SUPPLEMENTAL MATERIAL

Detailed 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. For the atherosclerosis study 1, female *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *sIgM*$^{-/-}$ 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*$^{-/-}$ or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ 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[10, 11]. 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 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 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 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% 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. 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 dH_20 (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 4°C. After two washing steps with FACS buffer (393 g for 3 minutes at 4°C), 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 *sIgM*$^{+/+}$ or *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ mice.

Primer list:

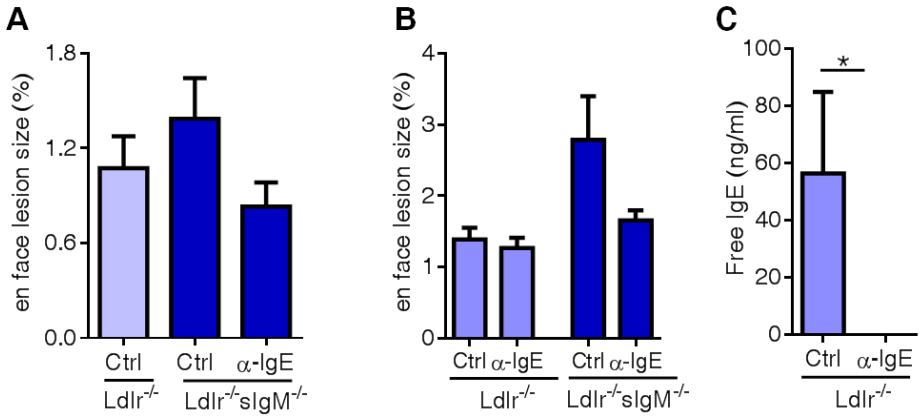
germline IgE forward: 5-TGGGCATGAATTAATGGTTACTAGAG-3 ,
germline IgE reverse: 5-TGGCCAGACTGTTCTTATTCGAA-3 ,
CD19 forward: 5-AAGAGGGAGGCAATGTTGTG-3,
CD19 reverse: 5-AAAAGCCACCAGAGAAACCA-3,
36B4 forward: 5- AGGGCGACCTGGAAGTCC-3,
36B4 reverse: 5- CCCACAATGAAGCATTTTGGA-3,
IL-6 forward: 5-CCACGGCCTTCCCTACTTCA-3,
IL-6 reverse: 5-TGCAAGTGCATCGTTGTTC-3

Online Table I

Study	experimental groups	final body weight (g)	total cholesterol (mg/dL)	triglycerides (mg/dL)
Study 1 / 16 weeks on atherogenic diet	$LDLR^{-/-}$	27 ±2.5	1762 ±355	767 ±310
	$LDLR^{-/-}$ $sIgM^{-/-}$	28 ±4.8	1770 ±236	757 ±277
Study 2 / 6 weeks on atherogenic diet	$LDLR^{-/-}$ +Ctrl Ab	22.5 ±2.9	1391 ±258	691 ±185
	$LDLR^{-/-}$ $sIgM^{-/-}$ +Ctrl Ab	22 ±2.9	1316 ±220	653 ±190
	$LDLR^{-/-}$ $sIgM^{-/-}$ +anti-IgE	21.5 ±1.5	1298 ±263	652 ±200
Study 3 / 8 weeks on atherogenic diet	$LDLR^{-/-}$ $sIgM^{-/-}$ +Ctrl Ab	23.7 ±2.3	1312 ±210	576 ±245
	$LDLR^{-/-}$ $sIgM^{-/-}$ +anti-IgE	23.2 ±2.3	1351 ±107	675 ±228

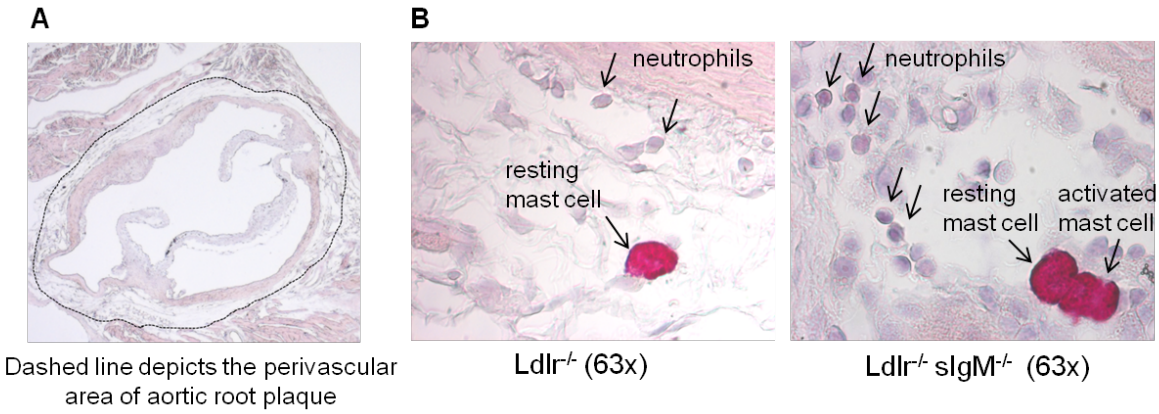
Online Table I. Whole body weight and serum/plasma total cholesterol and triglyceride quantification in *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice that were fed an atherogenic diet. Serum or plasma total cholesterol and triglycerides were measured under standardized conditions in an ISO 15189 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



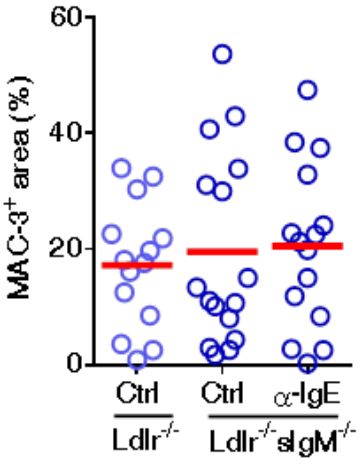
Online Figure I. En face lesions in *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice treated with an α-IgE or Ctrl Ab. Bar graphs show **(A,B)** the quantification of *en face* atherosclerotic lesion size expressed as percentage of total aortic area of Sudan VI stained aortas and (**C**) the free IgE in plasma of female *Ldlr*$^{-/-}$ (light blue bar) or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (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



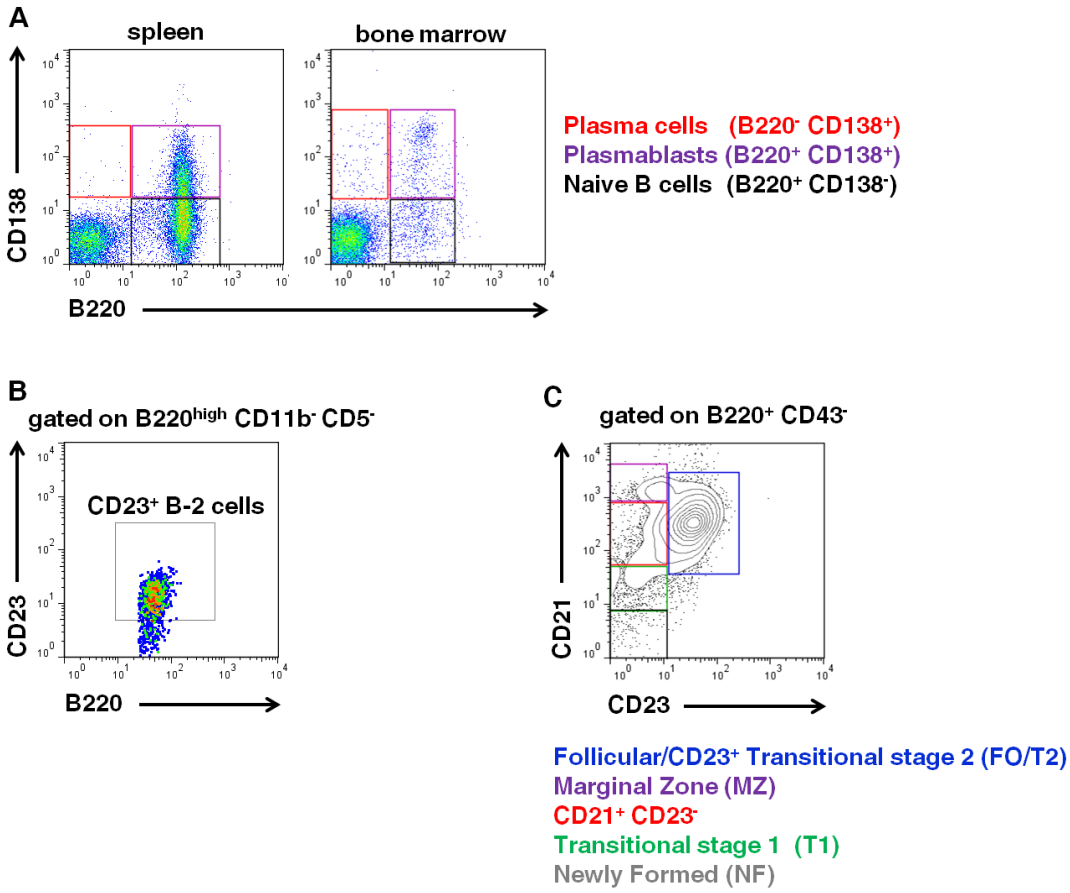
Online Figure II. Mast cells and neutrophils in the perivascular area of aortic root plaques of *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice. (**A**) Dashed line depicts the perivascular area of aortic root plaque in which mast cell activation and neutrophil numbers were quantified. (**B**) Representative 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



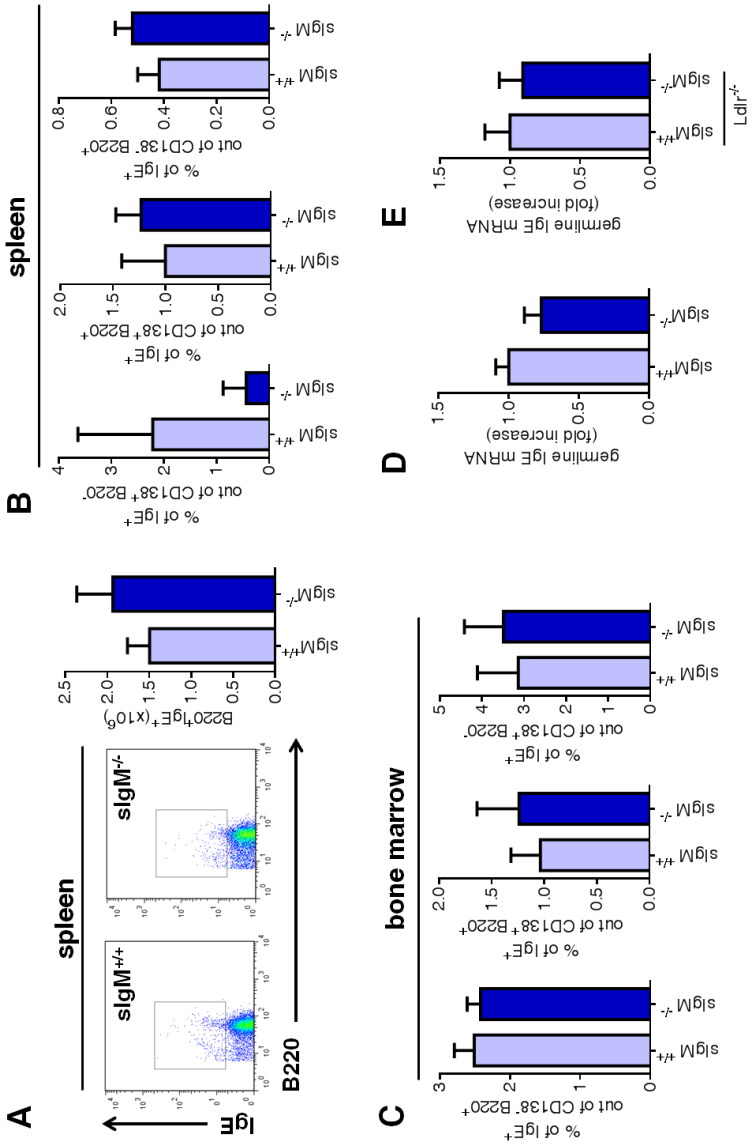
Online Figure III. Macrophage content in lesions of *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice treated with an α-IgE or Ctrl Ab. Dot plot of average MAC3-positive area in atherosclerotic lesions of female *Ldlr*$^{-/-}$ (light blue) or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue) mice fed an atherogenic 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



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



Online Figure V. IgE production is not altered in *sIgM*$^{-/-}$ mice. (**A**) Flow cytometry plots (left) and bars (right) represent absolute numbers of splenic IgE^{+} B cells in *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice (n=5 mice per group). Data shown are from one of two independent experiments Bars represent the frequency of IgE^{+} cells within plasma cells (CD138^{+} B220^{-}), plasmablasts (CD138^{+} B220^{+}) and naïve B cells (CD138^{-} B220^{+}) in the (**B**) spleen and (**C**) bone marrow (as defined in Online Figure IIIA) as determined by combined surface and intracellular staining for IgE reactivity (n=5-8 mice per group). Germline IgE mRNA in splenocytes of (**D**) *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice (n=6 mice per group; data shown are from one of two independent experiments) and (**E**) *Ldlr*$^{-/-}$ *sIgM*$^{+/+}$ (light blue bar) *and Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bar) mice (n=6 mice per group) quantified by Real-time PCR. All results are represented as mean ± SEM.