Increased Plasma IgE Accelerate Atherosclerosis in Secreted IgM Deficiency

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Tsiantoulas et al, Secreted IgM regulate proatherogenic IgE levels

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ABSTRACT

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 LDL 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 due to 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.

KEYWORDS: Atherosclerosis, secreted IgM, IgE, B cells

ABBREVIATIONS

sIgM; secreted IgM
Ldlr; low density lipoprotein receptor
MC; mast cells

INTRODUCTION

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 predominately 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 CVD[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/or 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 secreted IgM (*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[9]. This indicates that sIgM harbor currently unknown atheroprotective properties, which are responsible for the robust proatherogenic effect of sIgM deficiency.

MATERIALS AND 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). *SIgM*$^{-/-}$ 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.

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 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 (Biolegend). To determine free IgE serum levels, plates were coated with the anti-IgE antibody R1E4 antibody 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 RPMI 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 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 (Gibco) containing 1% FBS (Gibco) and 1% penicillin and streptomycin. Cells were plated in a 96-well (flat bottom) for at least 2 hours prior to stimulation to allow adherence. Then, cells were stimulated with 10% sterile 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 ell viability assay (Promega), respectively.

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[10].

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 *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 two tailed Student's unpaired or Mann-Whitney test as appropriate. To analyze multiple group data, either One-Way ANOVA test followed by Newman-Keuls or Dunn's or unpaired t test were performed as indicated. Data are presented as mean ± SEM or as mean ± SD where indicated. A P value of <0.05 was considered significant.

RESULTS

Secreted IgM 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 compared to *sIgM*$^{+/+}$ controls (Figure 1A, B). Similar to *sIgM*$^{-/-}$ mice, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice fed a regular chow also display robustly increased plasma IgE (>9 fold) (Figure 1C). Although atherogenic diet feeding itself has been shown to increase

plasma IgE levels[16], *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice that were fed an atherogenic diet for 16 weeks display much higher levels of IgE in their plasma (>5-fold) compared to the moderately increased levels in control *Ldlr*$^{-/-}$ mice (Figure 1D). *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice that were fed an atherogenic diet for 16 weeks developed increased atherosclerosis in the aortic arch, thoracic and abdominal aorta compared to control *Ldlr*$^{-/-}$ mice (Figure 1E) despite similar serum cholesterol and triglyceride levels (Online Table I; study 1), which is consistent with Lewis et al[9]. Because a proatherogenic role for IgE is supported by both experimental[16] 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 atherogenic 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 Lewis et al[9], *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice treated with the control antibody developed 30% increased atherosclerosis in the aortic root compared to *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). Furthermore, similar data were obtained when *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice were fed an atherogenic diet for 8 weeks (Online Table I, study 3; and Figures 2C,D) 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 and a 40% decrease of lesion size in the aortic origin (Figure 2C, D). A similar trend was found in the entire aorta by en face lesion size for both studies mentioned above (Online Figure IA,B). These data indicate that the pathogenesis of atherosclerotic lesion formation in mice lacking sIgM is prominently driven by IgE antibodies. Interestingly, anti-IgE treatment in *Ldlr*$^{-/-}$ mice fed an atherogenic diet for 8 weeks had no effect on the extent of lesion formation compared to Ctrl Ab treated mice (Online Figure IB) despite an efficient neutralization of plasma IgE (Online Figure IC) and similar body weights (anti-IgE; 25.2 ±1.6g; Ctrl: 24.4 ±1.3g) and total cholesterol levels (anti-IgE; 1506 ±194mg/dL; Ctrl: 1403 ±75mg/dL). Notably, plasma IgE levels in these mice were equivalent to wild-type mice, which suggests that the proatherogenic effect of IgE requires higher levels such as found in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice.

Because IgE antibodies robustly activate mast cells via the FcεRI and mast cells have been shown to promote atherosclerotic plaque formation[12, 18], we examined the activation status of mast cells in the perivascular area of aortic root lesions. We found that *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice displayed a significantly increased frequency of activated mast cells compared to *Ldlr*$^{-/-}$ mice, while this effect was reversed by anti-IgE treatment (Figure 3A and Online Figure IIA,B). Moreover, we have recently shown that neutrophils, which promote atherosclerosis[19] are recruited in the vessel wall upon mast cell activation[13]. In line with this, we found that the perivascular area of atherosclerotic lesions of *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice had significantly higher numbers of neutrophils compared to *Ldlr*$^{-/-}$ mice, which was fully reversed by anti-IgE treatment (Figure 3B and Online Figure IIA,B). To assess the proatherogenic properties of pooled plasma IgE from *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice *in vitro*, we performed cell-based stimulation assays using plasma from Ctrl and anti-IgE treated *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice (study 3) that was collected in a sterile manner. Stimulation of bone marrow derived mast cells with plasma collected from control antibody-treated *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice (study 3) at the end of the experiment resulted in robust IL-6 secretion (which was >9 fold higher compared to stimulation with *Ldlr*$^{-/-}$ plasma; data not shown), whereas stimulation with plasma from anti-IgE treated *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice failed to do so (Figure 3C). Moreover, stimulation of peritoneal macrophages with plasma from control antibody-treated *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice (study 3) resulted in higher IL-6 mRNA and decreased cell survival, compared to stimulation with plasma from anti-IgE treated *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice (Figure 3D,E). Interestingly, we found no difference in lesional macrophage content between *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (Online Figure III). These data indicate that sIgM 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 *sIgM*$^{-/-}$ mice

In order to investigate whether the increased IgE levels were due to increased IgE production, we quantified IgE producing B cells as defined in Online Figure IVA. We found that *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$ 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 bone marrow (Online Figure VB) and the spleen (Online Figure VC) between *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$ mice. Similar data were obtained for naïve B cells (Online Figure VB, C). In line with these data, germline IgE mRNA in the spleen was not different between *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$, or between *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ and *Ldlr*$^{-/-}$ *sIgM*$^{+/+}$ mice (Online Figure IVD, E).

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*$^{-/-}$*sIgM*$^{-/-}$ mice had reduced CD23-expressing B cells in the spleen (Figure 4A) and the peritoneal cavity (Figure 4B) compared to *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ controls. Similar data were obtained in the spleen and the peritoneal cavity of *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$ 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*$^{-/-}$*sIgM*$^{-/-}$ mice is due to 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 (NF) and transitional stage 1 (T1) B cells) are not altered in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice (Figure 4C), which suggests that secreted IgM 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 towards OxLDL and apoptotic cells[3] have been suggested to confer an atheroprotective effect by blocking OxLDL-induced foam cell formation and promoting apoptotic cell clearance[6]. 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 to their controls[9].

Kyaw et al demonstrated that splenectomy of atherosclerotic Apolipoprotein E deficient (*Apoe*$^{-/-}$) mice reduced plasma IgM levels by approximately 50% and resulted in increased atherosclerosis, which was reversed following administration of B-1a cells that are able to secrete IgM[23]. However, despite the fact that transfer of B-1a cells from IgM competent mice reversed the proatherogenic effect of splenectomy compared to transfer of B-1a cells from sIgM ko mice, the deposition of MDA-LDL and apoptotic cells was not different between the two 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 semi-synthetic diet compared to atherogenic diet[9]. 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 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 levels[24] and associates with increased risk for myocardial infarction[6], has been reported to result in elevated plasma IgE titers[25]. 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 upon 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 upon hypercholesterolemia in mice and correlate with CVD risk in humans[6, 16, 17], it is tempting to hypothesize that CD23^{+} B cells may be critically involved in preventing atherosclerosis progression by promoting the removal of IgE from the circulation.

Moreover, we demonstrate an increased portion of activated mast cells and neutrophils in the perivascular area of atherosclerotic lesions of *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice, which was driven by the increased IgE levels in these mice. Although the numbers of mast cells and neutrophils are relatively low compared to macrophages in plaques, we and others have previously established that both mast cells[12] and neutrophils[19] exhibit strong proatherogenic properties. Notably, we found even higher numbers of mast cells in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice compared to our previous studies[12]. Moreover and in line with a previous study[16], we demonstrate that IgE have the capacity to promote IL-6 production by macrophages while prolonged stimulation with IgE-containing plasma induces increased cell death in macrophages. This may provide an explanation why despite increased IgE-mediated vascular inflammation the number of lesional macrophage is not different.

Besides the proatherogenic role of absent or reduced IgM, previous studies reported atheroprotective effects in association with increased levels of OxLDL-specific IgM. For example, immunization of *Ldlr*$^{-/-}$ mice with heat inactivated pneumococcal extracts resulted in increased levels of the prototypic PC-specific natural IgM T15/E06 that binds OxLDL and apoptotic cells, and decreased atherosclerotic lesion formation[11]. Similarly, vein graft atherosclerosis was reduced when T15/E06 was passively infused into *Apoe*$^{-/-}$ mice[26]. Furthermore, we recently showed that mice lacking the sialic acid binding immunoglobulin-like lectin G, which display increased numbers of B-1 cells and elevated levels OxLDL-specific IgM, were less susceptible to atherosclerosis[10]. In addition, Rosenfeld et al, demonstrated that *Apoe*$^{-/-}$*Rag*$^{-/-}$ mice that were adoptively transferred with B-1b cells that produced IgM antibodies with specificity for OSE *in vivo,* showed reduced development of atherosclerosis compared to PBS injected controls[27]. Together these studies suggest a protective role of high levels of B-1 cell derived OSE-specific IgM antibodies that recognize OxLDL and apoptotic debris. However, we believe that the effects seen in mice lacking all sIgM address a more fundamental role of sIgM in immune regulation, which goes beyond the potential mechanisms by which high levels of natural IgM protect against atherosclerosis. Along this line, it is important to note that *sIgM*$^{-/-}$ mice lack the entire repertoire of soluble IgM antibodies, which includes natural IgM and IgM derived from conventional B-2 cells. In contrast to B-1 cells B-2 cells exhibit a more diverse repertoire of 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*$^{-/-}$*sIgM*$^{-/-}$ 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 CVD complications in individuals of the Anglo-Scandinavian Cardiac Outcomes Trial[29].

Interestingly, recent work indicates that IgE promote lupus like autoimmunity in mice[30] and that the presence of autoreactive IgE positively associates with disease activity in SLE patients[31]. Notably, lupus-prone mice lacking sIgM develop enhanced autoimmune pathology compared to their controls[32]. Our findings provide an alternative explanation in which increased

IgE in mice lacking sIgM may contribute to the aggravated autoimmunity. This is particularly interesting considering that SLE patients display premature atherosclerosis and are at high risk for CVD complications, which cannot be explained by the traditional risk factors[33]. Thus, our data suggest that anti-IgE treatment could be considered as a therapeutic option for accelerated atherosclerosis in autoimmune diseases.

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DISCLOSURES

None

NOVELTY AND SIGNIFICANCE

What is known?

- Deficiency of secreted IgM (sIgM) Abs results in accelerated atherosclerosis
- sIgM Abs regulate B cell development
- B cells are important players in the pathogenesis of atherosclerosis

What new information dies this article contribute?

- Deficiency of sIgM Abs leads to robustly increased IgE Abs in plasma
- Increased plasma IgE Abs drive accelerated atherosclerosis in mice lacking sIgM Abs
- sIgM Abs aggravate atherosclerosis by regulating splenic B cell development

Total and OxLDL-specific IgM Abs have been shown to inversely associate with CVD risk. In line with this, mice lacking sIgM Abs develop accelerated atherosclerosis. However, the underlying mechanism has been unknown. We show here that sIgM deficient mice display strongly increased IgE levels in plasma that are responsible for the increased atherosclerosis in these mice. These data suggest that sIgM impact atherosclerosis via regulation of B cell functions and humoral immunity.

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FIGURE LEGENDS

Figure 1. Increased plasma IgE titers in *sIgM*$^{-/-}$ mice. Total plasma IgE antibody levels in (**A**) 12-14 week old (n=9-11 mice per group) and (**B**) 28 week old (n=10 mice per group) *sIgM*$^{+/+}$ (light blue bars) and *sIgM*$^{-/-}$ (dark blue bars) mice measured by ELISA. **(C)** Total plasma IgE of *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (light blue bar) or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ 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) show the quantification of *en face* atherosclerotic lesion size expressed as percentage of total aortic area of Sudan VI stained aortas of female *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ (light blue bar) or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bar) mice that were fed an atherogenic diet for 16 weeks (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 or unpaired t test).

Figure 2. IgE neutralization reverses accelerated atherosclerosis in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice. (**A**) Levels of free plasma IgE antibodies and (**B**) representative photomicrographs of H&E-stained aortic root lesions (50x) and dot plot of the average lesion size in the aortic origin expressed as μm^2/section (400μm) 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). (**C**) Levels of free plasma IgE antibodies and (**D**) representative photomicrographs of H&E-stained aortic root lesions (50x) and dot plot of the average lesion size in the aortic origin expressed as μm^2/section (400μm) of female *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice fed an atherogenic diet for 8 weeks and injected intraperitoneally once every week with an anti-IgE neutralizing antibody (R1E4) or a control IgG. *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ + 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 (One-Way ANOVA followed by (A) Dunn's (B) Newman-Keuls test, and (C,D) unpaired t test), scale bar: 200μm.

Figure 3. Elevated plasma IgE promote mast cell activation, neutrophil recruitment and macrophage cell death in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice.
(**A**) Representative photomicrographs of chloroacetate esterase stained mast cells (MC) (upper left; 10x, bottom left; 40x) 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 *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=13; *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ + Ctrl IgG, n=15; *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ + anti-IgE, n=14 (Online Table I, study 2). (**C**) IL-6 secretion by bone marrow derived mast cells, (**D**) *il-6* mRNA production and (**E**) cell survival of peritoneal resident macrophages stimulated with pooled plasma of *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice (Online Table I, study 3), which were fed an atherogenic diet for 8 weeks and treated with a control antibody or a neutralizing anti-IgE measured by (**C**) ELISA, (**D**) RT-PCR, 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 (One-Way ANOVA followed by (A) Newman-Keuls, or (B) unpaired t test, and (C,D,E) unpaired t test).

Figure 4. Reduced CD23$^+$ B cells in *Ldlr$^{-/-}$sIgM$^{-/-}$* mice. (**A**) Flow cytometry plots (left) and bars (right) show the absolute numbers splenic CD23$^+$ B cells (defined as B220$^+$IgM$^+$CD43$^-$ CD23$^+$) in *Ldlr$^{-/-}$sIgM$^{+/+}$* (light blue bar) and *Ldlr$^{-/-}$sIgM$^{-/-}$* (dark blue bar) mice. (**B**) Flow cytometry plots (left) and bars (right) represent the frequency of peritoneal CD23$^+$ B cells within B-2 cells (as defined in Online Figure IIIB) in *Ldlr$^{-/-}$sIgM$^{+/+}$* (light blue bar) and *Ldlr$^{-/-}$sIgM$^{-/-}$* (dark blue bar) mice. (**C**) Flow cytometry plots (left) and bars (right) represent absolute numbers of FO/T2 (blue), MZ (purple), CD21$^+$CD23$^-$ B cells (red), T1 (green) and NF (grey) cells (as defined in Online Figure IIIC) in *Ldlr$^{-/-}$sIgM$^{+/+}$* (light blue bar) and *Ldlr$^{-/-}$sIgM$^{-/-}$* (dark blue bar) mice. (**D**) Bars show plasma soluble CD23 in *Ldlr$^{-/-}$sIgM$^{+/+}$* (light blue bar) and *Ldlr$^{-/-}$sIgM$^{-/-}$* (dark blue bar) mice. All results are represented as mean ± SEM, n= 6 mice per group, *P<0.05, **P<0.01, ***P<0.001 (Mann-Whitney or unpaired t test).