Materials and Methods

Mice. C57BL/6J WT and *ApoE*$^{-/-}$ mice were purchased from the Jackson laboratory and housed in the animal facilities of University of Jena and Ludwig-Maximilians-University Munich Germany. Mice were maintained in a pathogen-free environment on a 12 hour light/dark cycle and fed standard chow. 78-80 weeks old male WT and *ApoE*$^{-/-}$ mice were used. CD45.1/Ly5.1 mice were bred in the animal facility of the Leibniz-Institute for Age Research Jena, Germany. Animal procedures were conducted according to guidelines approved by the Animal Care and Use Committees.

Single cell preparation from aorta, spleen, RLN, blood, PerC, and BM. Single cell suspensions from aorta were prepared by enzymatic digestion as previously described[1] with further modifications as described[1, 2]. Briefly, WT and *ApoE*$^{-/-}$ mice were euthanized and the aorta was perfused with 2 mM EDTA in PBS, PBS, and FACS buffer. Care was taken to remove adjacent adipose tissue and small paraaortic lymph nodes to avoid contamination with cells present in adjacent connective tissues including fat associated lymphoid clusters. Thoracic and abdominal aorta segments were harvested, cut into small pieces and digested in 2.5 ml of enzyme cocktail (450 U/ml collagenase type I, 125 U/ml collagenase type XI, 60 U/ml hyaluronidase and 60 U/ml DNase1) in Dulbecco's phosphate buffered saline (DPBS) containing 20 mM HEPES. Digestion was carried out at 37° C for 1 hour in a water bath with shaking. The cell suspension was filtered through a 70 µm cell strainer by mashing aorta pieces using a syringe plunger. The cell suspension was centrifuged and resuspended in FACS buffer. Cell suspensions from spleen and RLNs was prepared using a 70 µm cell strainer and mashing spleen and RLNs with a syringe plunger and dissolved in FACS buffer. For blood samples, blood (0.2 ml) was collected into 5 mM Na_2-EDTA in an Eppendorf tube, mixed, centrifuged, and washed. For BM, femurs and tibiae were prepared and the muscle was removed. After cutting the ends of femur and tibia, BM was flushed with FACS buffer, centrifuged, and washed. Spleen, BM, and blood samples were resuspended in erythrocyte lysis buffer and washed. After centrifugation, cells were washed and resuspended in FACS buffer and cells were counted. For PerC, peritoneal lavage was collected, washed, centrifuged, and resuspended in FACS buffer and cells were counted.

Detection and quantification of ATLOs. ATLOs were identified and their size was determined as previously described[1-3].

Flow cytometry. Fc receptors were blocked by pretreatment with purified anti-mouse CD16/32 mAb for 10 min at 4° C. Cells were incubated with fluorescent labelled antibodies for 25 min at 4° C and washed twice. Cells were incubated with streptavidin conjugate for 20 min at 4° C. After washing, 8-color FACS measurements were performed on FACSCanto II^{TM} (BD Bioscience). Data were analysed using FlowJo (tree star). Labelled Abs that were used for FACS analyses: CD16/32 (93) (Fc-block); CD45-PerCpCy5.5 (30-F11); CD45.1-PerCpCy5.5 (A20); CD19-APC (ID3); CD19-eFluor 450 (ID3); CD23-FITC (B3B4), CD5-PE (53-7.3); CD43-Biotin (R2/60); CD11b-eFluor 450 (M1/70); CD38-PerCp eFluor-710 (90); PNA-FITC; GL7-Biotin (GL7); IgM-PECy7

(II/41); IgD-eFluor 450 (11-26c); IgG1-FITC (A85-1); IgA-PE (mA-6E1); IgE-Biotin (23G3) and Streptavidin-APC eFluor780; IL-10-PE(JES5-16E3); CD5-APC(53-7.3); TGF beta 1–PE; FasL-FITC; PD-L1-PE. All Abs are from eBioscience except PNA-FITC (Vector) and IgG1-FITC, CD138-APC (281-2) (BD Biosciences). All antisera were used at 1:200 dilutions for staining except PNA-FITC and GL7-Biotin at 1:300.

BrdU labeling and immunofluroscence staining. *ApoE*$^{-/-}$ mice (78-80 weeks) were fed with BrdU (1 mg/ml; Sigma-Aldrich) and 1% glucose (Merck) in drinking water for 2 weeks. Drinking water was protected from light and changed every 3 days[4]. After BrdU treatment, mice were euthanized and perfused with 3% paraformaldehyde (PFA) in 1x PBS at 120 Hg pressure for 30 min at RT. Fresh frozen tissue blocks were prepared for aorta and 10 µm sections were prepared for immunofluorescence analyses. Sections were washed with PBS for 10 min. To denature DNA, sections were incubated with 2 N HCl at 37° C for 15 min (only for BrdU staining) and washed 3 x 5 min with PBS. A blocking step was performed at RT for 1 hour in blocking solution (5% FBS, 1% BSA and 0.4% Triton X-100 in PBS). After blocking without washing, sections were incubated overnight with primary antibodies at 4° C. After primary antibody staining, sections were washed and incubated with fluorescent labelled secondary antibodies and DAPI at RT for 60 min. Sections were washed and mounted onto slides using fluorescence mounting medium (S3025, Dako). Immunofluorescence analyses were performed as described[1] using primary antibodies CD138 (1:50, R&D), BrdU (1:50, Oxford Biotechnology), CD45R/B220 (1:200, BD), IgG1-FITC (1:50, BD), IgM-FITC (1:50, BD), IgA-PE (1:50, eBioscience), IgE-Biotin (1:50, eBioscience), and corresponding fluorescent labelled secondary antibodies (1:300, all from Dianova).. For negative controls, stainings were performed without primary antibodies. Pictures were taken by LSM-510 META confocal scanning microscope (Zeiss) or SP5 (Leica) and analysed by Zeiss LSM image browser or Fiji image processing softwares.

MACS and ELISPOT assay. To identify constitutively IgM- and IgG-secreting B cells, ELISPOT assays were performed. Single cell suspensions of aortas were incubated with CD45 microbeads for 15 min at 4° C. Cells were washed, centrifuged and the pellet was resuspended in MACS running buffer. CD45$^+$ cells were separated using the POSSEL-S program in Auto-MACS (Miltenyi Biotec) and purified >97%. MultiScreen$_{\text{HTS}}$ IP-Plates (MSIPS4510, Millipore) were used. Each well was rinsed according to manufacturer’s protocol. After washing, wells were coated with unlabelled anti-mouse IgM or IgG antisera (10 µg/ml, Southern Biotech) and incubated overnight at 4° C. The antibody solution was decanted, wells were washed and blocked with RPMI-1640 + 10% FCS for 2 hours at 37° C. Cells were washed in cold complete RPMI-1640 medium to minimize background and a suspension of 10^6 cells/ml was prepared. After blocking, 0.25 x 10^6 cells from each tissue was added to the wells except for the aorta where total sample was added to the well and incubated overnight at 37° C. After 24 hours, cell culture dishes were decanted and washed with 0.1% Tween-20 in PBS. Biotin-labelled anti-mouse IgM or IgG antibody was (1:500, Southern Biotech) added to each well and incubated for 2 hours at 37° C. Plates were washed and streptavidin alkaline phosphatase (3:100, Abcam) was added to each well and incubated for 20-30 min at RT. Plates were washed, BCIP/NBT was added and incubated until spots became visible. Pictures were taken under the dissection microscope (AxioCam dissection microscope, Zeiss) and spots were counted manually.

Cell stimulation for IL-10 staining. Cell stimulation and IL-10 staining were performed as described[5].

Adoptive B cell transfer experiments. B-2 B cell migration into ATLOs were determined following transfer of FACS-sorted B-2 B cells (>98% purity). 8 - 10 weeks old male CD45.1 (Ly5.1) mice (n=3-4 mice/sort) were used as donors. Spleens from 3-4 mice were pooled and single cells were prepared. Cells were stained with anti-CD19 APC, anti-CD43 PE, incubated, washed and DAPI$^-$/CD19$^+$/CD43$^-$ B-2 B cells were sorted using a BD FACS Aria at a purity of >98% of total live cells. 30x10^6 cells per mouse were injected into aged *ApoE*$^{-/-}$ or WT mice by tail vein injection. After 36 hours, mice were analysed for migrated Ly5.1$^+$ B-2 B cells in PerC, spleen, RLNs, BM, and aorta.

Laser capture microdissection (LCM) and microarray analysis. LCM and microarray analysis has been done as previously described[3, 6, 7]. Briefly, total aorta microarray analyses were performed on Affymetrix mouse whole genome 430 2.0 microarrays. Laser capture-derived RNA microarray analyses were performed with Affymetrix mouse whole genome 430A 2.0 microarrays. Signal intensities were calculated from the raw data and scaled to an array trimmed mean of 500. All further steps were performed using R and Bioconductor (R Development Core Team; Gentleman RC, *et al*. 2004). Logarithmic signals were normalized across arrays of every analysis using quantile normalization. Data were filtered prior to statistical analysis to remove genes with low expression or without variability between 2 groups: Probe sets were included if at least 2 arrays per group were called present (detection $P \leq 0.05$) and 2 or 3 arrays showed a log signal ≥ log2 (200) conditioned on a foldchange of at least log2 (2.0) between groups. To correct for lamina media RNA contamination in LCM experiments (error caused by nearby media tissue) on adventitia or plaque a correction algorhythm was performed: Up-regulated genes in WT adventitia, *ApoE*$^{-/-}$ adventitia, ATLO or plaque data were obtained as described[6]. Using the filtered data a one-factor variance analysis (ANOVA) or a two-sided T-test for two independent samples was applied and corrected with Benjamini-Hochberg correction for multiple testing.

Serum Antibodies detection by ELISAs. For the detection of total IgM and IgG, microtitre plates (Thermo Scientific, England, United Kingdom) were coated with serially diluted serum from aged ApoE$^{-/-}$ and WT mice in PBS overnight at 4°C. The next day, plates were washed in PBS containing 0.05% tween and subsequently blocked using Animal-Free Blocker (Vector Laboratories, Peterborough, UK) for 1 hour at room temperature. Plates were subsequently washed and incubated with HRP conjugated rat anti-mouse IgM or IgG for 1 hour at room temperature. For the detection of total IgE a Ready-SET-Go Kit was used (Affymetrix, eBioscience). Briefly, plates were coated with 100 µl of anti-mouse IgE monoclonal antibody and incubated at 4°C degrees overnight. The next day, plates were washed in PBS containing 0.05% tween and incubated with 250 µl of blocking buffer at room temperature for 2 hours. After washing, plates were incubated with serial dilutions of serum from aged ApoE$^{-/-}$ and WT mice for 4h at room temperature. The plates were then washed and incubated with 100 µl of biotin conjugated anti-mouse IgE monoclonal antibody at room temperature for 1 hour. After the washing the plates were finally incubated with HRP conjugated Streptavidin for 30 minutes at room temperature. After a final washing step, 100 µl of SureBlueTM TMB microwell peroxidase substrate (KPL, Gaithersburg, MD, USA) was added to each well. To stop the reaction, 50 µl of 10% hydrochloric acid was added to each well. Using the sunrise ELISA reader (Tecan, Mannedorf, Switzerland), plates were read at 450 nm with a reference of 630 nm.

Serum *anti*-malondialdehyde-oxidized-low density lipoprotein (anti-MDA-LDL) antibody detection by ELISA. For the detection of anti-MDA-LDL-specific antibodies, microtitre plates were coated with 100 µl of 12.5 µg/ml MDA-LDL (Nordic Biosite, Propellervagen, Sweden) in PBS overnight at 4° C. Next day, plates were washed, blocked and incubated for 4 hours at room temperature with serially diluted serum from aged *ApoE*$^{-/-}$ and aged WT mice. Plates were washed and incubated with HRP-conjugated goat anti-mouse IgG and IgM antisera for 1 hour at room temperature. After a final washing step, 100 µl of SureBlue^{TM} TMB microwell peroxidase substrate (KPL, Gaithersburg, MD, USA) was added to each well. To stop the reaction, 50 µl of 10% hydrochloric acid was added to each well. Using the Sunrise ELISA reader (Tecan, Mannedorf, Switzerland), plates were read at 450 nm with a reference of 630 nm.

Statistics. Data are expressed as means ± SEM. Data were analysed by two-tailed unpaired Student’s t-test. One-way ANOVA with Bonferroni-Holm correction was done when multiple comparisons were made.

Supplemental References

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