Characterization of age-associated immunity in atherosclerosis

Smit, V.

Citation

Smit, V. (2024, September 12). *Characterization of age-associated immunity in atherosclerosis*. Retrieved from https://hdl.handle.net/1887/4083231

Chapter 4

Sexual dimorphism in atherosclerotic plaques of aged *Ldlr*$^{-/-}$ mice

Virginia Smit[1], Jill de Mol[1], Mireia N. A. Bernabé Kleijn[1], Marie A.C. Depuydt[1], Menno P.J. de Winther[2], Ilze Bot[1], Johan Kuiper[1], and Amanda C. Foks[1]

[1] Leiden Academic Centre for Drug Research, Division of BioTherapeutics, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands.
[2] Amsterdam University Medical Centers – location AMC, University of Amsterdam, Experimental Vascular Biology, Department of Medical Biochemistry, Amsterdam Cardiovascular Sciences, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

Published in Immunity & Ageing, 2024 May 2, doi: 10.1186/s12979-024-00434-3.

ABSTRACT

Background

Atherosclerosis, the main underlying pathology of cardiovascular disease, is a chronic inflammatory disease characterized by lipid accumulation and immune cell responses in the vascular wall, resulting in plaque formation. It is well-known that atherosclerosis prevalence and manifestation vary by sex. However, sexual dimorphism in the immune landscape of atherosclerotic plaques has up to date not been studied. In this study, we investigated sex-specific differences in atherosclerosis development and the immunological landscape of aortas at single-cell level in aged *Ldlr*$^{-/-}$ mice.

Methods

We compared plaque morphology between aged male and female chow diet-fed *Ldlr*$^{-/-}$ mice (22 months old) with histo logical analysis. Using single-cell RNA-sequencing and flow cytometry on CD45^{+} immune cells from aortas of aged *Ldlr*$^{-/-}$ mice, we explored the immune landscape in the atherosclerotic environment in males and females.

Results

We show that plaque volume is comparable in aged male and female mice, and that plaques in aged female mice contain more collagen and cholesterol crystals, but less necrotic core and macrophage content compared to males. We reveal increased immune cell infiltration in female aortas and found that expression of pro-atherogenic markers and inflammatory signaling pathways was enriched in plaque immune cells of female mice. Particularly, female aortas show enhanced activation of B cells (*Egr1*, *Cd83*, *Cd180*), including age-associated B cells, in addition to an increased M1/M2 macrophage ratio, where *Il1b*$^{+}$ M1-like macrophages display a more pro-inflammatory phenotype (*Nlrp3*, *Cxcl2, Mmp9*) compared to males. In contrast, increased numbers of age-associated Gzmk^{+}CD8^{+} T cells, dendritic cells, and *Trem2*$^{+}$ macrophages were observed in male aortas.

Conclusions

Altogether, our findings highlight that sex is a variable that contributes to immunological differences in the atherosclerotic plaque environment in mice and provide valuable insights for further preclinical studies into the impact of sex on the pathophysiology of atherosclerosis.

Keywords: cardiovascular disease, atherosclerosis, aging, sex, immunology, single-cell transcriptomics

INTRODUCTION

Atherosclerosis, a chronic inflammatory disease characterized by lipid accumulation and immune cell infiltration in the arterial wall, is the main underlying pathology of cardiovascular disease (CVD). Although CVD is the leading cause of death in both women and men, accounting for 45% and 39% of all deaths respectively[1,2], sex differences in atherosclerotic CVD prevalence and manifestation have been described. CVD develops about 10 years later in women than in men[3] but women have a poorer prognosis and are more likely to die following an acute cardiovascular event.[4] While acute cardiovascular events in women are mostly caused by stable atherosclerotic plaques that undergo erosion, in men, acute plaque rupture is often the culprit factor.[5] Moreover, women generally have smaller plaque area with decreased necrotic core volume compared to men.[6,7] Incidence of thin-cap fibroatheroma and large calcification area varies by sex, but only when stratified by age, since men younger than 70 years of age showed a higher prevalence of thin-cap fibroatheroma and large calcification, while women older than 70 years showed a higher prevalence.[8] Notably, CVD risk in women is often missed due to the assumption that women are "protected" against CVD at younger age. Combined with the underrepresentation of women in scientific research, these factors contribute to a knowledge gap regarding the pathophysiology of atherosclerotic CVD in women.[9]

Inflammation of the arterial wall is a key driver of atherosclerosis pathogenesis. Evidently, human and mouse studies that mapped the immune landscape of atherosclerotic plaques with single-cell technologies showed a heterogenous leukocyte pool within the plaque, including innate and adaptive immune cells.[10–14] Lymphoid cells, particularly T cells, were highly abundant in human atherosclerotic plaques and plaques of aged *Ldlr*$^{-/-}$ mice.[15] However, sexual dimorphism in the immune landscape of atherosclerotic plaques is seldomly studied. At a transcriptomic level, Hartman and colleagues reported significant sex-specific differences in sex-stratified gene regulatory networks from bulk RNA-sequencing derived from atherosclerotic aortic root tissue.[16] Genes that were more active in women were associated with mesenchymal and endothelial cells, while genes more active in men were associated with the immune system, particularly macrophages. Detailed profiling of plaque-residing immune cells is however lacking. Moreover, only few preclinical studies compared plaque immune cell numbers in the aortic root or arch between sexes, where either no differences were found either between sexes or increased infiltration of T cells in male chow diet-fed *ApoE*$^{-/-}$ mice.[17–20] None of them have taken aging into account, one of the most dominant risk factors of CVD.[21]

To bridge this knowledge gap, we investigated sex-specific differences in the atherosclerotic plaque of aged *Ldlr*$^{-/-}$ mice, a highly translational preclinical atherosclerosis model.[15] We compared plaque morphology between males and females with histological analysis. Using single-cell RNA-sequencing and flow cytometry on CD45$^+$ immune cells from aortas of aged *Ldlr*$^{-/-}$ mice, we explored the immune landscape in the atherosclerotic environment in males and females.

MATERIALS & METHODS

Animals

All animal experiments were approved by the Leiden University Animal Ethics Committee and were performed according to the guidelines of the European Parliament Directive 2010/63/EU of the European Parliament. Male and female *Ldlr*$^{-/-}$ mice on a C57Bl/6J genetic background (3 months or 20 months old at the start of the experiment) were bred and aged in-house and kept under standard laboratory conditions. Young (3 months old) mice were randomized according to weight and basal serum cholesterol levels, and fed a regular chow diet (CD) or a Western diet (WD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK) for 10 weeks. Diet and water were provided *ad libitum*. At the end of experiment, mice were anaesthetized by a subcutaneous injection of a cocktail containing ketamine (100 mg/kg), atropine (0.5 mg/kg), and xylazine (10 mg/kg). Mice were bled by retro-orbital bleeding, and tissues were harvested after *in situ* perfusion with phosphate buffered saline (PBS). One mouse was excluded from the experiment due to presence of tumors.

Histology

Hearts and aortas were embedded in O.C.T. compound (Sakura) and snap-frozen. To determine lesion size, cryosections (10 μm) of the aortic root were stained with Oil-Red-O and hematoxylin (Sigma-Aldrich). To quantify lesion volume, sections were collected from when aortic valves started to appear until a distance of 1.2 mm relative to the root was reached. The average of five sequential sections of the three-valve area of aortic roots, displaying the highest lesion content, was used to compare the vessel occlusion. Collagen content in the lesions was quantified using a Masson's trichrome staining (Sigma-Aldrich). The necrotic core was defined as the acellular, debris-rich lesion area as percentage of total plaque area. Corresponding sections on separate slides were stained for monocyte/macrophage content with a MOMA-2 antibody (1:1000, AbD Serotec) followed by a biotinylated goat anti-rat IgG antibody (1:200, Vector). Secondary antibodies were detected using the Vectastain ABC kit (Vector) and visualized with ImmPACT NovaRED HRP substrate (Vector). We categorized cholesterol crystallization of atherosclerotic lesions in the aortic root on a scale of 0 (no cholesterol crystallization) to 3 (>75% of the lesion area contains crystalline cholesterol). Presence of calcification was manually scored based on morphology. To quantify calcification area, sharp demarcated acellular dark pink to purple areas in the hematoxylin staining of three consecutive sections were divided by total plaque area.[22] Analysis and scoring were performed blinded. Mice with bicuspid aortic valves were excluded from histological analyses (n=3). Pictures were taken with a Mikrocam II

(Besser) linked to a Leica DM6000 Microscope. Stained sections were manually analyzed with ImageJ software.

Aortic CD45$^+$ cell isolation for single-cell RNA-sequencing

Atherosclerotic aortic arches, carefully detached from other surrounding organs, extensively flushed with PBS, and thoroughly cleaned from any residual perivascular adipose tissue , were isolated from aged chow diet-fed male *Ldlr*$^{-/-}$ mice (22 months old; n=23) and enzymatically digested as previously described.[15] Single cell suspensions were stained with Fixable Viability Dye eFluor™ 780 (1:2000, eBioscience) and CD45-PE (1:500, clone 30-F11, Biolegend). After removing doublets, alive CD45$^+$ cells were sorted (Supplementary Figure 1) using a 100μm nozzle in PBS supplemented with 0.04% BSA using a FACS Aria II SORP (BD Biosciences) and immediately processed for single-cell RNA-sequencing (scRNA-seq).

Single-cell library preparation

Aortic CD45$^+$ cell suspensions were loaded on a Chromium Single Cell instrument (10x Genomics) to generate single cell gel bead emulsions (GEMs). ScRNA-seq libraries were prepared using the Single Cell 3' Solution v2 Reagent Kit (10xGenomics). Sequencing was performed on an Illumina HiSeq2500 and the digital expression matrix was generated by de-multiplexing barcode processing and gene UMI (unique molecular index) counting using the Cell Ranger v6.0 pipeline (10x Genomics).

Single-cell data processing, integration, and analysis

The digital expression matrix of aortas isolated from chow diet-fed aged male *Ldlr*$^{-/-}$ mice and of the female *Ldlr*$^{-/-}$ mice, that was recently published[15], were analyzed using the R package Seurat (version 4). Cells were filtered by unique gene count per cell >200 and <6,000 for aged male, and >200 and <7500 for aged female. In addition, a cutoff was set to a maximum of 6%, and 12% mitochondrial gene expression for aged male and aged female samples, respectively. Doublets were identified and removed using the DoubletDecon package. The remaining 5294 (aged male) and 4674 (aged female) cells were log-normalized, integrated using canonical correlation analysis and scaled subjected to principal component analysis (PCA). Based on the elbow plot, Jackstraw functions and separation of marker genes, 16 PCA components were included for cluster detection at a resolution of 0.245, which were subsequently visualized through Uniform Manifold Approximation and Projection (UMAP).

The Seurat function FindAllMarkers was used to find the differentially expressed genes (DEGs) per cluster, which were examined to define the cell clusters. For the high-resolution re-clustering, (*Cd79b*$^+$) B-cell clusters, (*Cd3e*$^+$) T-cell clusters and (*Cd68*$^+$ and *Itgam*$^+$) myeloid clusters were selected and extracted from the main clustering. Thresholds were set to Cd19<0.3, Cd79b<0.3, Cd68<0.3 to exclude non-T-cells from the T-cell clustering, Cd3e<0.3, Cd68<0.3 to exclude non-B-cells from the B-cell clustering, and Cd3e<0.3, Cd19<0.3, Cd79b<0.3 to exclude non-myeloid cells from the myeloid clustering. The variable genes of these selected clusters were then used as input for dimensionality reduction and re-clustering. PCA analysis on rescaled transcripts was performed with the following dimensions and resolutions: T cells (3155 cells), dimensions 9, resolution 0.6; B cells (2746 cells), dimensions 11, resolution 0.25; myeloid cells (1818 cells), dimensions 12, resolution 0.5. Tregs (Foxp3>0.3) and non-Tregs (Foxp3<0.3) were selected from cluster 4 $CD4^+$ T cells (Cd8a<0.3, Cd8b1<0.3, Tcrg-C1<0.3, Cd4>0.4, Kit<0.3). UMAP plots, dot plots, violin plots, volcano plots were generated in R. Enrichment scores of the SenMayo geneset were calculated using the AUCell package.[23,24] Pathway analyses were performed using the Single Cell Pathway Analysis (SCPA) package.[25]

Flow cytometry

Immunostaining was performed as previously described on single cell suspensions derived from murine aortas to characterize immune cells.[15] To block Fc receptors, an unconjugated anti-CD16/32 antibody (clone 2.4G2, BD Bioscience) was used for mouse samples. Living cells were selected using Fixable Viability Dye eFluor™ 780 (1:2000, eBioscience) and different cell populations were defined using anti-mouse fluorochrome-conjugated antibodies (Supplementary Table S1). Antibody staining of transcription factors and cytokines was performed using transcription factor fixation/permeabilization concentrate and diluent solutions and cytofix/permeabilization solutions, respectively (BD Biosciences). Flow cytometry analysis was performed on a Cytoflex S (Beckman Coulter) and the acquired data were analyzed using FlowJo software (version 10.7).

Statistical analysis

Data are expressed as mean ± SEM. Outliers were identified and removed using Grubbs outlier tests (a = 0.05). Significance of data with more than 2 groups was tested using one-way ANOVA test followed by a Tukey multiple comparisons test. Statistical significance of data with 2 groups was tested using an unpaired two-tailed t-test or a nonparametric Mann-Whitney U test. Plotted comparisons are between males and females per age group. P-values of <0.05 were considered significant. Statistical analysis was performed using GraphPad Prism 9.0.

RESULTS

Atherosclerotic lesions of aged female mice are rich in collagen and cholesterol crystals

The *Ldlr*$^{-/-}$ mouse is a widely used experimental model to study atherosclerosis, but we and others have previously shown that severe hypercholesterolemia induced by a Western diet (WD) is needed to promote atherosclerosis in young (3 months) *Ldlr*$^{-/-}$ mice (Figure 1A-D).[15] Notably, as shown in Figure 1D-E, young female *Ldlr*$^{-/-}$ mice are more prone to develop atherosclerosis compared to young male *Ldlr*$^{-/-}$ mice upon WD feeding. However, this WD-accelerated induction of atherosclerosis in young mice diverges from the gradual buildup of atherosclerotic lesions and pathology that comes with aging as manifested in humans. We therefore investigated sex-related differences in atherosclerotic plaque development, composition, and the immune landscape in a more translational setting, using chow diet (CD)-fed aged (22 months) *Ldlr*$^{-/-}$ mice (~200-250 mg/dl serum cholesterol, Figure 1B) of both sexes. As opposed to the large discrepancy of lesion volume between sexes in the young WD-fed mice, lesion volume between aged male and female CD-fed mice did not statistically differ, although vessel occlusion was still slightly elevated in aged CD-fed females (Figure 1E-F). Atherosclerotic lesions of aged female *Ldlr*$^{-/-}$ mice were relatively enriched in collagen content and cholesterol crystals but showed less necrotic core and macrophage content compared to male mice (Figure 1I-J). Calcification incidence and content, which significantly increases in aged atherosclerotic mice[15], were comparable between both sexes (Figure 1K).

Single-cell profiling reveals increased immune cell infiltration in the aorta of aged female mice

Next, we sought out to explore sex differences in the immunological landscape of the aged atherosclerotic plaque and identify unique and conserved gene expression signatures of distinct plaque immune cell types between aged male and female mice. We performed single-cell RNA sequencing analysis on CD45^{+} cells obtained from the atherosclerotic aortic arch of aged female *Ldlr*$^{-/-}$ mice[15], and integrated this with scRNA-seq data of aged male *Ldlr*$^{-/-}$ mice (Figure 2A). To identify distinct immune cell types in the atherosclerotic plaque of males and females, we performed dimensionality reduction and unsupervised cell clustering on a total of 9968 cells (male: 5294 cells, and female: 4674 cells). We observed overlapping alignment of the male and female immune cell clusters (Figure 2B), indicating proper batch effect correction and consistency in cluster definition across sexes. Immune cell clusters were defined by canonical marker genes and visualized in a UMAP plot and proportional abundance barplot (Figure 2C-D and Supplementary Figure S2A-C and Table S2). Proportionally, we observed increased abundance of CD8^{+} T cells in male aortas, while populations of CD4^{+}CD8^{+} double positive (DP) T cells, CD21^{-}CD23^{-} B cells and *Il1b*$^{+}$ macrophages (MF) were increased in female aortas (Figure 2D). We also measured sex-specific changes in major immune cell abundance with flow cytometry and found increased immune cell infiltration in the aortic arches of female mice (Figure 2E). In agreement with the scRNA-seq data, male aortic arches showed increased numbers of CD8^{+} T cells and myeloid cells, whereas female aortic arches contained more CD19^{+} B cells and CD4^{+}CD8^{+} DP T cells (Figure 2E and Supplementary Figure S2D and S3).

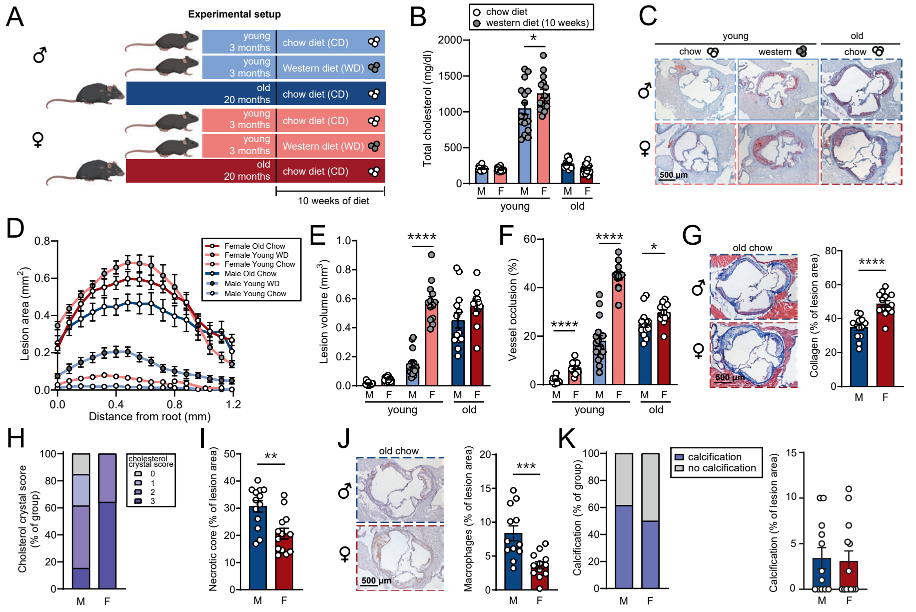


Figure 1. **Plaque size and composition of aged male and female *Ldlr*$^{-/-}$ mice.**
A) Experimental setup: young male (light blue) and young female[15] (light red) *Ldlr*$^{-/-}$ mice were randomized according to weight and basal serum cholesterol levels and fed a chow diet (white circles) or western diet (grey circles) for 10 weeks, and old male (dark blue) and old female[15] (dark red) *Ldlr*$^{-/-}$ were fed a chow diet. B) Total serum cholesterol levels at sacrifice were measured. C) Cross sections of the aortic root were stained for lipid and collagen content. D) Atherosclerotic lesion area over distance, E) lesion volume, and F) vessel occlusion were quantified. G) Collagen content was quantified as percentage of lesion area. H) Cholesterol crystallization in atherosclerotic lesions was categorized on a scale of 0 (no cholesterol crystallization) to 3. I) Necrotic cores and J) macrophage content (MOMA-2) were measured as percentage of lesion area. K) Presence of calcification (purple) or no calcification (grey) was presented as percentage of the group and measured as percentage of lesion area. Data are from n = 12–16 mice per group. Statistical significance was tested by one-way ANOVA. Mean ± S.E.M. plotted. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

To investigate potential sex-specific differences of senescence in the aortic leukocytes of these aged mice, we performed enrichment of the SenMayo senescence gene set.[23] Although B and T cells did not show any sex-specific difference, NK cells and to a lesser extent myeloid cells of female aortas displayed enrichment of senescence (Figure 2F). In line with this, we observed increased expression of senescence-associated secretory phenotype (SASP) genes (e.g. chemokines, *Mmp9*, *Il1b, Tnf*), intracellular (e.g. *Gem*, *Icam1*, *Jun*) and transmembrane senescence-associated genes (e.g. *Cxcr2*, and STAT3 target genes *Tnfrsf1a/b*) in NK cells and myeloid cells of females (Figure 2G). To gain further insight into possible sex differences within the subsets, we next performed reclustering of each major immune cell population (B cells, T cells and myeloid cells).

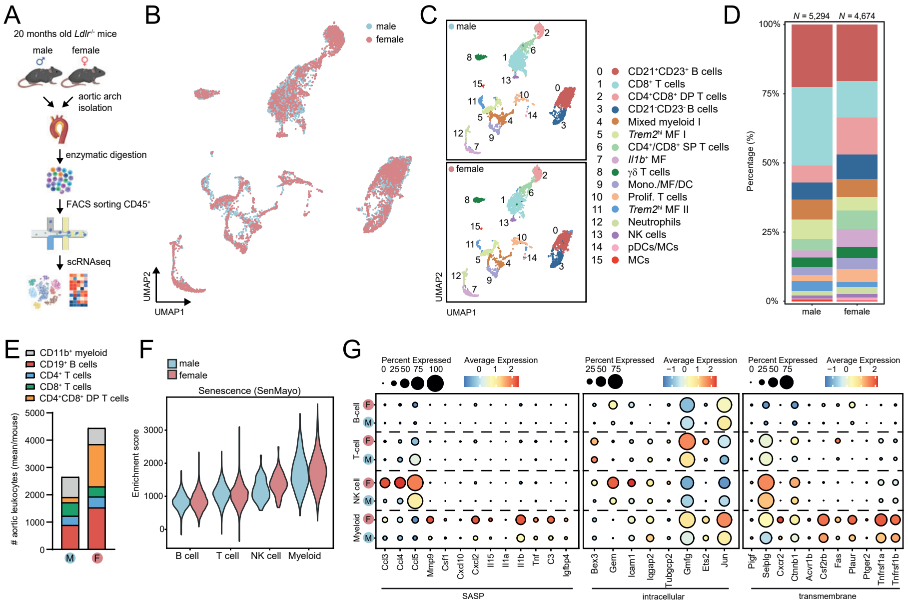


Figure 2. Immune cell landscape of aortas from aged male and female *Ldlr*$^{-/-}$ mice.
A) Workflow of scRNA-seq on aortic CD45$^+$ cells of chow diet-fed 20-months-old male (n = 23) and female (n = 12) *Ldlr*$^{-/-}$ mice. UMAP visualization of clustered aortic leucocytes grouped by (B) sex or (C) immune cell clusters. D) Stacked diagram showing the relative proportions of major immune cell subtypes within CD45$^+$ cells of *Ldlr*$^{-/-}$ aortas measured by scRNA-seq. E) Stacked diagram showing the number of major immune cell types in the aorta of aged male and female *Ldlr*$^{-/-}$, measured as mean per mouse with flow cytometry. F) Violin plot showing the senescence (SenMayo gene set) enrichment score of major immune cell types per sex. G) Average expression of SASP, intracellular and transmembrane genes from the SenMayo gene set in major immune cell types split by sex. DP, double positive; MF, macrophages; SP, single positive; DC, dendritic cell; NK, natural killer; pDC, plasmacytoid dendritic cell; MC, mast cell.

Activated age-associated B cells are enriched in aortas of female mice

Proportionally, B2-like cells (cluster 0; *Ighd*, *Fcer2a*, *Cr2*) comprised the largest B cell cluster in aortas of both sexes (males 66% and females 54%) (Figure 3A, Supplementary Figure S4 and B, and Supplementary Table S3). We further detected B1-like cells and regulatory B cells (cluster 1; *Zbtb32*, *S100a6*, *Cd9*), age-associated B cells (ABCs, cluster 2; *Zbtb20*, *Tbx21*, *Fas*), Ifn-induced B cells (cluster 3; *Ifit2*, *Ifit3*, *Ifi213*), activated B cells enriched for Myc-target genes (cluster 4; *Nme2*, *Mif*), immature B cells (cluster 5; *Cd93*, *Cd24a*), plasma cells (cluster 6; *Sdc1*, *Jchain*, *Prdm1*) and undefined B cells (cluster 7).

Not surprisingly, DEG analysis showed upregulation of X-chromosomal genes (*Xist*, *Tsix*, *Gm6377*) in female B cells, while Y-chromosomal genes *Eif2s3y* and *Ddx3y* were upregulated in male B cells (Figure 3B). Female B cells displayed upregulation of activation-related genes *Cd40*, *Cd80*, *Cd83*, *Cd86*, *Egr1*, *Cd180*[26–30], while B cells in male aortas exhibited high expression of *Ets1*, a transcription factor that negatively controls B cell activation and concomitant antibody-secreting cell function[31], suggesting that B cells in males are less likely to contribute to humoral immunity (Figure 3B and C). Additionally, expression of genes encoding co-inhibitory molecules was relatively higher in females compared to males, where *Havcr1* expression (Tim-1) was particularly high in the ABC cluster. Although hard to detect, both pro- and anti-inflammatory cytokine genes were mostly expressed at higher levels in female B cells (Supplementary Figure S4C). A similar pattern was seen in antigen-processing and presentation-related genes, particularly in ABCs. Strikingly, ABCs were more abundant in atherosclerotic aortic arches of aged female than in aged male *Ldlr*$^{-/-}$ mice, which was confirmed with flow cytometry (386±53 vs. 161±26 cell count, $P<0.01$; Figure 3A and 3D). ABCs in females showed high expression levels of ABC-characteristic marker genes *Tbx21* (T-bet), *Fas*, and particularly *Itgax* (CD11c; Figure 3E). Also, *Itgb2* (encoding CD18 that forms the functional CR4 complex with CD11c) and *Cd72* (encoding a transmembrane molecule that can regulate B cell activation) were upregulated in female ABCs (Figure 3F).[32,33] *Cxcr3*, a chemokine receptor that is likely to be involved in the migration of B cells to the site of inflammation and differentiation into antibody-secreting plasma cells[34,35], is almost exclusively expressed by the ABC cluster, but expression levels were comparable between sexes (Figure 3C).

Certain zinc finger genes (*Zbtb20*, *Zbtb32*) in B cells are associated with plasma cell differentiation[36,37], and expression levels of these genes were elevated in female ABCs (Figure 3G). In line with this, plasma cells characterized by high expression of immunoglobulin-encoding genes and *Ly6c2*[38], were more abundantly present in female aortas (Figure 3A, H and Supplementary Figure S4D). Overall, immunoglobulin-encoding genes were more expressed in B cell clusters of females, of which ABCs showed high expression of *Ighg1* and *Ighg3* compared to other B cell clusters (excluding plasma cells; Figure 3H). Notably, gene expression of *Ctla4* (co-inhibitory molecule), *Slamf9* (upregulated by inflammatory stimulus on B1 cells) and *Itgb7* (involved in homing of B cells) was increased in the female B1/Breg cluster[39–41], suggesting a more inflammatory and activated profile of the B1/Breg cluster in female mice (Figure 3I).

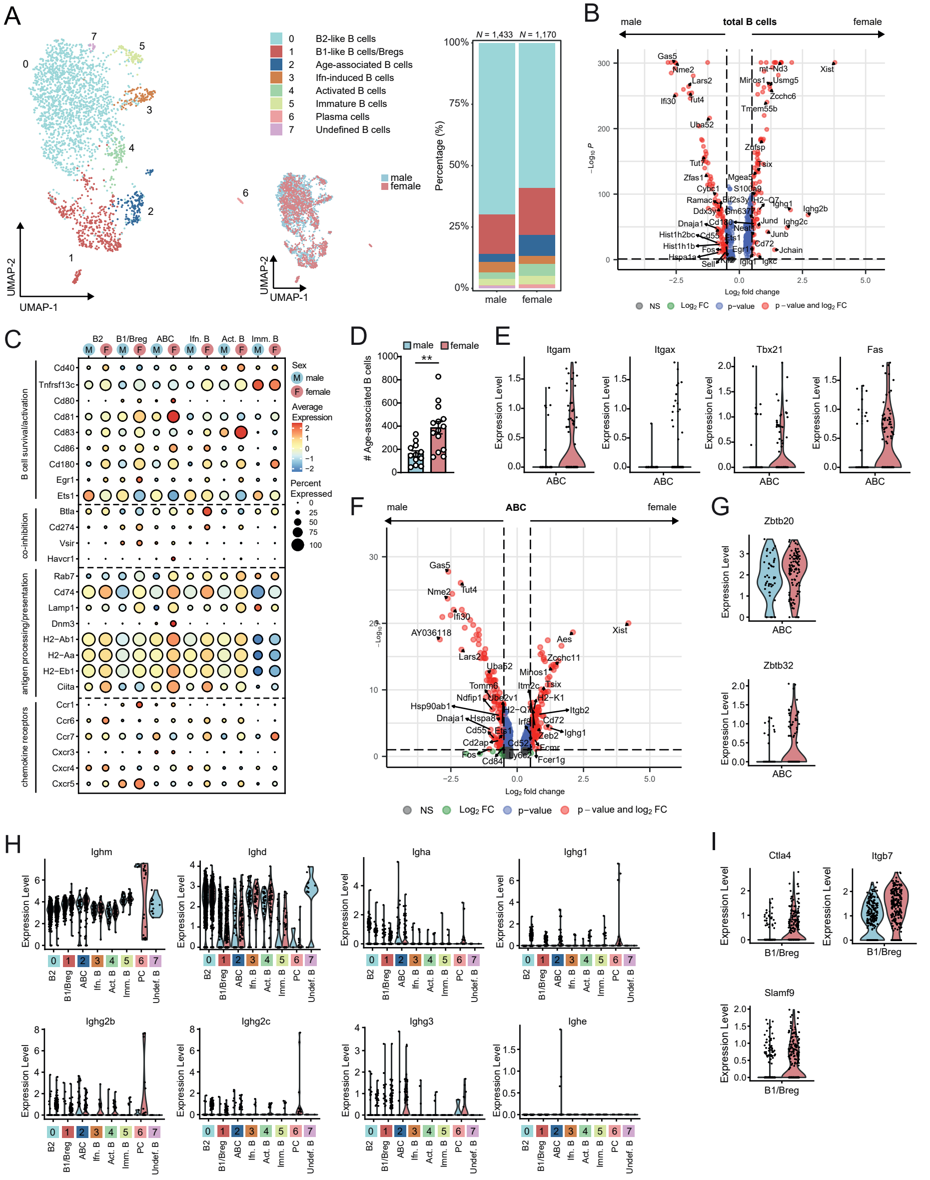


Figure 3. Sex-specific gene signatures of aortic B cells in aged *Ldlr*$^{-/-}$ mice.
A) UMAP plots and stacked diagram of B cell clusters in the aorta of *Ldlr*$^{-/-}$ mice. B) Volcano plot displaying differentially expressed genes of the total B cell subclustering between aged male and female *Ldlr*$^{-/-}$ mice. C) Average expression of biological process-associated genes in B cell clusters split by sex. D) Absolute number of age-associated B cells in the aortas of aged male and female *Ldlr*$^{-/-}$ mice was measured with flow cytometry. E) Sex-specific gene expression level of age-associated B cell-specific markers in cluster 2. F) Volcano plot of cluster 2 displaying differentially expressed genes between male and female. Sex-specific expression level of G) zinc-finger protein genes in cluster 2, H) immunoglobulin isotype genes in all B cell clusters, and I) genes differentially expressed in cluster 1. Flow cytometry data are from n = 12–14 mice per group. Statistical significance was tested by a t-test. Mean ± S.E.M. plotted. **P < 0.01.

Granzyme-expressing effector CD8$^+$ T cells are enriched in atherosclerotic aortas of aged males

The aortic T cell pool of aged *Ldlr*$^{-/-}$ mice contained 3 CD8$^+$ T cell clusters, specifically *Gzmk*$^+$CD8$^+$ T cells (cluster 0; *Gzmk*, *Nkg7*, *Eomes*), *Gzmb*$^+$CD8$^+$ T cells (cluster 2; *Gzmb*, *Klrk1*, *Ly6c2*), and *Sell*$^+$CD8$^+$ T cells (cluster 3; *Sell*, *Klf2*, *Foxp1*; Figure 4A and Supplementary Figure S5A and B and Table S4). CD8$^+$ T cells comprised 63.3% of aortic T cells in males compared to 28.3% of aortic T cells in females (Figure 4A). While CD4$^+$CD8$^+$ double positive (DP) T cells (cluster 1; *Rag1*, *Arpp21*, *Ccr9*) were the largest T cell cluster in the female aortic arches, the proportion of CD4$^+$ T cells (cluster 4; *Tnfrsf4*, *Izumo1r*, *Icos*) did not differ between the sexes. These sex-specific frequencies of main T cell populations were also confirmed with flow cytometry (Figure 4B). Additionally, we identified proliferating T cells (cluster 5; *Mki67*, *Pclaf*, *Nusap1*), *Tox*^{hi} T cells (cluster 6; *Tox*, *Itm2a*, *Nab2*), gd T cells (cluster 7; *Tcrg-C1*, *Serpinb1a*, *Tmem176a/b*) and a cluster of mixed cells (cluster 8; *Malat1*, *Lck*).

Analysis on total T cells showed that compared to females, the male T cell compartment was enriched in the natural killer pathway and cytotoxic/effector-related genes (*Nkg7*, *Ccl5*, *Klrd1*, *Gzmb*, *Gzmk*) and (Figure 4C-D). In contrast, CTLA-4, TCR and interleukin-related pathways (red dots in Figure 4C) were more enriched in female T cells. Moreover, effector molecules (*Prf1*, *Ccl4*) and cytokines (*Ifng, Tnf, Il2, Tgfb1*) were expressed at higher levels in T cells from females than from males, particularly in the *Gzmb*$^+$CD8$^+$ T cells (Figure 4E). *Gzmk*$^+$CD8$^+$ T cells were more abundant in males, as measured by scRNA-seq as well as with flow cytometry (Figure 4A and F) and showed comparable gene expression of effector molecules and cytokines, but increased expression of some exhaustion markers (*Lag3*, *Ctla4*) in females (Figure 4E). In addition, *Ccl5* and the gene encoding its receptor *Ccr5* were expressed on the majority of the *Gzmb*$^+$ and *Gzmk*$^+$CD8$^+$ T cells. Interestingly, genes associated with T cell migration (*Itga1*, *Itga4*) and activation marker *Cd69* were expressed at higher levels in both granzyme-expressing CD8$^+$ T cell clusters of males compared to females.

Cluster 4 mainly consisted of CD4$^+$ T cells, including regulatory *Foxp3*$^+$CD4$^+$ T cells (Treg), but also contained some remainder *Kit*$^+$ mast cells (Supplementary Figure S5B). In female atherosclerotic aortic arches, this cluster was enriched for *Lag3*, *Ctla4*, *Tnfsf8* (CD30L; Figure 4E). Upon division of cells from cluster 4 into *Foxp3*$^+$CD4$^+$ Tregs and *Foxp3*$^-$CD4$^+$ non-Tregs, we found that Tregs in females showed higher expression of *Pdcd1* (PD-1), *Ctla4* and *Tnfrsf4* (OX40) but lower expression of *Havcr2* (TIM-3), while non-Treg CD4$^+$ T cells in females specifically showed higher expression of *Tox*, *Lag3*, and *Tnfsf8* (Figure 4G). This may indicate increased presence of the recently described CD30L$^+$PD-1$^+$CD44$^+$CD4$^+$ senescence-associated T cells[42] in aged aortas of female compared to male mice (Supplementary Figure S5C). Additionally, Tregs in females displayed higher expression of anti-inflammatory cytokine genes

Tgfb1 and *Ebi3* (IL-35), whereas non-Tregs in females showed elevated expression levels of *Tnf*, *Il18*, and *Il21* compared to non-Tregs in males (Figure 4H).

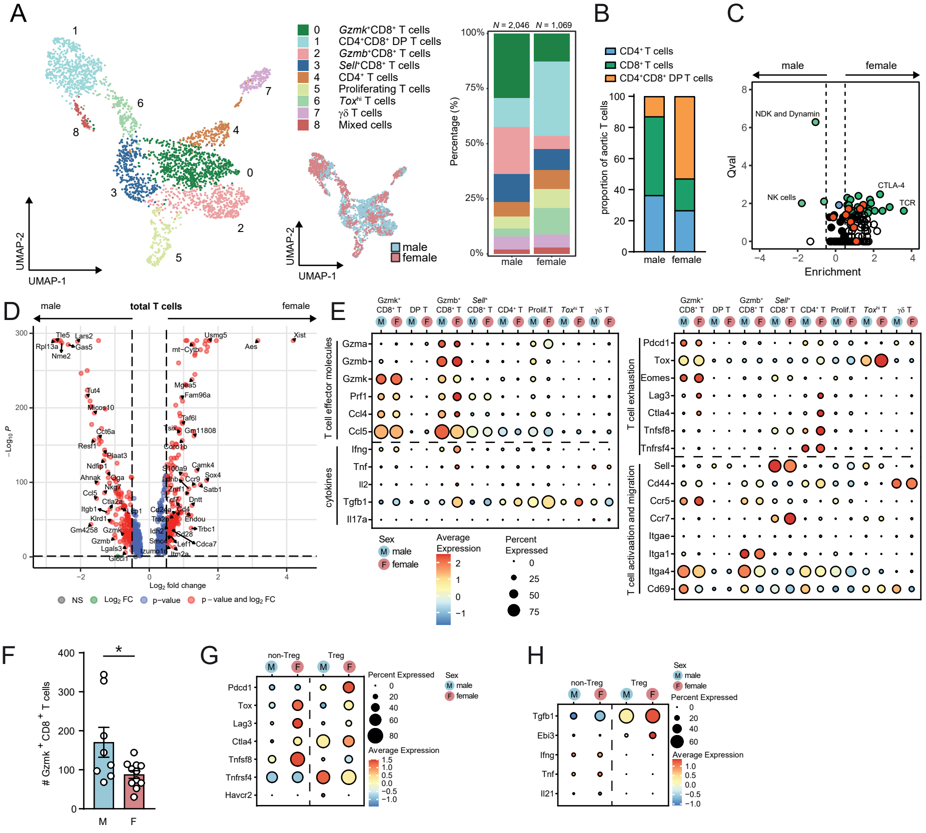


Figure 4. Transcriptomic comparison of T cells in aortas of aged male and female *Ldlr*$^{-/-}$ mice.
A) UMAP plots and stacked diagram of T cell clusters in the aorta of *Ldlr*$^{-/-}$ mice. B) Stacked diagram showing the relative proportions of CD4^{+}, CD8^{+} and CD4^{+}CD8^{+} T cells within aged male and female *Ldlr*$^{-/-}$ aortas, measured by flow cytometry. C) Pathway enrichment of T cells in male and female mice. Green dots: significantly enriched pathways; red dots: interleukin-related pathways; white dots: insignificantly enriched pathways; black dots: insignificantly unenriched pathways. D) Volcano plot displaying differentially expressed genes of the total T cell subclustering between aged male and female *Ldlr*$^{-/-}$ mice. E) Dot plot displaying the sex-specific expression of biological process-associated genes in T cell clusters. F) Absolute number of Gzmk^{+}CD8^{+} T cells in the aortas of aged male and female *Ldlr*$^{-/-}$ mice was measured with flow cytometry. Average gene expression of G) costimulatory and coinhibitory molecules and H) cytokines in Tregs and non-Tregs from CD4^{+} T cells in cluster 4, split by sex.

Female bias towards inflammatory M1-like macrophages in the aorta

The aortic myeloid cell compartment contained M1- and M2-like macrophages, resident macrophages, dendritic cells, monocytes, neutrophils, and mast cells (Figure 5A and Supplementary Figure 6A and B). DEG analysis showed upregulation of *Cxcl2*, *Il1b* and *Ccl3* in aortic myeloid cells of *Ldlr*$^{-/-}$ females, while *Fabp5*, *Apoe*, *Cd5l* and *Spp1* were upregulated in myeloid cells from *Ldlr*$^{-/-}$ males (Figure 5B). *Il1b*$^{+}$ M1-like macrophages (*Il1b*, *Csf3r*, *Cxcr2*) were the most abundant myeloid population in females (~30% in females vs. ~10% in males). Moreover, expression of M1-like specific markers *Nlrp3*, *Cxcl2, Mmp9* was elevated in females, suggesting that these macrophages have an enhanced inflammatory phenotype in the atherosclerotic aorta of females compared to males (Figure 5C). Males, on the other hand, show increased presence of *Trem2*$^+$ myeloid cells including non-foamy M2-like macrophages (*Trem2*, *Mmp12*), foamy macrophages (*Fabp5*, *Cd5l*), resident M2 macrophages (*Lyve1*, *Mrc1*) and mixed *Trem2*$^+$ macrophages (*Emp1*, *Lpl*; Figure 5A and Supplementary Table S5). Foamy macrophages are characteristic of atherosclerotic plaques and are considered to be rather anti-inflammatory than pro-inflammatory[43]. *Cd36*, *Apoe, Fabp5*, and *Cd5l* expression was higher in male Trem2$^+$ foamy macrophages, which mediate lipid-uptake and promote foam cell survival in lesions (Figure 5D and E).[44,45] Additionally, male foamy macrophages showed increased expression of *Tgfb1* and *Gpnmb* (encoding a glycoprotein that is upregulated in foamy macrophages)[46], which have been described to regulate lesion development (Figure 5E). Cluster 7 consists of a mix of foamy and non-foamy macrophages with differential expression of *Lpl* and *Spp1* between males and females (Supplementary Figure S6C).

Conventional dendritic cells (cDCs; *Xcr1*, *Ppt1*) and migratory dendritic cells (mDCs; *Ccr7, Ccl5, Ccl17, Cccl22*) were more abundant in males (Figure 5A). Interestingly, male mDCs showed higher expression of chemokines *Ccl5* and *Ccl22* compared to females (Supplementary Figure S6D). Expression of MHCII-related genes *(H2-Aa*, *H2-Ab1*, *H2-Eb1*, *Ciita*) among the DC clusters was highest in the cDCs, but comparable between sexes (Supplementary Figure S6E). We identified cluster 5 and 10 as neutrophils (*Ly6g*, *Cd177*), of which cluster 10 seemed to be proliferating based on high expression of *Mki67* and histone-encoding genes (Supplementary Table S5). Although neutrophils in females showed elevated expression of pro-inflammatory gene *S100a8*, expression of other neutrophil markers were comparable (Supplementary Figure S5D). Mast cells (MCs) in cluster 9 showed comparable gene expression levels of MC-markers *Fcer1a* and *Cpa3*, while c-Kit (*Kit*) was more expressed in females (Figure 5F). Although MC-specific protease genes encoding chymase (*Cma1*) and tryptase (*Tpsab1*) were barely detected, genes encoding secretory molecules *Ctsg*, *Ccl3*, *Ccl4* and antigen-presentation-associated molecules (*H2-Aa*/*Ab*/*Eb1*, *Cd74*) were increased in female MCs, suggesting a more pro-atherogenic signature of MCs in female compared to male aortas.

Lastly, pathway analysis showed enrichment of the phagocytosis-associated NDK Dynamin pathway (*Nme2*, *Dnm1*), and the migration-associated mCalpain pathway (*Cxcr3*, *Itgb1*, *Tln1*) in myeloid cells of males, while inflammatory signaling, such as interleukin-related (red dots: IL6, IL1R, IL2, IL2RB pathways), MAPK and TNFR pathways, was enriched in female myeloid cells (Figure 5G).

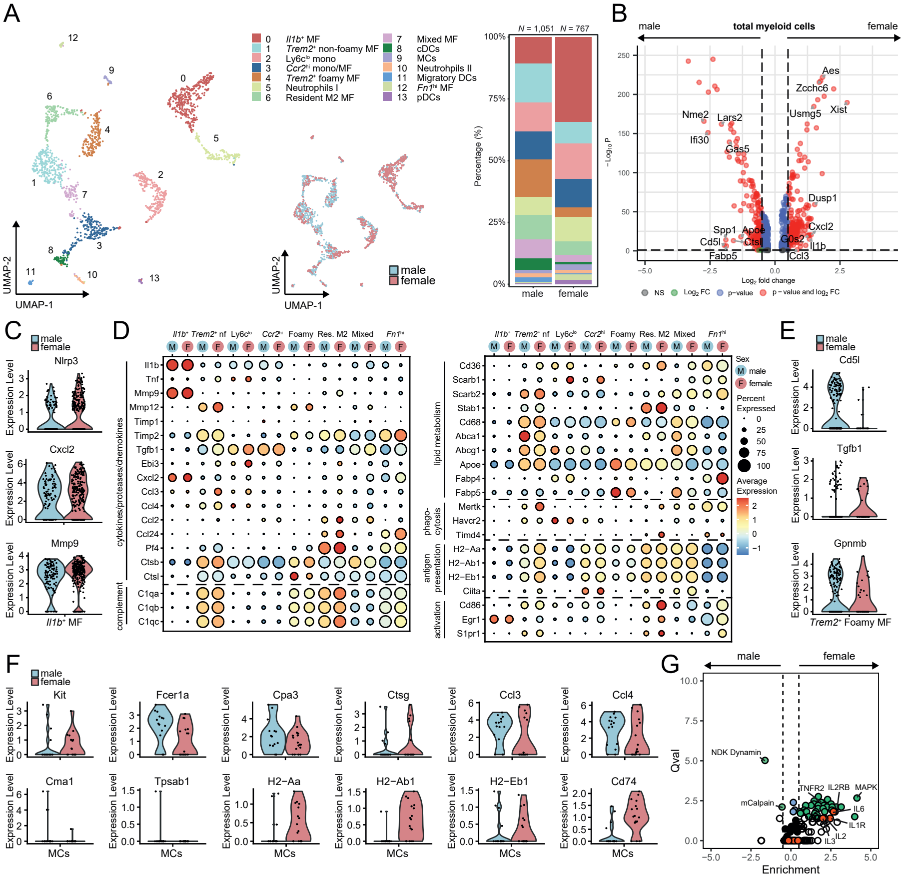


Figure 5. Plaques of aged male and female *Ldlr*$^{-/-}$ mice differ in myeloid cell proportions.
A) UMAP plots and stacked diagram of myeloid cell clusters in the aorta of *Ldlr*$^{-/-}$ mice. B) Volcano plot displaying differentially expressed genes of the total myeloid cell cells between aged male and female *Ldlr*$^{-/-}$ mice. C) Sex-specific expression levels of *Il1b*$^{+}$ M1-associated genes. D) Dot plot displaying the sex-specific expression of biological process-associated genes in macrophage clusters. Sex-specific gene expression of E) *Trem2*$^{+}$ M2-related genes in cluster 4 and F) mast cell associated-markers in cluster 9. D) Pathway enrichment of myeloid cells in male and female mice. Green dots: significantly enriched pathways; red dots: interleukin-related pathways; white dots: insignificantly enriched pathways; black dots: insignificantly unenriched pathways.

DISCUSSION

Advances in single-cell technologies have enabled comprehensive profiling of immune cell populations in the atherosclerotic plaque. While sex is known to impact immune responses and atherosclerotic CVD prevalence and manifestation, studied investigating sex differences in the immune landscape of the plaque are rarely studied. Our study reveals sexual dimorphism in plaque composition and immune cell proportions and gene signatures in aged *Ldlr*$^{-/-}$ mice.

While plaques of young WD-fed male and female *Ldlr*$^{-/-}$ mice show no difference in collagen and necrotic core area[47,48], our data shows that plaques of aged female *Ldlr*$^{-/-}$ mice were relatively more stable compared to plaques of aged male *Ldlr*$^{-/-}$ mice, due to increased collagen content, and less necrotic core area and macrophages. This corroborates with studies in humans, in which female CVD patients display similar signs of plaque stability compared to male.[49] We did however observe a higher influx of immune cells in female compared to male atherosclerotic aortas of aged *Ldlr*$^{-/-}$ mice and found that gene expression of pro-atherogenic markers and inflammatory signaling pathways were more enriched in female aortas. In line with these findings, women are known to elicit stronger innate and adaptive immune responses compared to men, contributing to their increased susceptibility for inflammatory and autoimmune diseases.[50]

We report a striking increase of ABCs in aortas of atherosclerotic female mice, displaying enhanced expression of genes involved in B cell activation and antigen presentation, compared to ABCs in aortas of male atherosclerotic mice, which illustrates sex differences in B cell immunity that could contribute to atherosclerosis. We see enrichment of immunoglobulin genes in ABCs in addition to female-biased expression of *Tbx21* (T-bet) and *Cd72*, which are associated with autoantibody production.[51–53] Furthermore, expression of genes associated with plasma cell differentiation was elevated in female ABCs, suggesting that ABCs in females are more likely to become antibody-secreting cells in atherosclerosis. Accordingly, although only few plasma cells were found in the atherosclerotic aortas, their abundance was increased in females. High frequencies of ABCs in women have previously been linked to the susceptibility of autoimmune diseases, such as systemic lupus erythematosus , rheumatoid arthritis and multiple sclerosis.[34,35,54–57] Interestingly, both the TLR7 gene, crucial for ABC activation, and the gene for CD40L, which is involved in immunoglobulin class switching, are located on the X chromosome.[58] Since almost 15% of X-linked genes escape silencing, this may clarify the increased ABC frequency observed in females compared to males.[59] In addition, estrogen has been shown to stimulate the survival and activation of autoreactive B cells.[60–62] These findings contribute to the increasing body of evidence that atherosclerosis pathology involves autoimmune-like components[63–65], but where these age-associated B cells are precisely located in the atherosclerotic plaque environment remains to be investigated.

The presence of clonally expanded, activated T cells in the plaque of cardiovascular disease patients and mice also supports the concept of atherosclerosis as an inflammatory disease with autoimmune-like features.[66,67] Depuydt et al. showed that clonally expanded CD8$^+$ T cells in the plaque of male CVD patients had increased expression of granzymes (GZMB, GZMK and GZMA) compared to CD8$^+$ T cells in the blood.[67] Although we did not investigate clonality of T cells in this study, we show that the immune landscape in males is more CD8$^+$ T cell-driven, illustrated by the large male-specific increase in *Gzmk*$^+$CD8$^+$ T cells and *Gzmb*$^+$CD8$^+$

T cells. Both CD8$^+$ T cell populations express high levels of *Ccr5* and its ligand *Ccl5*, and in males show more expression of genes associated with activation and migration. Research has demonstrated that both antagonism and deficiency of the CCR5/CCL5-axis attenuate atherosclerosis in advanced stages by decreasing lesion size, promoting plaque stability, and reducing monocyte, macrophage, and T cell infiltration.[68,69] The male-specific increase in *Ccr5*-expressing CD8$^+$ T cells may contribute to the relatively increased macrophage content and reduced collagen that we observed in aged *Ldlr*$^{-/-}$ male mice. Furthermore, we observed elevated gene expression of activation marker *Cd69* across multiple T cell types in males[70], corroborating with high expression of *Cd69* on clonally expanded T cells and a large proportion of CD69$^+$ cells among T cells in plaques of male CVD patients.[67] CD4$^+$CD8$^+$ DP T cells accounted for the majority (~36%) of the T cells in females and have been previously found in murine and human plaques.[13,71] Possibly, these cells escaped from the thymus into the periphery promoted by age-induced thymic involution.[72] However, in contrast to immature CD4$^+$CD8$^+$ thymocytes[73], CD4$^+$CD8$^+$ DP T cells in the plaque show high expression of the cytolytic factor GzmA and memory markers. In line with these findings, CD4$^+$CD8$^+$ DP T cells with cytotoxic or regulatory functions have been described in viral infections[74,75], cancer[76,77] and rheumatoid arthritis.[78] Although some studies show that sex hormones can influence thymic involution and the number of CD4$^+$ CD8$^+$ DP T cells[79–83], Aspinall et al. have shown a sex hormone-independent increase in CD4$^+$ CD8$^+$ DP T cells in females.[84]

We found an increase in CD11b$^+$ myeloid cell numbers, including a larger proportion of conventional and migratory DCs, as well as *Trem2*$^+$ non-foamy M2-like macrophages in aortas of aged males, while the female myeloid compartment largely contained pro-inflammatory *Il1b*$^+$ M1-like macrophages. Elevated expression of foam cell survival genes in the male Trem2$^+$ foamy macrophage cluster may explain the increase in foamy macrophage proportion and larger macrophage area observed in male lesions. In addition, increased expression of *Tgfb1* and *Gpnmb* in this cluster may contribute to regulating plaque development in the male mice.[85] We observed a higher M1/M2 macrophage ratio in atherosclerotic plaques of females than in males. In autoimmune diseases such as SLE and RA, females also show a bias towards M1 polarization, however the underlying mechanism is unclear.[86] Notably, mast cells displayed a more pro-atherogenic gene profile in female compared to male mice as illustrated by increased expression of proteases, chemokines and MHC class II molecules. This is in line with a previous study which showed that mast cells in females store and secrete more inflammatory mediators and are more likely to initiate an immune response.[87]

Although limited conclusive information is available on how hormonal and chromosomal sex differences affect inflammation in atherosclerosis, a variety of studies highlighted the impact of estrogen on leukocyte migration. Estrogen inhibited IL-1-induced upregulation of ICAM-1 and VCAM-1 human endothelial cells[88], and reduced MCP-1 expression in rabbits.[89] These estrogen-related effects might decrease monocyte chemotaxis in atherosclerosis, thereby possibly leading to the lower macrophage content in females compared to males. Furthermore, men with androgen deficiencies have higher IL-1β concentrations than men with normal testosterone levels[90,91], which might contribute to the lower proportion of inflammatory Il1b$^+$ macrophages in males compared to females.

It should however be noted that female mice do not experience a dramatic reduction in estrogen levels that resembles human menopause, but have comparable estrogen levels during aging.[92] These endocrinologic differences between mice and men, in addition to dissimilarities in the aging environment between laboratory mice and humans, are limitations of using preclinical models.[93–96]Apart from biological differences, it is important to keep in mind that our study faced several technical limitations. The limited number of aortic immune cells demands pooling of multiple samples to obtain enough events for single-cell RNA sequencing analysis, which restrained us from performing statistical analysis and may affect differential gene expression profiles. In addition, although we thoroughly cleaned and flushed the aorta, we cannot exclude contamination with a few circulating leukocytes. Nevertheless, our single-cell RNA sequencing analysis and validation at protein level using flow cytometry reveal an elaborate insight into immunological differences between aged atherosclerotic male and female mice, which should be taken into account in preclinical atherosclerosis research.

CONCLUSION

Our data can be utilized as a valuable tool for future preclinical studies, including target validation in experimental mice for intervention studies, but also in refining study design and rationale for choosing the appropriate sex. Although we cannot not directly extrapolate the observed sex differences in the murine atherosclerotic immune landscape to that of humans, we do see similarities between the aged *Ldlr*$^{-/-}$ mouse model and human atherosclerosis pathology, illustrating the relevance of our data set.

Taken together, our study shows that sex is a variable that influences plaque characteristics and immune cell composition at single-cell resolution in aged *Ldlr*$^{-/-}$ mice. These immunological sex differences may contribute to sex-based clinical differences in atherosclerotic CVD and highlight potential future areas of sex-specific immunomodulating therapies to combat atherosclerosis. To investigate this, further research into sex differences of the immune landscape of atherosclerotic plaques of cardiovascular disease patients is needed.

DATA AVAILABILITY

In silico data analysis was performed using custom R scripts (R version 4.1.2) designed especially for this research and/or based on the recommended pipelines from the pre-existing packages listed in the individual segments above. Single-cell RNA sequencing data are available upon personal request from the corresponding author (a.c.foks@lacdr.leidenuniv.nl).

COMPETING INTERESTS

The authors declare that they have no competing interests.

FUNDING

This work was supported by the Dutch Heart Foundation grant number 2018T051 to A.C.F., 2019T067 to I.B., CVON2017-20: Generating the best evidence-based pharmaceutical targets and drugs for atherosclerosis (GENIUS II) to J.K, and the ERA-CVD B-eatATHERO consortium; Dutch Heart Foundation grant number 2019T107 to A.C.F.

AUTHORS’ CONTRIBUTIONS

V.S. and A.C.F. participated in the conceptualization and performed data analysis. V.S., J.d.M. and A.C.F. drafted the manuscript and designed the figures. V.S., J.d.M., M.N.A.B.K., M.A.C.D., I.B., and A.C.F executed the animal experiments. All authors provided feedback on the research, analyses and manuscript.

ACKNOWLEDGEMENTS

We would like to thank Berend from the *Flow Cytometry Core Facility* and Marja from the *Genomics Core Facility* of the AMC for flow sorting and processing the samples for sequencing. Graphical abstract and experimental set-up in Figure 1 and 2 were created with BioRender.com.

SUPPLEMENTARY MATERIAL

Supplementary Figures 1-5
Supplementary Tables 1-5

REFERENCES

1. Timmis A, Vardas P, Townsend N, Torbica A, Katus H, Smedt D De, Gale CP, Maggioni AP, Petersen SE, Huculeci R, Kazakiewicz D, Benito Rubio V de, Ignatiuk B, Raisi-Estabragh Z, Pawlak A, Karagiannidis E, Treskes R, Gaita D, Beltrame JF, McConnachie A, Bardinet I, Graham I, Flather M, Elliott P, Mossialos EA, Weidinger F, Achenbach S. European Society of Cardiology: cardiovascular disease statistics 2021. *European heart journal* 2022;**43**:716–799.
2. Towfighi A, Zheng L, Ovbiagele B. Sex-specific trends in midlife coronary heart disease risk and prevalence. *Archives of internal medicine* 2009;**169**:1762–1766.
3. Tsao CW, Aday AW, Almarzooq ZI, Anderson CAM, Arora P, Avery CL, Baker-Smith CM, Beaton AZ, Boehme AK, Buxton AE, Commodore-Mensah Y, Elkind MSV, Evenson KR, Eze-Nliam C, Fugar S, Generoso G, Heard DG, Hiremath S, Ho JE, Kalani R, Kazi DS, Ko D, Levine DA, Liu J, Ma J, Magnani JW, Michos ED, Mussolino ME, Navaneethan SD, Parikh NI, Poudel R, Rezk-Hanna M, Roth GA, Shah NS, St-Onge MP, Thacker EL, Virani SS, Voeks JH, Wang NY, Wong ND, Wong SS, Yaffe K, Martin SS. Heart Disease and Stroke Statistics-2023 Update: A Report From the American Heart Association. *Circulation* 2023;**147**:E93–E621.
4. Giosia P Di, Passacquale G, Petrarca M, Giorgini P, Marra AM, Ferro A. Gender differences in cardiovascular prophylaxis: Focus on antiplatelet treatment. *Pharmacological research* 2017;**119**:36–47.
5. Vrijenhoek JEP, Ruijter HM Den, Borst GJ De, Kleijn DPV De, Vries JPPM De, Bots ML, Weg SM Van De, Vink A, Moll FL, Pasterkamp G. Sex is associated with the presence of atherosclerotic plaque hemorrhage and modifies the relation between plaque hemorrhage and cardiovascular outcome. *Stroke* 2013;**44**:3318–3323.
6. Lansky AJ, Ng VG, Maehara A, Weisz G, Lerman A, Mintz GS, Bruyne B De, Farhat N, Niess G, Jankovic I, Lazar D, Xu K, Fahy M, Serruys PW, Stone GW. Gender and the extent of coronary atherosclerosis, plaque composition, and clinical outcomes in acute coronary syndromes. *JACC Cardiovascular imaging* 2012;**5**.
7. Halvorsen DS, Johnsen SH, Mathiesen EB, Njølstad I. The association between inflammatory markers and carotid atherosclerosis is sex dependent: the Tromsø Study. *Cerebrovascular diseases (Basel, Switzerland)* 2009;**27**:392–397.
8. Sato T, Minami Y, Asakura K, Katamine M, Kato A, Katsura A, Muramatsu Y, Kakizaki R, Nemoto T, Hashimoto T, Fujiyoshi K, Kameda R, Meguro K, Shimohama T, Ako J. Age- and Gender-Related Differences in Coronary Lesion Plaque Composition on Optical Coherence Tomography. *Circulation journal : official journal of the Japanese Circulation Society* 2020;**84**:463–470.
9. Maas AHEM, Appelman YEA. Gender differences in coronary heart disease. *Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation* 2010;**18**:598–603.
10. Winkels H, Ehinger E, Vassallo M, Buscher K, Dinh HQ, Kobiyama K, Hamers AAJ, Cochain C, Vafadarnejad E, Saliba AE, Zernecke A, Pramod AB, Ghosh AK, Michel NA, Hoppe N, Hilgendorf I, Zirlik A, Hedrick CC, Ley K, Wolf D. Atlas of the immune cell repertoire in mouse atherosclerosis defined by single-cell RNA-sequencing and mass cytometry. *Circulation Research* 2018;**122**:1675–1688.
11. Cochain C, Vafadarnejad E, Arampatzi P, Pelisek J, Winkels H, Ley K, Wolf D, Saliba AE, Zernecke A. Single-Cell RNA-Seq Reveals the Transcriptional Landscape and Heterogeneity of Aortic Macrophages in Murine Atherosclerosis. *Circulation research* 2018;**122**:1661–1674.
12. Kim K, Shim D, Lee JS, Zaitsev K, Williams JW, Kim KW, Jang MY, Jang HS, Yun TJ, Lee SH, Yoon WK, Prat A, Seidah NG, Choi J, Lee SP, Yoon SH, Nam JW, Seong JK, Oh GT, Randolph GJ, Artyomov MN, Cheong C, Choi JH. Transcriptome Analysis Reveals Nonfoamy Rather Than Foamy

Plaque Macrophages Are Proinflammatory in Atherosclerotic Murine Models. *Circulation research* 2018;**123**:1127–1142.

13. Fernandez DM, Rahman AH, Fernandez NF, Chudnovskiy A, Amir E ad D, Amadori L, Khan NS, Wong CK, Shamailova R, Hill CA, Wang Z, Remark R, Li JR, Pina C, Faries C, Awad AJ, Moss N, Bjorkegren JLM, Kim-Schulze S, Gnjatic S, Ma'ayan A, Mocco J, Faries P, Merad M, Giannarelli C. Single-cell immune landscape of human atherosclerotic plaques. *Nature Medicine* 2019;**25**:1576–1588.
14. Depuydt MAC, Prange KHM, Slenders L, Örd T, Elbersen D, Boltjes A, Jager SCA De, Asselbergs FW, Borst GJ De, Aavik E, Lönnberg T, Lutgens E, Glass CK, Ruijter HM Den, Kaikkonen MU, Bot I, Slütter B, Laan SW Van Der, Yla-Herttuala S, Mokry M, Kuiper J, Winther MPJ De, Pasterkamp G. Microanatomy of the Human Atherosclerotic Plaque by Single-Cell Transcriptomics. *Circ Res* 2020;**127**:1437–1455.
15. Smit V, Mol J de, Schaftenaar FH, Depuydt MAC, Postel RJ, Smeets D, Verheijen FWM, Bogers L, Duijn J van, Verwilligen RAF, Grievink HW, Bernabé Kleijn MNA, Ingen E van, Jong MJM de, Goncalves L, Peeters JAHM, Smeets HJ, Wezel A, Polansky JK, Winther MPJ de, Binder CJ, Tsiantoulas D, Bot I, Kuiper J, Foks AC. Single-cell profiling reveals age-associated immunity in atherosclerosis. *Cardiovascular Research* 2023.
16. Hartman RJG, Owsiany K, Ma L, Koplev S, Hao K, Slenders L, Civelek M, Mokry M, Kovacic JC, Pasterkamp G, Owens G, Björkegren JLM, Ruijter HM Den. Sex-Stratified Gene Regulatory Networks Reveal Female Key Driver Genes of Atherosclerosis Involved in Smooth Muscle Cell Phenotype Switching. *Circulation* 2021;**143**:713–726.
17. Elizabeth Moss M, Lu Q, Iyer SL, Engelbertsen D, Marzolla V, Caprio M, Lichtman AH, Jaffe IZ. Endothelial Mineralocorticoid Receptors Contribute to Vascular Inflammation in Atherosclerosis in a Sex-Specific Manner. *Arteriosclerosis, thrombosis, and vascular biology* 2019;**39**:1588–1601.
18. Engel D, Beckers L, Wijnands E, Seijkens T, Lievens D, Drechsler M, Gerdes N, Soehnlein O, Mat †, Daemen JAP, Stan R V, Biessen EAL, Lutgens E. Caveolin-1 deficiency decreases atherosclerosis by hampering leukocyte influx into the arterial wall and generating a regulatory T-cell response. *The FASEB Journal* 2011;**25**:3838–3848.
19. Hernández-Vargas P, Ortiz-Muñoz G, López-Franco O, Suzuki Y, Gallego-Delgado J, Sanjuán G, Lázaro A, López-Parra V, Ortega L, Egido J, Gómez-Guerrero C. Fcγ Receptor Deficiency Confers Protection Against Atherosclerosis in Apolipoprotein E Knockout Mice. *Circulation Research* 2006;**99**:1188–1196.
20. Smith DD, Tan X, Tawfik O, Milne G, Stechschulte DJ, Dileepan KN. Increased Aortic Atherosclerotic Plaque Development In Female Apolipoprotein E-null Mice Is Associated With Elevated Thromboxane A2 And Decreased Prostacyclin Production. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* 2010;**61**:309.
21. Rodgers JL, Jones J, Bolleddu SI, Vanthenapalli S, Rodgers LE, Shah K, Karia K, Panguluri SK. Cardiovascular Risks Associated with Gender and Aging. *Journal of Cardiovascular Development and Disease* 2019;**6**:19.
22. Vos A, Jong PA de, Verdoorn D, Mali WPTM, Bleys RLAW, Vink A. Histopathological characterization of intimal lesions and arterial wall calcification in the arteries of the leg of elderly cadavers. *Clinical Anatomy (New York, N.y)* 2021;**34**:835.
23. Saul D, Kosinsky RL, Atkinson EJ, Doolittle ML, Zhang X, LeBrasseur NK, Pignolo RJ, Robbins PD, Niedernhofer LJ, Ikeno Y, Jurk D, Passos JF, Hickson LTJ, Xue A, Monroe DG, Tchkonia T, Kirkland JL, Farr JN, Khosla S. A new gene set identifies senescent cells and predicts senescence-associated pathways across tissues. *Nature communications* 2022;**13**.

24. Aibar S, González-Blas CB, Moerman T, Huynh-Thu VA, Imrichova H, Hulselmans G, Rambow F, Marine JC, Geurts P, Aerts J, Oord J Van Den, Atak ZK, Wouters J, Aerts S. SCENIC: single-cell regulatory network inference and clustering. *Nature methods* 2017;**14**:1083–1086.
25. Bibby JA, Agarwal D, Freiwald T, Kunz N, Merle NS, West EE, Singh P, Larochelle A, Chinian F, Mukherjee S, Afzali B, Kemper C, Zhang NR. Systematic single-cell pathway analysis to characterize early T cell activation. *Cell reports* 2022;**41**.
26. Elgueta R, Benson MJ, Vries VC De, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunological Reviews*.
27. Krzyzak L, Seitz C, Urbat A, Hutzler S, Ostalecki C, Gläsner J, Hiergeist A, Gessner A, Winkler TH, Steinkasserer A, Nitschke L. CD83 Modulates B Cell Activation and Germinal Center Responses. *Journal of immunology (Baltimore, Md : 1950)* 2016;**196**:3581–3594.
28. Axelsson S, Magnuson A, Lange A, Alshamari A, Hörnquist EH, Hultgren O. A combination of the activation marker CD86 and the immune checkpoint marker B and T lymphocyte attenuator (BTLA) indicates a putative permissive activation state of B cell subtypes in healthy blood donors independent of age and sex. *BMC immunology* 2020;**21**.
29. Gururajan M, Simmons A, Dasu T, Spear BT, Calulot C, Robertson DA, Wiest DL, Monroe JG, Bondada S. Early growth response genes regulate B cell development, proliferation, and immune response. *Journal of Immunology (Baltimore, Md : 1950)* 2008;**181**:4590–4602.
30. Chaplin JW, Kasahara S, Clark EA, Ledbetter JA. Anti-CD180 (RP105) activates B cells to rapidly produce polyclonal Ig via a T cell and MyD88-independent pathway. *Journal of Immunology (Baltimore, Md : 1950)* 2011;**187**:4199.
31. Sunshine A, Goich D, Stith A, Sortino K, Dalton J, Metcalfe S, Svensson EC, Garrett-Sinha LA. Ets1 Controls the Development of B Cell Autoimmune Responses in a Cell-Intrinsic Manner. *Immunohorizons* 2019;**3**:331–340.
32. Schittenhelm L, Hilkens CM, Morrison VL. β2 Integrins As Regulators of Dendritic Cell, Monocyte, and Macrophage Function. *Frontiers in immunology* 2017;**8**.
33. Wu HJ, Bondada S. CD72, a coreceptor with both positive and negative effects on B lymphocyte development and function. *Journal of clinical immunology* 2009;**29**:12–21.
34. Mouat IC, Goldberg E, Horwitz MS. Age-associated B cells in autoimmune diseases. *Cellular and molecular life sciences : CMLS* 2022;**79**.
35. Langelaar J van, Rijvers L, Janssen M, Wierenga-Wolf AF, Melief MJ, Siepman TA, Vries HE de, Unger PPA, Ham SM van, Hintzen RQ, Luijn MM van. Induction of brain-infiltrating T-bet–expressing B cells in multiple sclerosis. *Ann Neurol* 2019;**86**:264–278.
36. Yoon HS, Scharer CD, Majumder P, Davis CW, Butler R, Zinzow-Kramer W, Skountzou I, Koutsonanos DG, Ahmed R, Boss JM. ZBTB32 is an early repressor of the CIITA and MHC class II gene expression during B cell differentiation to plasma cells. *Journal of immunology (Baltimore, Md : 1950)* 2012;**189**:2393–2403.
37. Chevrier S, Emslie D, Shi W, Kratina T, Wellard C, Karnowski A, Erikci E, Smyth GK, Chowdhury K, Tarlinton D, Corcoran LM. The BTB-ZF transcription factor Zbtb20 is driven by Irf4 to promote plasma cell differentiation and longevity. *The Journal of experimental medicine* 2014;**211**:827–840.
38. Wrammert J, Källberg E, Agace WW, Leanderson T. Ly6C expression differentiates plasma cells from other B cell subsets in mice
39. Yang Y, Li X, Ma Z, Wang C, Yang Q, Byrne-Steele M, Hong R, Min Q, Zhou G, Cheng Y, Qin G, Youngyunpipatkul J V., Wing JB, Sakaguchi S, Toonstra C, Wang LX, Vilches-Moure JG, Wang D, Snyder MP, Wang JY, Han J, Herzenberg LA. CTLA-4 expression by B-1a B cells is essential for immune tolerance. *Nature communications* 2021;**12**.

40. Wilson TJ, Clare S, Mikulin J, Johnson CM, Harcourt K, Lyons PA, Dougan G, Smith KGC. Signalling lymphocyte activation molecule family member 9 is found on select subsets of antigen-presenting cells and promotes resistance to Salmonella infection. *Immunology* 2020;**159**:393.
41. Gorfu G, Rivera-Nieves J, Hoang S, Abbott DW, Arbenz-Smith K, Azar DW, Pizarro TT, Cominelli F, McDuffie M, Ley K. Beta7 integrin deficiency suppresses B cell homing and attenuates chronic ileitis in SAMP1/YitFc mice. *Journal of immunology (Baltimore, Md : 1950)* 2010;**185**:5561–5568.
42. Sato Y, Oguchi A, Fukushima Y, Masuda K, Toriu N, Taniguchi K, Yoshikawa T, Cui X, Kondo M, Hosoi T, Komidori S, Shimizu Y, Fujita H, Jiang L, Kong Y, Yamanashi T, Seita J, Yamamoto T, Toyokuni S, Hamazaki Y, Hattori M, Yoshikai Y, Boor P, Floege J, Kawamoto H, Murakawa Y, Minato N, Yanagita M. CD153/CD30 signaling promotes age-dependent tertiary lymphoid tissue expansion and kidney injury. *The Journal of clinical investigation* 2022;**132**.
43. Kim K, Shim D, Lee JS, Zaitsev K, Williams JW, Kim K-W, Jang M-Y, Seok Jang H, Yun TJ, Lee SH, Yoon WK, Prat A, Seidah NG, Choi J, Lee S-P, Yoon S-H, Nam JW, Seong JK, Oh GT, Randolph GJ, Artyomov MN, Cheong C, Choi J-H. Transcriptome Analysis Reveals Nonfoamy Rather Than Foamy Plaque Macrophages Are Proinflammatory in Atherosclerotic Murine Models. *Circ Res* 2018;**123**:1127–1142.
44. Arai S, Shelton JM, Chen M, Bradley MN, Castrillo A, Bookout AL, Mak PA, Edwards PA, Mangelsdorf DJ, Tontonoz P, Miyazaki T. A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development. *Cell metabolism* 2005;**1**:201–213.
45. Amézaga N, Sanjurjo L, Julve J, Aran G, Pérez-Cabezas B, Bastos-Amador P, Armengol C, Vilella R, Escolà-Gil JC, Blanco-Vaca F, Borràs FE, Valledor AF, Sarrias M-R. Human scavenger protein AIM increases foam cell formation and CD36-mediated oxLDL uptake. *Journal of leukocyte biology* 2014;**95**:509–520.
46. Nickl B, Qadri F, Bader M. Role of Gpnmb in atherosclerosis of female mice. *Biochemical and biophysical research communications* 2022;**621**:20–24.
47. Petrovan RJ, Kaplan CD, Reisfeld RA, Curtiss LK. DNA vaccination against VEGF receptor 2 reduces atherosclerosis in LDL receptor-deficient mice. *Arteriosclerosis, thrombosis, and vascular biology* 2007;**27**:1095–1100.
48. Sala F, Aranda JF, Rotllan N, Ramírez CM, Aryal B, Elia L, Condorelli G, Catapano AL, Fernández-Hernando C, Norata GD. MiR-143/145 deficiency attenuates the progression of atherosclerosis in Ldlr-/-mice. *Thrombosis and Haemostasis* 2014;**112**:796–802.
49. Man JJ, Beckman JA, Jaffe IZ. Sex as a Biological Variable in Atherosclerosis. *Circulation research* 2020;**126**:1297–1319.
50. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* 2016;**16**:626–638.
51. Shen Y, Ma Y, Xie J, Lin L, Shi Y, Li X, Shen P, Pan X, Ren H. A regulatory role for CD72 expression on B cells and increased soluble CD72 in primary Sjogren's syndrome. *BMC Immunology* 2020;**21**:1–6.
52. Peng SL, Szabo SJ, Glimcher LH. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proceedings of the National Academy of Sciences of the United States of America* 2002;**99**:5545–5550.
53. Rubtsova K, Rubtsov A V., Thurman JM, Mennona JM, Kappler JW, Marrack P. B cells expressing the transcription factor T-bet drive lupus-like autoimmunity. *Journal of Clinical Investigation* 2017;**127**:1392–1404.
54. Ricker E, Manni M, Flores-Castro D, Jenkins D, Gupta S, Rivera-Correa J, Meng W, Rosenfeld AM, Pannellini T, Bachu M, Chinenov Y, Sculco PK, Jessberger R, Prak ETL, Pernis AB. Altered function and differentiation of age-associated B cells contribute to the female bias in lupus mice. *Nature communications* 2021;**12**.

55. Rubtsov A V., Rubtsova K, Fischer A, Meehan RT, Gillis JZ, Kappler JW, Marrack P. Toll-like receptor 7 (TLR7)-driven accumulation of a novel CD11c+ B-cell population is important for the development of autoimmunity. *Blood* 2011;**118**:1305–1315.
56. Wang S, Wang J, Kumar V, Karnell JL, Naiman B, Gross PS, Rahman S, Zerrouki K, Hanna R, Morehouse C, Holoweckyj N, Liu H, Casey K, Smith M, Parker M, White N, Riggs J, Ward B, Bhat G, Rajan B, Grady R, Groves C, Manna Z, Goldbach-Mansky R, Hasni S, Siegel R, Sanjuan M, Streicher K, Cancro MP, Kolbeck R, Ettinger R. IL-21 drives expansion and plasma cell differentiation of autoreactive CD11chiT-bet+ B cells in SLE. *Nature Communications* 2018;**9**:1–14.
57. Qin Y, Cai ML, Jin HZ, Huang W, Zhu C, Bozec A, Huang J, Chen Z. Age-associated B cells contribute to the pathogenesis of rheumatoid arthritis by inducing activation of fibroblast-like synoviocytes via TNF-α-mediated ERK1/2 and JAK-STAT1 pathways. *Annals of the rheumatic diseases* 2022;**81**:1504–1514.
58. Sarmiento L, Svensson J, Barchetta I, Giwercman A, Cilio CM. Copy number of the X-linked genes TLR7 and CD40L influences innate and adaptive immune responses. *Scandinavian journal of immunology* 2019;**90**.
59. Pinheiro I, Dejager L, Libert C. X-chromosome-located microRNAs in immunity: might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. *BioEssays : news and reviews in molecular, cellular and developmental biology* 2011;**33**:791–802.
60. Jeganathan V, Peeva E, Diamond B. Hormonal milieu at time of B cell activation controls duration of autoantibody response. *Journal of autoimmunity* 2014;**53**:46–54.
61. Grimaldi CM, Jeganathan V, Diamond B. Hormonal regulation of B cell development: 17 beta-estradiol impairs negative selection of high-affinity DNA-reactive B cells at more than one developmental checkpoint. *Journal of immunology (Baltimore, Md : 1950)* 2006;**176**:2703–2710.
62. Grimaldi CM, Michael DJ, Diamond B. Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus. *Journal of immunology (Baltimore, Md : 1950)* 2001;**167**:1886–1890.
63. Lorenzo C, Delgado P, Busse CE, Sanz-Bravo A, Martos-Folgado I, Bonzon-Kulichenko E, Ferrarini A, Gonzalez-Valdes IB, Mur SM, Roldán-Montero R, Martinez-Lopez D, Martin-Ventura JL, Vázquez J, Wardemann H, Ramiro AR. ALDH4A1 is an atherosclerosis auto-antigen targeted by protective antibodies. *Nature* 2021;**589**.
64. Palinski W, Hörkkö S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK, Witztum JL. Cloning of Monoclonal Autoantibodies to Epitopes of Oxidized Lipoproteins from Apolipoprotein E-deficient Mice Demonstration of Epitopes of Oxidized Low Density Lipoprotein in Human Plasma modified lipo-proteins • autoantibodies • atherosclerosis • immune system. *J. Clin. Invest.*
65. Greco TP, Conti-Kelly AM, Anthony JR, Greco T, Doyle R, Boisen M, Kojima K, Matsuura E, Lopez LR. Oxidized-LDL/beta(2)-glycoprotein I complexes are associated with disease severity and increased risk for adverse outcomes in patients with acute coronary syndromes. *American journal of clinical pathology* 2010;**133**:737–743.
66. Wang Z, Zhang X, Lu S, Zhang C, Ma Z, Su R, Li Y, Sun T, Li Y, Hong M, Deng X, Rafiee Monjezi M, Hristov M, Steffens S, Santovito D, Dornmair K, Ley K, Weber C, Mohanta SK, Habenicht AJR, Yin C. Pairing of single-cell RNA analysis and T cell antigen receptor profiling indicates breakdown of T cell tolerance checkpoints in atherosclerosis. *Nature Cardiovascular Research 2023 2:3* 2023;**2**:290–306.
67. Depuydt MAC, Schaftenaar FH, Prange KHM, Boltjes A, Hemme E, Delfos L, Mol J de, Jong MJM de, Bernabé Kleijn MNA, Peeters JAHM, Goncalves L, Wezel A, Smeets HJ, Borst GJ de, Foks AC, Pasterkamp G, Winther MPJ de, Kuiper J, Bot I, Slütter B. Single-cell T cell receptor sequencing of

paired human atherosclerotic plaques and blood reveals autoimmune-like features of expanded effector T cells. *Nature Cardiovascular Research 2023 2:2* 2023;**2**:112–125.

68. Braunersreuther V, Zernecke A, Arnaud C, Liehn EA, Steffens S, Shagdarsuren E, Bidzhekov K, Burger F, Pelli G, Luckow B, Mach F, Weber C. Ccr5 but not Ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. *Arteriosclerosis, thrombosis, and vascular biology* 2007;**27**:373–379.
69. Veillard NR, Kwak B, Pelli G, Mulhaupt F, James RW, Proudfoot AEI, Mach F. Antagonism of RANTES Receptors Reduces Atherosclerotic Plaque Formation in Mice. 2004.
70. Cibrián D, Sánchez-Madrid F. CD69: from activation marker to metabolic gatekeeper. *European journal of immunology* 2017;**47**:946–953.
71. Zernecke A, Winkels H, Cochain C, Williams JW, Wolf D, Soehnlein O, Robbins CS, Monaco C, Park I, McNamara CA, Binder CJ, Cybulsky MI, Scipione CA, Hedrick CC, Galkina E V., Kyaw T, Ghosheh Y, Dinh HQ, Ley K. Meta-Analysis of Leukocyte Diversity in Atherosclerotic Mouse Aortas. *Circulation research* 2020;**127**:402–426.
72. Lee W-W, Nam K-H, Terao K, Akari H, Yoshikawa Y. Age-related increase of peripheral CD4+ CD8+ double-positive T lymphocytes in cynomolgus monkeys: longitudinal study in relation to thymic involution. *Immunology* 2003;**109**:217–225.
73. Ebnet K, Levelt CN, Tran TT, Eichmann K, Simon MM. Transcription of granzyme A and B genes is differentially regulated during lymphoid ontogeny. *Journal of Experimental Medicine* 1995;**181**:755–763.
74. Kitchen SG, Whitmire JK, Jones NR, Galic Z, Kitchen CMR, Ahmed R, Zack JA. The CD4 molecule on CD8+ T lymphocytes directly enhances the immune response to viral and cellular antigens. *Proceedings of the National Academy of Sciences of the United States of America* 2005;**102**:3794–3799.
75. Kitchen SG, Jones NR, LaForge S, Whitmire JK, Vu BA, Galic Z, Brooks DG, Brown SJ, Kitchen CMR, Zack JA. CD4 on CD8+ T cells directly enhances effector function and is a target for HIV infection. *Proceedings of the National Academy of Sciences of the United States of America* 2004;**101**:8727–8732.
76. Desfrançois J, Moreau-Aubry A, Vignard V, Godet Y, Khammari A, Dréno B, Jotereau F, Gervois N. Double Positive CD4CD8 αβ T Cells: A New Tumor-Reactive Population in Human Melanomas. Lowenstein PR, ed. *PLoS ONE* 2010;**5**:e8437.
77. Rahemtullah A, Reichard KK, Preffer FI, Harris NL, Hasserjian RP. A Double-Positive CD4+CD8+ T-Cell Population Is Commonly Found in Nodular Lymphocyte Predominant Hodgkin Lymphoma. *Am J Clin Pathol* 2006;**126**:805.
78. Quandt D, Rothe K, Scholz R, Baerwald CW, Wagner U. Peripheral CD4CD8 Double Positive T Cells with a Distinct Helper Cytokine Profile Are Increased in Rheumatoid Arthritis. Frey O, ed. *PLoS ONE* 2014;**9**:e93293.
79. Zoller AL, Kersh GJ. Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of beta-selected thymocytes. *Journal of immunology (Baltimore, Md : 1950)* 2006;**176**:7371–7378.
80. Okasha SA, Ryu S, Do Y, McKallip RJ, Nagarkatti M, Nagarkatti PS. Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus. *Toxicology* 2001;**163**:49–62.
81. Bernardi AI, Andersson A, Stubelius A, Grahnemo L, Carlsten H, Islander U. Selective estrogen receptor modulators in T cell development and T cell dependent inflammation. *Immunobiology* 2015;**220**:1122–1128.
82. Erlandsson MC, Ohlsson C, Gustafsson JÅ, Carlsten H. Role of oestrogen receptors alpha and beta in immune organ development and in oestrogen-mediated effects on thymus. *Immunology* 2001;**103**:17–25.
83. Guevara Patiño JA, Marino MW, Ivanov VN, Nikolich-ˇ Zugich J. Sex steroids induce apoptosis of CD8 + CD4 + double-positive thymocytes via TNF-alpha. *European heart journal* **30**:2586–2592.

84. Aspinall R, Andrew D, London R, 9nh S. Gender-Related Differences in the Rates of Age Associated Thymic Atrophy. *Developmental Immunology* 2001;**8**:95–106.

85. Nickl B, Qadri F, Bader M. Role of Gpnmb in atherosclerosis of female mice. *Biochem Biophys Res Commun* 2022;**621**:20–24.

86. Funes SC, Rios M, Escobar-Vera J, Kalergis AM. Implications of macrophage polarization in autoimmunity. *Immunology* 2018;**154**:186–195.

87. Mackey E, Ayyadurai S, Pohl CS, D'Costa S, Li Y, Moeser AJ. Sexual dimorphism in the mast cell transcriptome and the pathophysiological responses to immunological and psychological stress. *Biology of sex differences* 2016;**7**:1–19.

88. Caulin-Glaser T, Watson CA, Pardi R, Bender JR. Effects of 17beta-estradiol on cytokine-induced endothelial cell adhesion molecule expression. *The Journal of clinical investigation* 1996;**98**:36–42.

89. Pervin S, Singh R, Rosenfeld ME, Navab M, Chaudhuri G, Nathan L. Estradiol suppresses MCP-1 expression In vivo : implications for atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* 1998;**18**:1575–1582.

90. Mohamad NV, Wong SK, Wan Hasan WN, Jolly JJ, Nur-Farhana MF, Ima-Nirwana S, Chin KY. The relationship between circulating testosterone and inflammatory cytokines in men. *The Aging Male* 2019;**22**:129–140.

91. Chin KY, Ima-Nirwana S. The Effects of Testosterone Deficiency and Its Replacement on Inflammatory Markers in Rats: A Pilot Study. *International Journal of Endocrinology and Metabolism 2017 15:1* 2017;**15**:43053.

92. Dubal DB, Broestl L, Worden K. Sex and gonadal hormones in mouse models of Alzheimer's disease: what is relevant to the human condition? *Biology of Sex Differences* 2012;**3**:24.

93. Huggins MA, Jameson SC, Hamilton SE. Embracing microbial exposure in mouse research. *Journal of leukocyte biology* 2019;**105**:73.

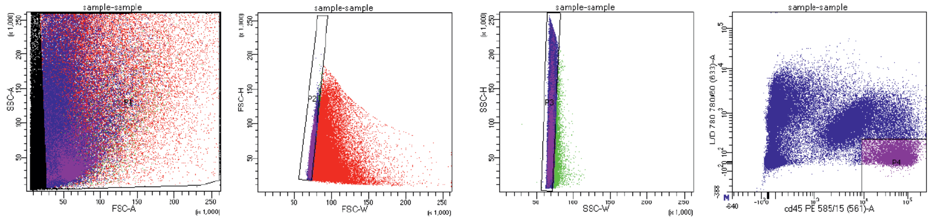
94. Li Y, Baldridge MT. Modelling human immune responses using microbial exposures in rodents. *Nature Microbiology 2023 8:3* 2023;**8**:363–366.

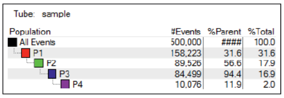
95. Tao L, Reese TA. Making Mouse Models That Reflect Human Immune Responses. *Trends in Immunology* 2017;**38**:181–193.

96. Sanders AE, Arnesen H, Shepherd FK, Putri DS, Fiege JK, Pierson MJ, Roach SN, Carlsen H, Masopust D, Boysen P, Langlois RA. Comparison of mouse models of microbial experience reveals differences in microbial diversity and response to vaccination. *mSphere* 2024;**9**.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURES



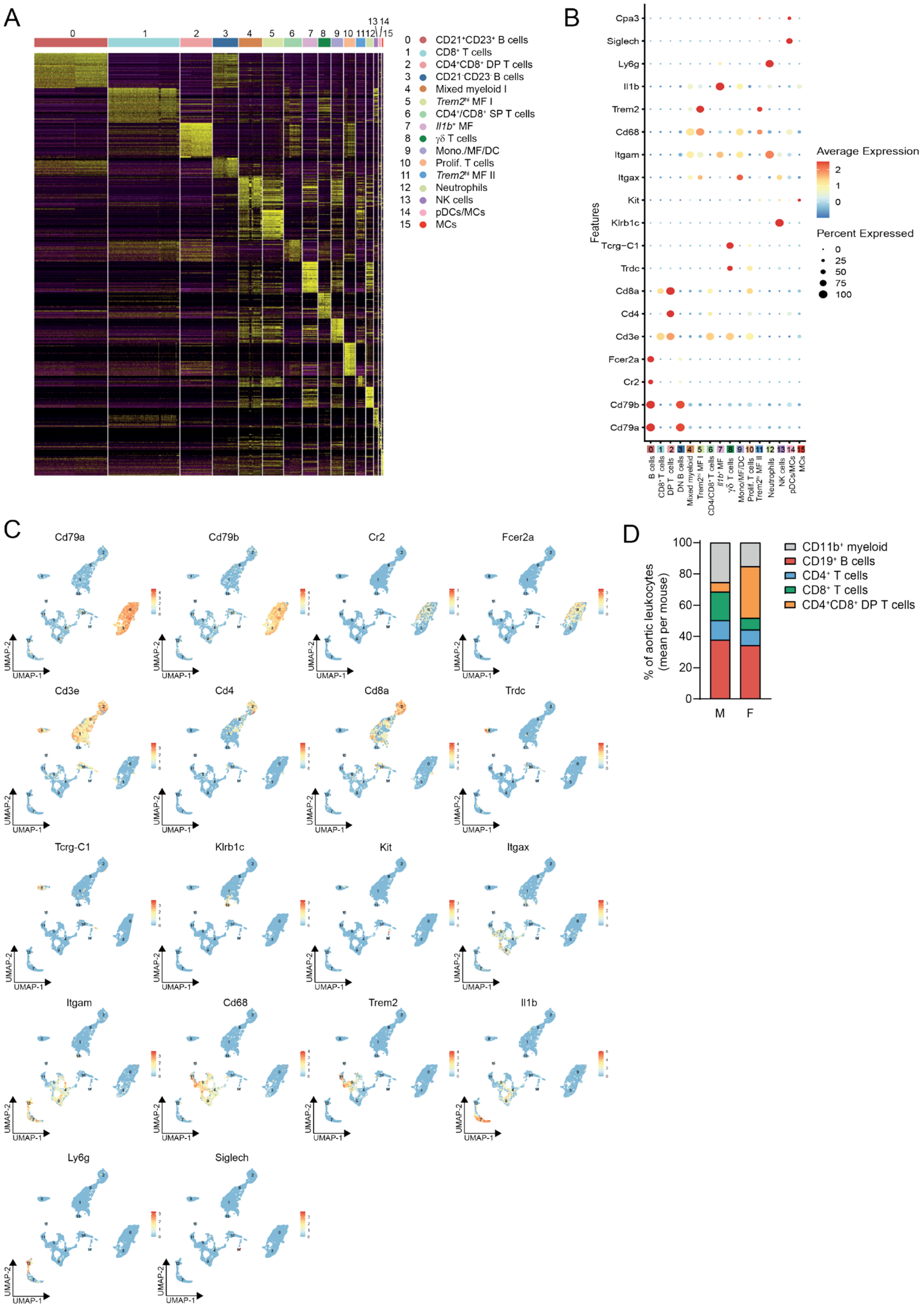


Tube: sample

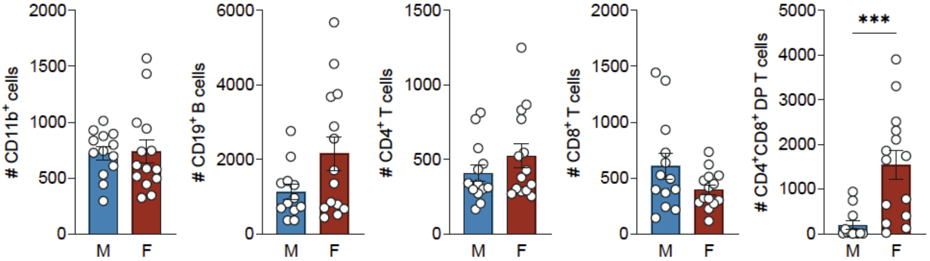
Population	#Events	%Parent	%Total
■ All Events	500,000	####	100.0
🟥 P1	158,223	31.6	31.6
🟩 P2	89,526	56.6	17.9
🟦 P3	84,499	94.4	16.9
🟪 P4	10,076	11.9	2.0

Supplementary Figure S1. Gating scheme of aortic CD45$^+$ cells from male aged *Ldlr*$^{-/-}$ mice before single-cell RNA sequencing.

Gating strategy of alive aortic CD45$^+$ cells for sorting from chow diet-fed aged male *Ldlr*$^{-/-}$ mice.

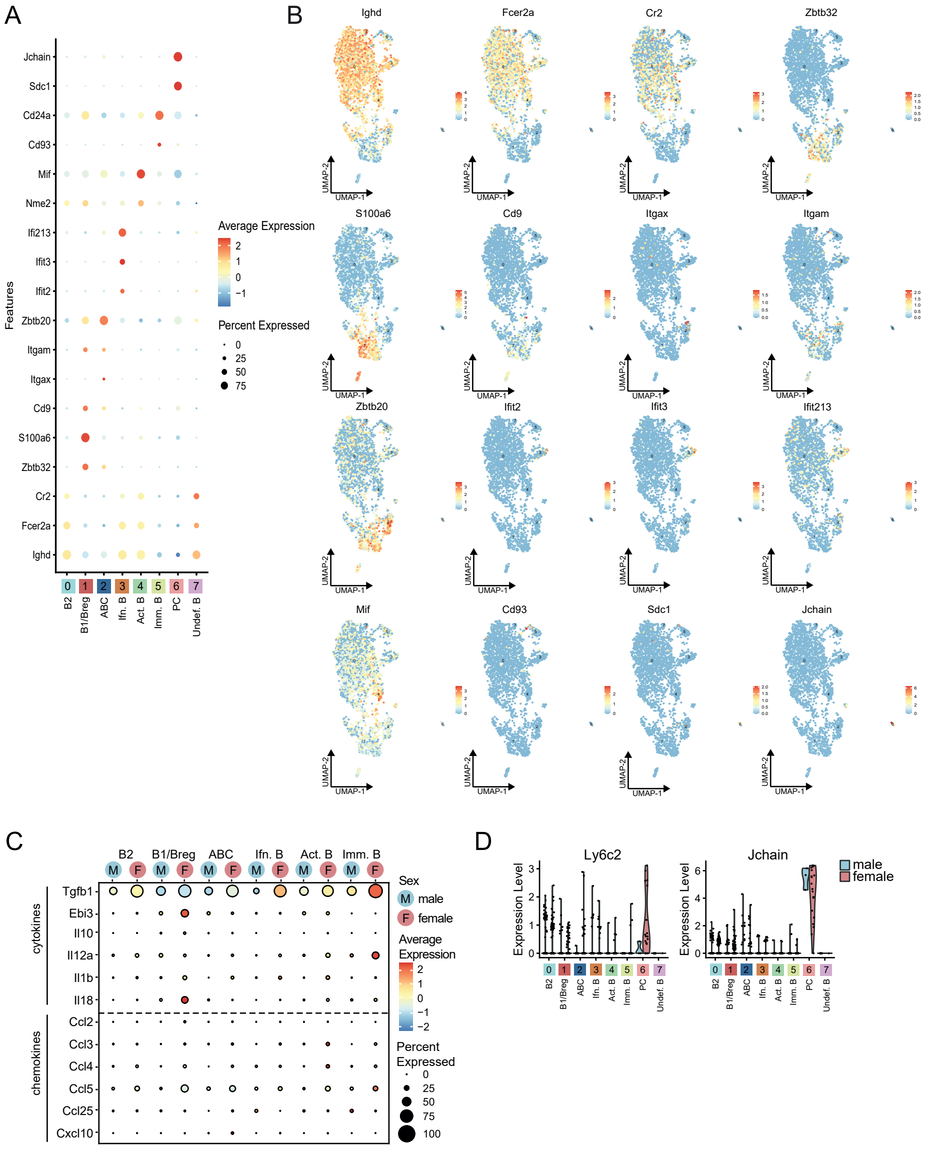


Supplementary Figure S2. Immune cell clustering and frequency in aortas of aged $Ldlr^{-/-}$ mice.
A) Heatmap of the top 50 differentially expressed genes (normalized single-cell gene expression shown) per cluster. B) Feature Dot Plot and C) Feature UMAP of the marker genes used for cluster annotation. D) Stacked diagram showing the relative proportions of major immune cell subtypes within aged male and female $Ldlr^{-/-}$ aortas, measured by flow cytometry.

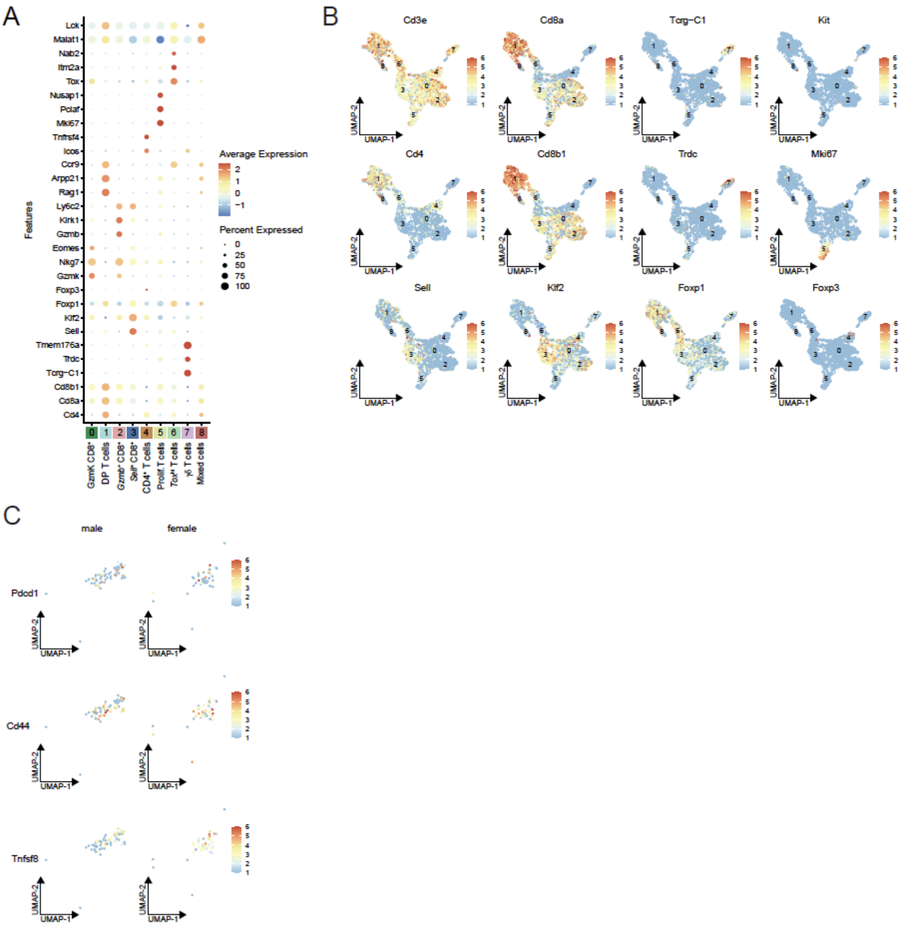


Supplementary Figure S3. Biological distribution of immune cells in aged *Ldlr$^{-/-}$* mice.

Flow cytometry analysis of CD11b^{+} myeloid, CD19^{+} B cells, CD4^{+} T cells, CD8^{+} T cells and double positive CD4^{+} CD8^{+} T cells in chow diet-fed aged male and female Ldlr$^{-/-}$ mice. Data are from n = 12–14 mice per group. Statistical significance was tested by a t-test. Mean ± S.E.M. plotted. ***$P < 0.001$.

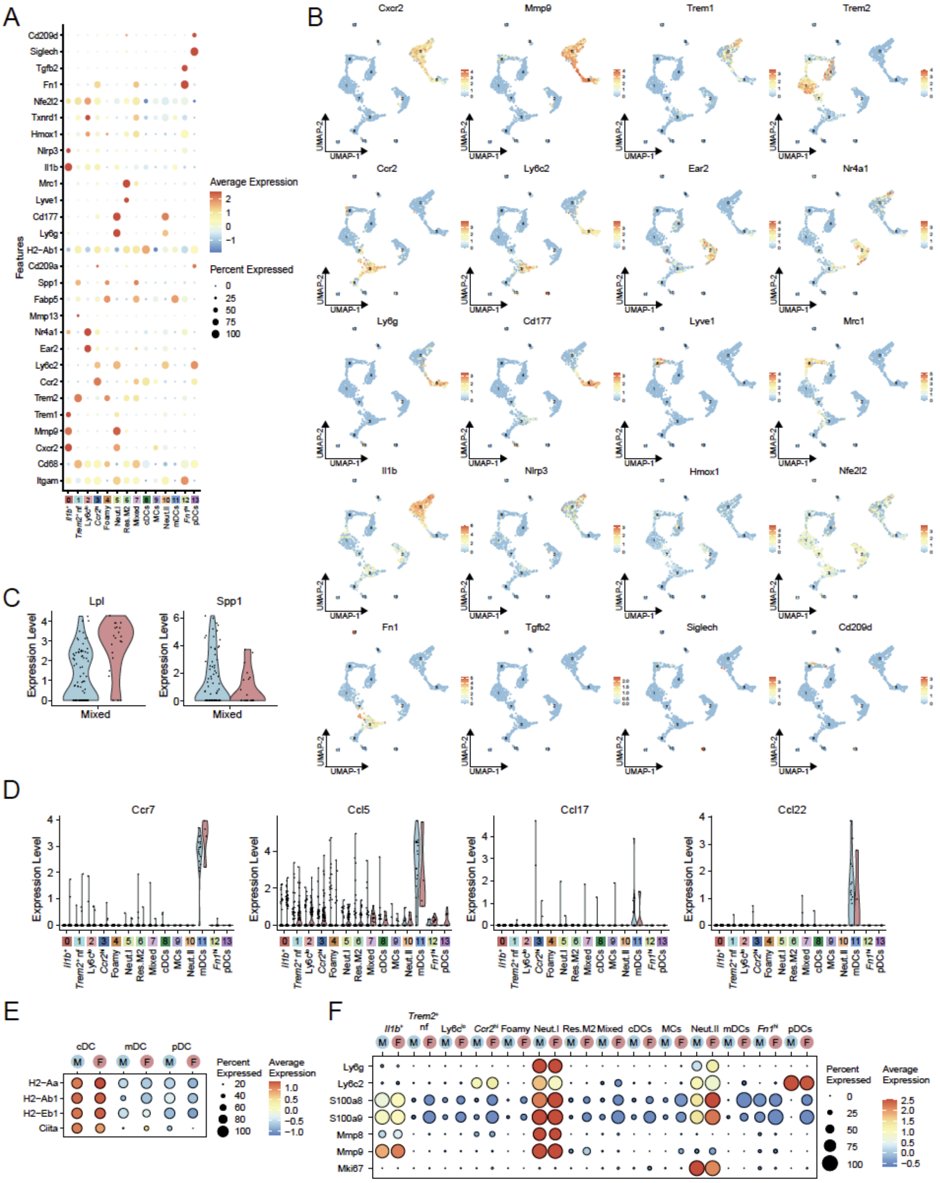


Supplementary Figure S4. Sex-specific differences in aortic B cells of aged *Ldlr*$^{-/-}$ mice.
A) Average expression of cytokine and chemokine genes in B cell clusters split by sex. B) Feature Dot Plot and C) Feature UMAP of the marker genes used for cluster annotation. D) Sex-specific gene expression level of plasma cell-associated genes in B cell clusters.



Supplementary Figure S5. Characterization of aortic T cells in aged *Ldlr*$^{-/-}$ mice.

A) Feature Dot Plot of the marker genes used for cluster annotation. B) Average expression of canonical markers in T cell clusters projected on the UMAP plot. C) UMAP projection displaying sex-specific expression level of genes characteristic for senescence-associated CD4⁺ T cells.



Supplementary Figure S6. Comparison of aortic myeloid cells between aged male and female *Ldlr*$^{-/-}$ mice.
A) Feature Dot Plot and B) Feature UMAP of the marker genes used for cluster annotation. Sex-specific expression of C) differentially expressed genes in cluster 7 and D) chemotaxis genes specific for migratory dendritic cells. E) Average expression of MHCII-related genes in dendritic cell clusters split by sex. F) Average expression of neutrophil markers in myeloid cell clusters split by sex.

SUPPLEMENTARY TABLES

Supplementary Table S1. Major Resources Table

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mouse, *Ldlr*$^{-/-}$	Jackson	C57BL/6J	Male	www.jax.org
Mouse, *Ldlr*$^{-/-}$	Jackson	C57BL/6J	Female	www.jax.org

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Persistent ID / URL
Mouse				
MOMA-2	Bio-Rad (formerly AbD Serotec)	MCA519G	1:1000	www.bio-rad-antibodies.com
α-Rat	Vector	BA-4001	1:200	www.vectorlabs.com
CD4 – V500	BD Biosciences	560782	1:1000	www.bdbiosciences.com
CD8a – AF700	Biolegend	100730	1:500	www.biolegend.com
CD19 – BV605	Biolegend	115540	1:500	www.biolegend.com
CD11b – PE	eBioscience	12-0112-82	1:1000	www.thermofisher.com/ebioscience
CD11c – FITC	Biolegend	117306	1:800	www.biolegend.com
CD8a – PE-Texas Red	Invitrogen	MCD0817	1:1000	www.thermofisher.com/invitrogen
CD45 – AF700	Biolegend	103128	1:1000	www.biolegend.com
CD3 – eFluor450	eBioscience	48-0032-82	1:200	www.thermofisher.com/ebioscience
CD11b – BV605	Biolegend	101257	1:500	www.biolegend.com
PD-1 – PerCP.Cy5.5	Biolegend	109120	1:400	www.biolegend.com
Ly6C – APC	eBioscience	17-5932-82	1:500	www.thermofisher.com/ebioscience
CD45 – AF700	Biolegend	103128	1:1000	www.biolegend.com
Tox – PE	Miltenyi Biotec	130-120-716	1:400	www.miltenyibiotec.com

CD45 – PE	Biolegend	103106	1:500	www.biolegend.com
CD16/32 (Fc Block)	BD Biosciences	553142	1:250	www.bdbiosciences.com
Fixable viability dye – eFluor 450	eBioscience	65-0865-18	1:2000	www.thermofisher.com/ebioscience

Other

Description	Source / Repository	Persistent ID / URL
Trichrome Stain (Masson) Kit	Sigma Aldrich	www.sigmaaldrich.com
Oil Red O	Sigma Aldrich	www.sigmaaldrich.com
Hematoxylin Solution, Mayer's	Sigma Aldrich	www.sigmaaldrich.com
RPMI 1640	Gibco	www.thermofisher.com
Vectastain ABC kit (PK-4000)	Vector	www.vectorlabs.com
ImmPact NovaRed kit	Vector	www.vectorlabs.com
Collagenase I	Sigma Aldrich	www.sigmaaldrich.com
Collagenase XI	Sigma Aldrich	www.sigmaaldrich.com
Hyaluronidase	Sigma Aldrich	www.sigmaaldrich.com
DNAse I	Sigma Aldrich	www.sigmaaldrich.com
Fetal Bovine Serum	Greiner Bio-One	www.gbo.com

Supplementary Table S2. Top 25 differentially expressed genes Main clustering

Main.00	Main.01	Main.02	Main.03	Main.04	Main.05	Main.06	Main.07
Ighd	Ccl5	Rag1	Ighm	Fn1	C1qa	Lef1	Il1b
Ebf1	Nkg7	Arpp21	Mzb1	Ifitm3	C1qb	Itm2a	Retnlg
Cd79a	Ms4a4b	Dntt	Iglc2	Ccr2	C1qc	Tcf7	Csf3r
Cd79b	AW112010	Satb1	Ms4a1	Psap	Apoe	Tmsb10	S100a8
Mef2c	Ctla2a	Ccr9	Iglc1	Tgfbi	Pf4	Trac	Ifitm1
Ms4a1	Ctsw	Cd8b1	C130026I21Rik	Ms4a6c	Ms4a7	Satb1	Slpi
Fcmr	Hcst	Endou	AC168977.1	Ccl9	Selenop	Cd27	Msrb1
Bank1	Ms4a6b	Ldhb	Pkig	Lgals3	Mrc1	Cd28	Cxcr2
Iglc3	H2-Q7	Rmnd5a	Iglc3	Plbd1	Mafb	Cd3d	G0s2
Iglc2	Klrd1	Tcf7	Cd79a	Naaa	Ctsb	Cd5	Mmp9
Ly6d	Il2rb	Trbc2	Ly6a	Mpeg1	Trem2	Prkcq	Hdc
Fcer2a	Xcl1	Sox4	H2-DMb2	Gm2a	Trf	Rgs10	Stfa2l1
H2-DMb2	Gzmk	Cd8a	Cd79b	Alox5ap	Dab2	Tox	Hp
Cd74	Gzmb	Themis	Napsa	Ifitm2	Cd63	Trbc2	Ccl6
H2-Ob	Cxcr6	Ssbp2	Tcf4	Ctss	Lgmn	Txk	Mxd1
Iglc1	Gimap4	Lck	Igkc	H2-DMb1	Cxcl16	Cd3g	Ifitm2
Vpreb3	Cd3g	Myb	Plac8	Cst3	Csf1r	Bcl11b	Tnfaip2
Cd55	Klrk1	Cd4	Fcmr	Lyz2	Fcrls	Cd6	Grina
Pax5	Bcl2	Arl5c	Ly6d	S100a4	Cd14	Ccr9	Slc7a11
Gm31243	Gimap3	Aqp11	Zbtb20	Vim	Timp2	Cd2	Ccr1
Ralgps2	Gm2682	Mier1	D10Wsu102e	Fabp5	Lpl	Chd3	Gsr
H2-Aa	Gimap7	Bcl11b	Dnajc7	Lyz1	Adgre1	Sox4	Dusp1
Tnfrsf13c	Trac	Tcf12	Zcwpw1	Ifi30	Pltp	Ets1	S100a9
H2-Oa	Itgb1	Trbc1	Tppp3	Plac8	Hexb	Trbc1	Cxcl2
H2-Eb1	Ly6c2	Gm4258	Crip1	Retnla	Mmp12	Ikzf2	S100a11

Main.08	**Main.09**	**Main.10**	**Main.11**	**Main.12**	**Main.13**	**Main.14**	**Main.15**
Trdc	Ear2	Stmn1	Cd5l	Camp	Ncr1	Siglech	Fscn1
Tmem176a	Ace	Top2a	Cd63	Ngp	Klrb1c	Cox6a2	Cacnb3
Tmem176b	Clec4a3	Mki67	Gpnmb	Ltf	Klra4	Klk1	Ccl22
Tcrg-C1	Eno3	Hist1h2ae	C1qb	Lcn2	Klra8	Gm21762	Socs2
Cd163l1	Treml4	Hist1h1b	C1qc	Wfdc21	Klrb1b	Fcer1a	Zmynd15
Cxcr6	Cd300e	Pclaf	C1qa	Chil3	Klrb1a	Gata2	Tbc1d4
Il18r1	Csf1r	Ube2c	Fabp5	Mmp8	Klra9	Cpa3	Anxa3
Actn2	Grk3	Hist1h3c	Trem2	Ifitm6	Klre1	Smim5	Rogdi
Trdv4	Clec4a1	Birc5	Mmp12	Cd177	Klrc2	Atp1b1	Cxcl16
Podnl1	Adgre4	Rrm2	Apoe	Mmp9	Klra7	Cd7	Serpinb6b
Kcnk1	Dusp16	Cenpf	Ftl1	Ly6g	Klrk1	Cyp11a1	Ccr7
Ltb4r1	Cx3cr1	Nusap1	Ms4a7	Adpgk	Prf1	Plac8	Relb
Ly6g5b	Lst1	Hist1h2ap	Fth1	Mcemp1	Gzma	Tyrobp	Basp1
Il7r	Pla2g7	H2afx	Lgals3	Cebpe	Serpinb9	Csf1	Fabp5
Tmem64	Clec4e	Dut	Wfdc17	Mgst1	Gzmb	Rnase6	Gadd45b
Maf	Ms4a6c	Hmgn2	Spp1	Hp	Klrd1	Ctsl	Tmem176a
Ckb	Tnfrsf1b	Anp32e	Ctsb	Retnlg	Il2rb	Tcf4	Etv3
Ramp1	Gngt2	Ptma	Ctsl	Anxa1	Serpinb6b	Ifitm1	Syngr2
S100a4	Cebpb	Hmgb1	Cd68	Dstn	Ctsw	Bst2	Traf1
Serpinb1a	Nr4a1	H2afz	Serpinb6a	Pglyrp1	Nkg7	Hdc	Samsn1
Lmo4	Fcer1g	Hmgb2	Ctsd	S100a8	Cd7	Ccl4	Tmem123
Blk	Cybb	Tuba1b	Selenop	S100a9	AW112010	Irf8	Marcks
Emb	Sat1	H2afv	Cstb	Prdx5	Xcl1	Ccl3	Map4k4
Ifngr1	Plek	Ube2s	S100a1	Cybb	Ccl5	Ccl9	Cst3
Cd3g	Prdx1	Tubb5	Ctss	Lyz2	Ccl4	Ccl6	Ifi30

Supplementary Table S3. Top 25 differentially expressed genes B cell clustering

B.00	B.01	B.02	B.03	B.04	B.05	B.06	B.07
Ighd	S100a6	Nt5e	Ifit3	Srm	Akap12	Derl3	Fchsd2
Fcer2a	Ahnak	AC168977.1	Ifi213	Nop58	Atp1b1	Fam46c	Gm20559
Mef2c	Zbtb32	AC133103.1	Usp18	Ncl	Fam129c	Jchain	Zfp329
Satb1	Ahnak2	Itm2c	Ifit2	C1qbp	Vpreb3	Iglv1	Chd2
Neurl3	Lmna	C130026I21Rik	Irf7	Nme1	Myb	Slpi	C1qa
Ralgps2	Anxa2	Itgb1	Slfn5	Hsp90ab1	Pafah1b3	Mt1	BE692007
Stk17b	Rassf4	Adgre1	Ifi206	Eif4a1	Cd79b	Ly6c2	Slc38a2
Fchsd2	Vim	Zbtb20	Ifi203	Ddx21	Sox4	Creld2	Gm31243
Cd55	Tppp3	Bhlhe41	Zbp1	Mif	Ly6d	Tmem176b	Cd69
Cr2	Myadm	Fgl2	Ifi27l2a	Nhp2	Cd24a	Prdx4	Macf1
Sell	Itgb1	Ighg3	Ifi214	Gnl3	Spib	Edem1	Plk2
H2-Ob	Pdlim1	Zeb2	Trim30a	Ranbp1	Cecr2	Xbp1	Zfp869
Pxdc1	Lgals1	Txn1	Stat1	Npm1	Iglc1	Sdf2l1	Atxn2
Pxk	S100a4	H2-Q7	Ms4a4c	Ppp1r14b	Hck	Txndc5	Zcchc7
Zfp318	Crip1	Tcf4	Shisa5	Hspe1	Siglecg	Pdia4	Pan2
Pold4	Tagln2	Fcgr2b	Ifi208	Dkc1	Chchd10	Igkc	Mycbp2
Gm31243	Atf3	Lgals1	Xaf1	Set	Arl5c	Ighg2c	Kidins220
Smad7	Plac8	Pld4	Ifi209	Eif5a	Cnp	Ssr4	2810013P06Rik
Icosl	Ccnd2	Apoe	Ifi47	Fbl	Dnajc7	Sec11c	Slc12a6
Gm8369	S100a11	Ptpn1	Rnf213	Hspd1	Tifa	Hsp90b1	Usp15
Bmyc	S100a10	Igha	Parp14	Pa2g4	Tcf3	Manf	Txndc16
Dmxl1	Zcwpw1	Tnfaip8	Phf11b	Psme2	Bcl7a	Mzb1	Akap9
Lmo2	Cd2	Fcer1g	Samhd1	Hspa5	Rgs2	Slc3a2	Tut7
H2-Eb2	Zbtb20	Cd72	Bst2	Il4i1	Marcks	Ighg1	Arid1a
Tecpr1	D10Wsu102e	Xist	Tor3a	Ccnd2	Ifi30	Ighg2b	Cxcr4

Supplementary Table S4. Top 25 differentially expressed genes T cell clustering

T.00	T.01	T.02	T.03	T.04	T.05	T.06	T.07	T.08
Gzmk	Rag1	Gzmb	Sell	Tnfrsf4	Pclaf	Itm2a	Tmem176a	Malat1
Nkg7	Arpp21	Klrk1	Rps20	Gpm6b	Hist1h3c	Rgs10	Tmem176b	Lck
Ctla2a	Dntt	Itga1	Rps19	Izumo1r	Rrm2	Lef1	Tcrg-C1	Prrc2c
Ccl5	Endou	Klrd1	Ccr7	Maf	Birc5	Satb1	Serpinb1a	Ptprc
Ms4a4b	Rmnd5a	Ccl5	Rpl23	Ifi27l2a	Nusap1	Sox4	Blk	Mbnl1
Eomes	Ldhb	Nkg7	Rps16	Ly6a	Cenpf	Tox	Actn2	mt-Nd4
Hcst	Ccr9	Ly6c2	Rplp1	Capg	Mki67	Gsn	Pxdc1	mt-Cytb
AW112010	Aqp11	AW112010	Rpl13	Icos	Hist1h2ab	Ccr9	Kcnk1	Ccr9
Rpl13a	Myb	Fgl2	Rps28	Tnfsf8	Ube2c	Cnn3	Ltb4r1	Mier1
Itga4	Satb1	Ctsw	Rps24	Tnfrsf18	Lig1	Tmsb10	Trdv4	Arpp21
H2-K1	Ssbp2	Lgals3	Rps7	Pou2f2	Top2a	Basp1	Cd163l1	Ets1
Rgs1	Arl5c	Zeb2	Rps18	Shisa5	Stmn1	Cd28	Ckb	Srrm2
H2-Q7	Sox4	Ahnak	Dapl1	Hif1a	Dut	Cd2	Podnl1	Tnrc6b
Ccr5	Cd4	Itgb1	Ms4a4c	Eea1	Hist1h2ae	Id3	Igf1r	Cd164
Ms4a6b	Dgkeos	Cx3cr1	Rpl36a	Nrp1	Tuba1b	Ikzf1	Capg	Son
Pdcd1	Mier1	Klre1	Rpl12	Cd82	Hmgn2	Cd5	Maf	Akap13
Tox	Ets2	Gzma	Rpl39	Ctla4	Ptma	Ikzf2	Trdc	Clk1
Gimap7	Tcf7	Klrc1	Rps12	Tbc1d4	Hist1h1b	Cd27	Il18r1	Thrap3
Bcl2	Themis	Cd226	Rps5	S100a11	H2afx	Hivep3	Selenop	Ppp2ca
Gm8369	Gtf2h4	Id2	Eef1b2	Ltb	Ran	Xist	Ccr2	Kpna4
Ccl4	Xrcc6	S100a6	S1pr1	Srgn	Hist1h2ap	Etnk1	Lmo4	Selenos
Zfp36l2	Tcf12	Lgals1	Klf2	Odc1	Hmgb2	Cytip	S100a4	Itgb1
Runx3	Cyb5a	Cd48	Igfbp4	Junb	H2afz	Sept7	Il7r	Lyz2
BE692007	Ly6d	Ccl4	Ly6c2	Ikzf2	Tubb5	Egr1	Cxcr6	Tnrc6c
Cst7	Gm4258	Xcl1	Gzmm	Samhd1	Hist1h4d	Trbc1	S100a6	Wbp11

Supplementary Table S5. Top 25 differentially expressed genes myeloid cell clustering

My.00	My.01	My.02	My.03	My.04	My.05	My.06
Cxcr2	Nes	Cd300e	Ms4a4c	Cd5l	Itgb2l	Cbr2
Csf3r	Cd72	Treml4	Ccr2	Ftl1	Ly6g	Mgl2
Hdc	Cadm1	Ace	Vcan	Fth1	Cd177	Cd163
Il1b	Ms4a7	Eno3	S100a4	Atp6v0d2	Ngp	Mrc1
H2-Q10	Myo1e	Adgre4	Fn1	Fabp5	Ltf	Lyve1
Mmp9	Zmynd15	Ear2	Plac8	Lgals3	Wfdc21	Igfbp4
Mxd1	Cxcl16	Spn	Ms4a6c	Gpnmb	Adpgk	Gas6
Msrb1	C3ar1	Dusp16	Tmsb10	Apoe	Chil1	Pf4
Junb	Trem2	Pou2f2	Itgb7	Syngr1	Lcn2	C4b
Slpi	Igf1	Grk3	Ms4a6b	Cd63	Camp	Folr2
Dusp1	Slamf9	Gngt2	Ifitm3	Mmp12	Cebpe	Stab1
Ccr1	Mafb	Nr4a1	Napsa	Trem2	Ckap4	Maf
Srgn	C1qa	Itgal	Slfn5	Serpinb6a	Ly6c2	F13a1
S100a11	C1qc	Itga4	Ly6c2	Lgals1	Mmp8	Fcrls
Fgl2	Hexb	Myo1g	H2-DMa	Cstb	Serpinb1a	Cd209f
Sell	Gatm	Cybb	H2afy	Ctsd	Ifitm6	Dab2
Ifitm1	C1qb	Clec4a3	Ifi209	Ctsl	S100a8	Ccl8
Grina	Mmp12	Clec4a1	Ahnak	Wfdc17	S100a9	Ccl12
Slc7a11	Clic4	Stap1	S100a10	Igf1	Pglyrp1	Tmem176b
Il1r2	Anxa5	Tnfrsf1b	Crip1	Spp1	Syne1	Cfh
Lrg1	Lmna	Bcl2a1d	Vim	Il18bp	Mmp9	Selenop
Stfa2l1	Lpl	Ms4a6c	Ifi27l2a	Pld3	Anxa1	Fcgrt

Ptgs2	Rgs1	Apoc2	Lyz2	AY036118	Chil3	Ctsc
G0s2	Ctsb	Txnrd1	Ifi30	Ftl1-ps1	Retnlg	Trf
Cxcl2	Apoe	Prdx1	Thbs1	S100a1	Hmgn2	Ccl7

My.07	**My.08**	**My.09**	**My.10**	**My.11**	**My.12**	**My.13**
Emp1	Xcr1	Gata2	Birc5	Cacnb3	Tgfb2	Gm21762
S100a10	Sept3	Cpa3	Hist1h3c	Fscn1	Ltbp1	Klk1
Fn1	Itgae	Csrp3	Fcnb	Ccr7	Prg4	Atp1b1
Psap	Ifi205	Fcer1a	Pclaf	Ccl22	Ptgis	Cox6a2
Capg	Olfm1	Ms4a2	Ube2c	Socs2	Alox15	Siglech
F10	Wdfy4	Cyp11a1	Mki67	Serpinb6b	Selp	Upb1
Apoc2	Naaa	Cd200r3	Top2a	Apol7c	Ednrb	Spib
Cstb	Naga	Sytl3	Elane	Serpinb9	Saa3	Ccr9
Lgals3	Irf8	Mcpt8	Cebpe	Tbc1d4	Fcna	Iglc3
Gpnmb	Plbd1	Il6	Hist1h1b	Clu	Serpinb2	Cd7
Plin2	Psmb9	Nedd4	Hist1h2ae	Il4i1	Icam2	Smim5
Ctss	Cst3	Cd7	Stmn1	Relb	Flnb	Bcl11a
Ifi30	Gng10	Ctsg	Serpinb1a	Zmynd15	C4b	Rpgrip1
Lgals1	H2-DMb1	Il18r1	Ltf	Traf1	Ltc4s	Ly6d
Ahnak	Ckb	Cd69	Camp	Birc2	Ecm1	Runx2
Atp6v0c	H2-DMa	Csf1	Hist1h2ap	Ccl5	Fn1	Fyn
Lgmn	Ppt1	Ifitm1	Ngp	Tmem123	Fabp4	Rnase6
Lpl	Psmb8	Ccl4	Chil3	Anxa3	Itga6	Ly6a
Ccl9	Eef1b2	Ets1	Prtn3	Etv3	Cxcl13	Tcf4
Fabp5	H2-Ab1	Serpinb1a	Hmgn2	Syngr2	Cfp	Dnajc7
Prdx1	H2-Eb1	Jun	Wfdc21	Map4k4	Fam46a	Bst2

Clec4n	Id2	Tmem71	Lcn2	Tmem176a	Emilin2	Irf8
Spp1	H2-Aa	Ccl3	Hmgb2	AW112010	Pycard	Ly6c2
Retnla	Tmsb10	Rgs1	H2afz	Fabp5	Tagln2	Plac8
Lyz1	Cd74	Ccl9	S100a8	Epsti1	Slpi	Xist