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SUPPLEMENTAL MATERIAL

Detailed Methods

Mice, treatments and diets

Ldlr<sup>−/−</sup> mice (on C57BL/6 background), slgM<sup>−/−</sup> (on 129 background) and Rag1<sup>−/−</sup> mice were originally bought from The Jackson Laboratories (USA). SlgM<sup>−/−</sup> mice were backcrossed onto C57BL/6 background for at least 10 generations. Ldlr<sup>−/−</sup>slgM<sup>−/−</sup> mice were generated by intercrossing Ldlr<sup>−/−</sup> and slgM<sup>−/−</sup> mice. For the atherosclerosis study 1, female Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup> slgM<sup>−/−</sup> were fed an atherogenic diet (0.2% cholesterol, 21% fat; E15721-347 bought from Ssniff, Germany) for 16 weeks starting at the age of 12 weeks. For atherosclerosis studies 2 and 3, 13–18 week old female Ldlr<sup>−/−</sup> or Ldlr<sup>−/−</sup>slgM<sup>−/−</sup> were fed an atherogenic diet for 6 or 8 weeks respectively. During the diet feeding period, the mice were injected intraperitoneally once every week, with 25µg diluted in 100µl DPBS (Sigma) of either an anti-IgE neutralizing antibody (clone R1E4), which binds free IgE only or a control IgG (Jackson Immunoresearch Inc.). All mice included in atherosclerosis studies were matched for age and body weight. All experimental studies were approved by the Animal Ethics Committee of the Medical University of Vienna (Austria) BMWF-66.009/0157-II/3b/2013 and BMWFW-66.009/0030-WF/V/3b/2016.

Plasma or serum cholesterol and triglyceride quantification

Fresh blood was collected from the vena cava at the time of sacrifice in MiniCollect Gold cap TUBE or MiniCollect K3EDTA TUBE (both from Greiner Bio-One). Blood was centrifuged at 1000g for 30 minutes at room temperature. Serum or plasma total cholesterol and triglycerides were measured under standardized conditions in an ISO 15189 accredited medical laboratory on Beckman Coulter AU5400 (Beckman Coulter) or Roche Cobas 8000 (Roche) instruments using the Beckman Coulter OSR6516 and OSR60118 or Roche CHOL2 and TRIGL reagents, respectively.

Quantification of size and macrophage content of atherosclerotic lesions

Atherosclerotic lesion size was evaluated by computer assisted image analysis using Adobe Photoshop Elements 6.0 and ImageJ software as described previously<sup>10, 11</sup>. Lesion size in the aortic arch, descending thoracic and abdominal aorta was quantified in Sudan IV stained en face preparations of the entire aorta and is expressed as percentage of the whole aortic surface area. Lesion size in the aortic root was quantified in Hematoxylin and Eosin (H&E) stained cross sections (n=9/mouse) with 50µm distance that were collected starting with the appearance of the valves. For macrophage content sections of paraffin embedded aortic root lesions were stained with an anti-MAC-3 antibody (BD) and a biotinylated goat anti-rat IgG (VectorLabs) and were developed with streptavidin-peroxidase polymer (Sigma).

Mast cell and neutrophil staining

Mast cells and neutrophils were visualized in the perivascular area of cross sections (1-2/mouse) by staining with a naphthol AS-D chloroacetate esterase staining kit (Sigma) and counted manually. A mast cell was considered resting when all granula were maintained inside the cell, while mast cells were assessed as activated when granula were deposited in the tissue surrounding the mast cell. Neutrophils were identified as round cells with a characteristic lobular nucleus and pink granular cytoplasm (Online Figure II). Perivascular size was measured using a Leica image analysis system (Leica Ltd, UK). Morphometric analyses were performed by a blinded independent operator.

Total and free IgE antibody quantification by ELISA

Total and free IgE serum titers were quantified by ELISA with the Mouse IgE ELISA MAX kit (Biolegend). To determine free IgE serum levels, plates were coated with the anti-IgE antibody R1E4 at 5 µg/mL.
Bone marrow derived mast cell stimulation
Bone marrow derived mast cells (BMMCs) were grown by culturing bone marrow cells at a density of 0.25x10^6 cells in RPMI1640 containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all from PAA) and 10% murine Interleukin-3 containing supernatant (supernatant from WEHI cells overexpressing murine Interleukin-3) for 4 weeks. BMMCs (5x10^5) were incubated with plasma (1:12 dilution in medium) from Ldlr^-/-sIgM^-/- mice of study 3 (Online Table I) for 2 hours at 37°C, after which supernatant was collected. IL-6 was determined by ELISA according to the manufacturer’s protocol (BD Biosciences).

Peritoneal macrophage stimulation
Macrophages were isolated from the peritoneum of Rag1^-/- mice by peritoneal lavage with RPMI1640 (Gibco) containing 1% FBS (Gibco) and 1% penicillin and streptomycin. Cells were plated in a 96-well (flat bottom) plate for at least 2 hours prior to stimulation to allow adherence. Then, cells were stimulated with 10% plasma from Ldlr^-/-sIgM^-/- mice of study 3 (Online Table I) for either 4 or 48 hours at 37°C, after which il-6 mRNA (4 hours) or cell viability (48 hours) were determined by Real-time PCR and CellTiter glo luminescent cell viability assay (Promega), respectively.

Soluble CD23 quantification by ELISA
Serum soluble CD23 was determined by ELISA. Briefly, 96-well white round-bottomed MicroFluor microtiter plates (Thermo Lab systems) plates were coated with 10 µg/mL of 2H10 mAb overnight and then washed 3 times with PBS/EDTA and blocked with Tris-buffered saline containing 1% BSA (TBS/BSA) for 1 h at room temperature. After washing the plates as before, diluted murine plasma and recombinant CD23 were added in TBS/BSA to the wells and incubated for 1 hour at room temperature. Plates were washed and bound soluble CD23 was detected with a rabbit anti-CD23 antibody. Following an incubation time for 1 hour at room temperature and a washing step as before, an anti-rabbit IgG conjugated to alkaline phosphatase (Sigma; A3687) was added for 1 hour at room temperature. Wells were washed again as before and rinsed once with distilled water, and 25 μl of a 30% LumiPhos Plus solution in dH₂O (Lumigen Inc) was added. After 75 min the light emission was measured with a Synergy 2 luminometer (BIO-TEK) and expressed as RLU per 100ms.

Flow cytometry
Peritoneal lavage with HBSS (Gibco) media containing 2% FBS was performed to collect peritoneal cells. Bone marrow cells were isolated from the tibia and the femur bones and single cell suspensions were prepared using cell strainers with 100 µm diameter (BD Biosciences). Whole spleens were isolated and single cell suspensions were obtained using cell strainers with 100 µm diameter (BD Biosciences). Erythrocytes were lysed with erythrocyte lysis buffer (MORPHISTO). Cells were incubated in a 96 well V-bottom plate (Thermo Scientific) with 2.5 µg/ml of a blocking anti-CD16/32 antibody (clone 93; eBiosciences) diluted in DPBS (Sigma) containing 10% FBS (FACS buffer) for 20 min at 4ºC. After two washing steps with FACS buffer (393 g for 3 minutes at 4ºC), cells were stained with the following antibodies in different combinations: anti-B220 PercP-Cy5.5 (clone RA3-6B2; eBiosciences), anti-CD23 FITC (clone B3B4; eBiosciences), anti-CD43 PE (clone S7; BD Biosciences), anti-IgM APC ( clone II/41; eBiosciences), anti-CD21 biotinylated (clone 7E9; Biolegend), anti-CD11b APC (clone M1/70; eBiosciences), anti-CD5 (clone 53-7;3; eBiosciences), anti-IgE PE (clone 23G3; eBiosciences), anti-CD138 biotinylated (clone 281-2; Biolegend) and streptavidin APC (eBiosciences). For intracellular IgE staining, cells were fixed and permeabilized with fixation and permeabilization solution (eBiosciences) for 30 minutes at 4°C and then stained intracellularly with anti-IgE PE (clone 23G3; eBiosciences) in permeabilization buffer (eBiosciences). A FACS Calibur (Becton Dickinson) was used to acquire the data, which then were analyzed using Flow Jo software 7.6 (Treestar).
Total RNA extraction, cDNA synthesis and Real-time PCR analysis

Total RNA was extracted with the peqGold total RNA kit (Peqlab) and cDNA was synthesized using the High capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative Real-time PCR analysis was performed with the KAPA SYBR green FAST BioRad iCycler kit (Peqlab). For il-6 and germline IgE mRNA quantification, 36B4 or CD19 were used as reference genes respectively and the data are expressed as fold change over slgM$^{+/+}$ or Ldlr$^{-/-}$ slgM$^{+/+}$ mice.

Primer list:
- germline IgE forward: 5'-TGGGCATGAATTAATGGTTACTAGAG-3',
- germline IgE reverse: 5'-TGGCCAGACTGTTCTTATTCGAA-3',
- CD19 forward: 5'-AAGAGGGAGCAATGTTGTG-3',
- CD19 reverse: 5'-AAAAGCCACCAGAGAAACCA-3',
- 36B4 forward: 5'-AGGGCGACCTGGAAGTCC-3',
- 36B4 reverse: 5'-CCCACAATGAAGCATTTTGG-3',
- IL-6 forward: 5'-CCACCGGCTTCCCTACTTCA-3',
- IL-6 reverse: 5'-TGCAAGTCATCGTTGTTC-3'
Online Table I

<table>
<thead>
<tr>
<th>Study</th>
<th>experimental groups</th>
<th>final body weight (g)</th>
<th>total cholesterol (mg/dL)</th>
<th>triglycerides (mg/dL)</th>
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<tr>
<td>Study 1 / 16 weeks on atherogenic diet</td>
<td>LDLR(^{-/-})</td>
<td>27 ±2.5</td>
<td>1762 ±355</td>
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<td>LDLR(^{-/-}) sIgM(^{-/-})</td>
<td>28 ±4.8</td>
<td>1770 ±236</td>
<td>757 ±277</td>
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<td>Study 2 / 6 weeks on atherogenic diet</td>
<td>LDLR(^{-/-}) +Ctrl Ab</td>
<td>22.5 ±2.9</td>
<td>1391 ±258</td>
<td>691 ±185</td>
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<td></td>
<td>LDLR(^{-/-}) sIgM(^{-/-}) +Ctrl Ab</td>
<td>22 ±2.9</td>
<td>1316 ±220</td>
<td>653 ±190</td>
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<td>LDLR(^{-/-}) sIgM(^{-/-}) +anti-IgE</td>
<td>21.5 ±1.5</td>
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<td>652 ±200</td>
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<td>23.2 ±2.3</td>
<td>1351 ±107</td>
<td>675 ±228</td>
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</tbody>
</table>

Online Table I. Whole body weight and serum/plasma total cholesterol and triglyceride quantification in LDLR\(^{-/-}\) and LDLR\(^{-/-}\) sIgM\(^{-/-}\) mice that were fed an atherogenic diet. Serum or plasma total cholesterol and triglycerides were measured under standardized conditions in an ISO 15189 accredited medical laboratory on Beckman Coulter AU5400 (Beckman Coulter) or Roche Cobas 8000 (Roche) instruments. All results are represented as mean±SD.
Online Figure I. En face lesions in *Ldlr*<sup>−/−</sup> and *Ldlr*<sup>−/−</sup>*sIgM*<sup>−/−</sup> mice treated with an α-IgE or Ctrl Ab. Bar graphs show (A,B) the quantification of en face atherosclerotic lesion size expressed as percentage of total aortic area of Sudan VI stained aortas and (C) the free IgE in plasma of female *Ldlr*<sup>−/−</sup> (light blue bar) or *Ldlr*<sup>−/−</sup>*sIgM*<sup>−/−</sup> (dark blue bar) mice that were fed an atherogenic diet for (A) 6 weeks (14-16 mice per group) or (B,C) 8 weeks (4-10 mice per group) and were treated with an α-IgE or Ctrl Ab as described in Methods. Results are represented as mean±SEM. *P<0.05 (unpaired t test).
Online Figure II

**Online Figure II.** Mast cells and neutrophils in the perivascular area of aortic root plaques of \(Ldlr^{-/-}\) and \(Ldlr^{-/-}sIgM^{-/-}\) mice. (A) Dashed line depicts the perivascular area of aortic root plaque in which mast cell activation and neutrophil numbers were quantified. (B) Representative examples of neutrophils, resting and activated mast cells in the perivascular area of aortic root plaques of \(Ldlr^{-/-}\) (left) and \(Ldlr^{-/-}sIgM^{-/-}\) (right) mice. A mast cell was considered resting when all granula were maintained inside the cell, while mast cells were assessed as activated when granula were deposited in the tissue surrounding the mast cell. Neutrophils were identified as round cells with a characteristic lobular nucleus and pink granular cytoplasm.
Online Figure III. Macrophage content in lesions of \(Ldlr^{-/-}\) and \(Ldlr^{-/-}sIgM^{-/-}\) mice treated with an \(\alpha\)-IgE or Ctrl Ab. Dot plot of average MAC3-positive area in atherosclerotic lesions of female \(Ldlr^{-/-}\) (light blue) or \(Ldlr^{-/-}sIgM^{-/-}\) (dark blue) mice fed an atherogenic diet for 6 weeks and injected intraperitoneally once every week with an anti-IgE neutralizing antibody (R1E4) or a control IgG. \(Ldlr^{-/-} +\) Ctrl IgG, n=14; \(Ldlr^{-/-}sIgM^{-/-} +\) Ctrl IgG, n=16; \(Ldlr^{-/-}sIgM^{-/-} +\) anti-IgE, n=15 (Online Table I, study 2).
Online Figure IV. Flow cytometry gating strategy of splenic, bone marrow and peritoneal B cells. (A) Splenic and bone marrow plasma cells are defined as B220^CD138^+ (red), plasmablasts as B220^CD138^- (purple) and naïve B cells as B220^CD138^- (black). (B) Peritoneal CD23^+ B-2 cells are defined as B220^{high^}CD11b^-CD5^-CD23^+. (C) Splenic B cell subsets: follicular and CD23^+ transitional stage 2 B cells (FO/T2; blue) are defined as B220^-CD21^-CD23^-CD43^-, marginal zone (MZ; purple) B cells as B220^CD21^{high^}CD23^-CD43^-, CD21^-CD23^- B cells as B220^-CD21^-CD23^-CD43^- (red), transitional stage 1 (T1; green) B cells as B220^-CD21^{low^}CD23^-CD43^- and newly formed (NF; grey) B cells as B220^-CD21^-CD23^-CD43^-.
Online Figure V. IgE production is not altered in slgM<sup>−/−</sup> mice. (A) Flow cytometry plots (left) and bars (right) represent absolute numbers of splenic IgE<sup>+</sup> B cells in slgM<sup>+/+</sup> (light blue bar) and slgM<sup>−/−</sup> (dark blue bar) mice (n=5 mice per group). Data shown are from one of two independent experiments. Bars represent the frequency of IgE<sup>+</sup> cells within plasma cells (CD138<sup>+</sup> B220<sup>−</sup>), plasmablasts (CD138<sup>+</sup> B220<sup>+</sup>) and naïve B cells (CD138<sup>−</sup> B220<sup>+</sup>) in the (B) spleen and (C) bone marrow (as defined in Online Figure IVA) as determined by combined surface and intracellular staining for IgE reactivity (n=5-8 mice per group). Germline IgE mRNA in splenocytes of (D) slgM<sup>+/+</sup> (light blue bar) and slgM<sup>−/−</sup> (dark blue bar) mice (n=6 mice per group; data shown are from one of two independent experiments) and (E) Ldlr<sup>−/−</sup> slgM<sup>+/+</sup> (light blue bar) and Ldlr<sup>−/−</sup> slgM<sup>−/−</sup> (dark blue bar) mice (n=6 mice per group) quantified by Real-time PCR. All results are represented as mean±SEM.