SUPPLEMENTARY INFORMATION

Disruption of the CCL1-CCR8 axis inhibits vascular Treg recruitment and function and promotes atherosclerosis in mice

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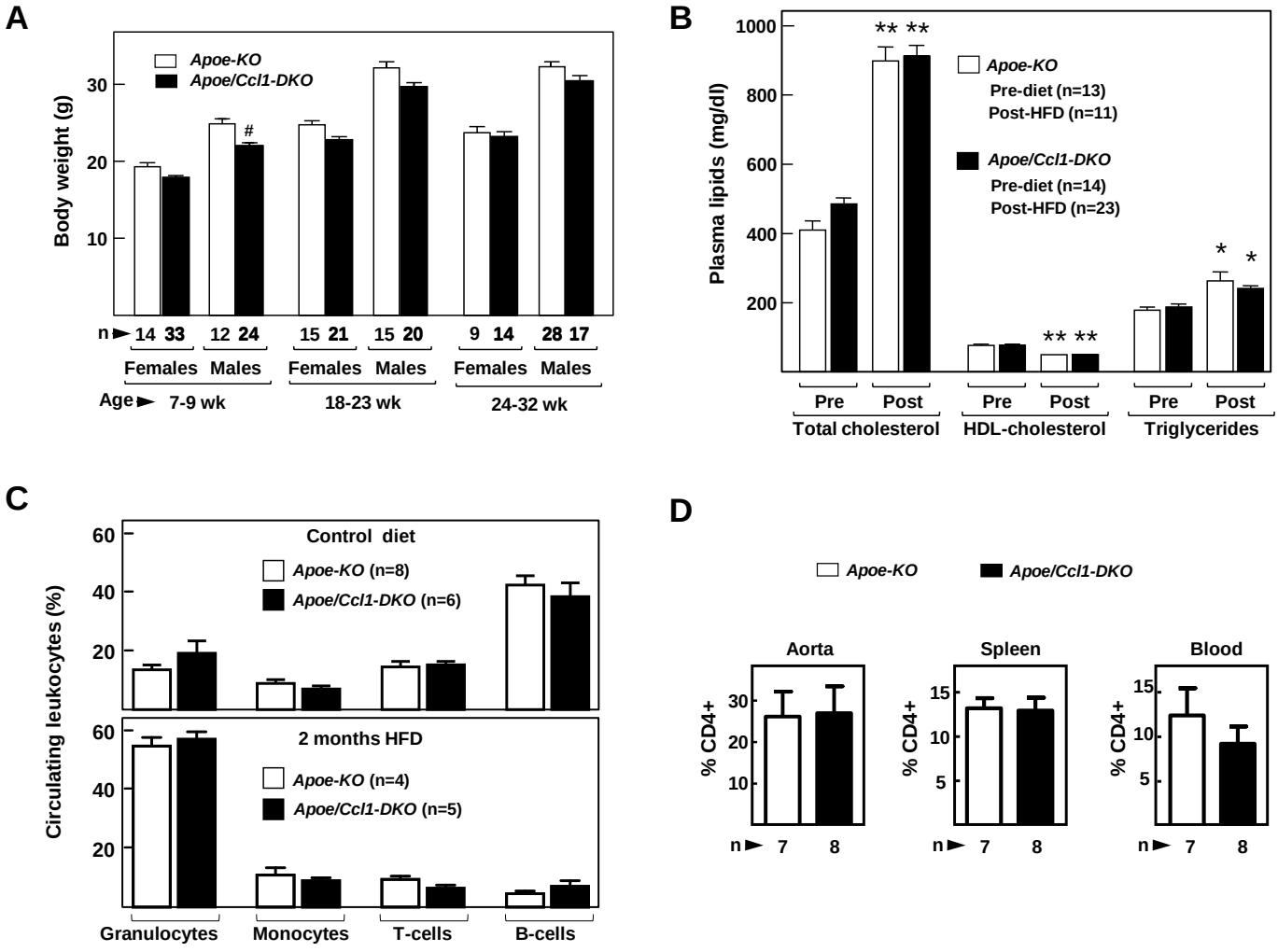
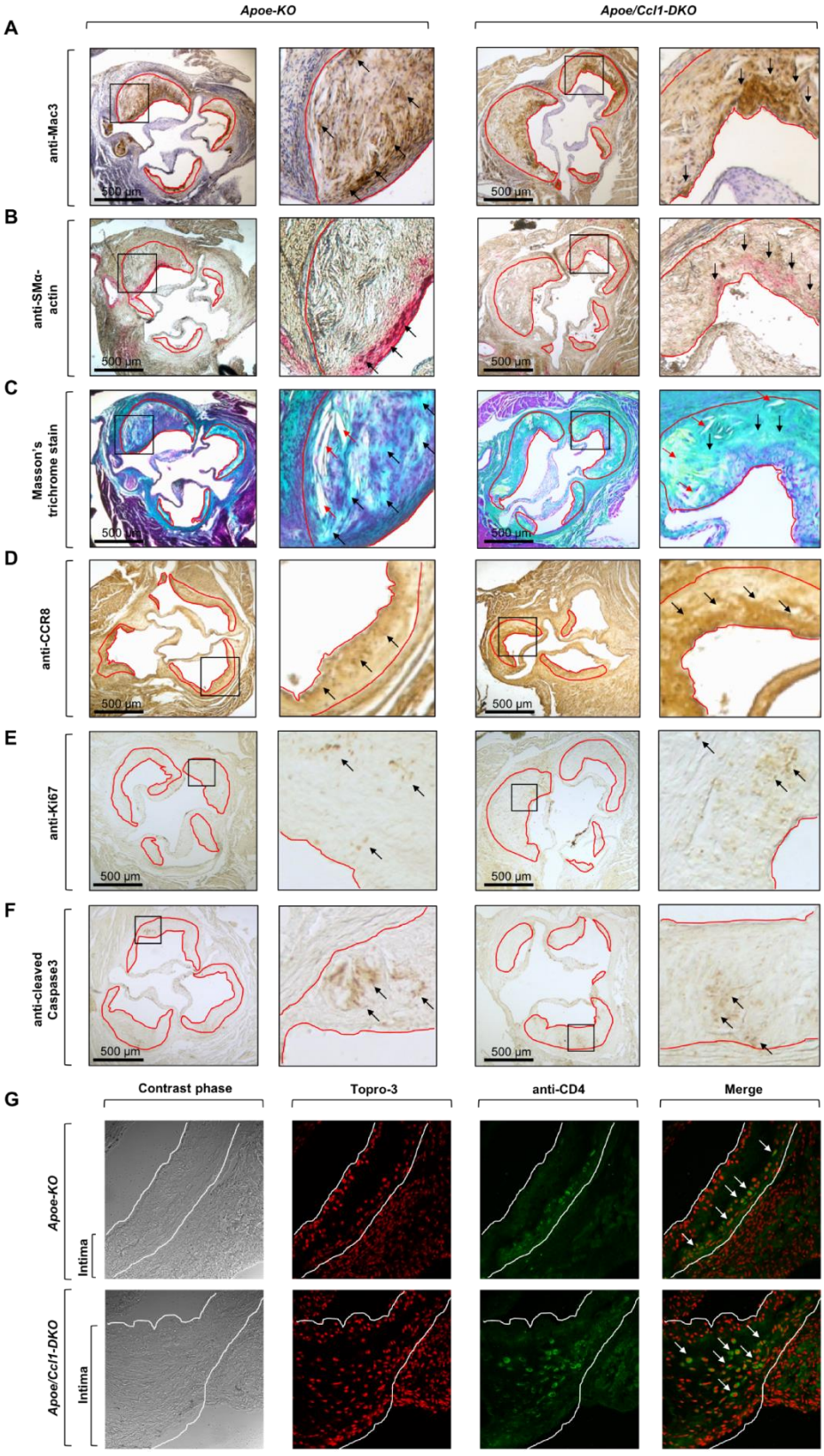
Short tittle:

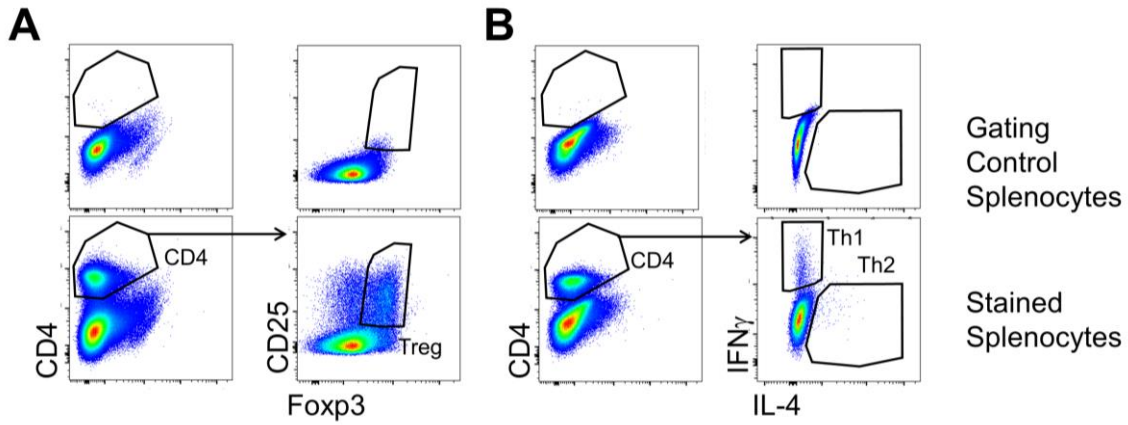
CCL1-CCR8 inhibition aggravates atherosclerosis



Supplementary Figure S1: Characterization of Apoe/Ccl1-DKO mice. (A) Body weight of mice of the indicated ages, gender and genotype. #, p <0.05 *vs*. male *Apoe-KO* (same age). **(B)** Plasma lipids measured at baseline (pre-diet) and after 2 months of HFD (post-diet). *, p<0.001 and **, p<0.0001 *vs*. pre-diet (same genotype). **(C)** Percentage of circulating leukocyte populations in male mice fed control or HFD for 2 months. **(D)** Percentage of CD4+ T-cells in aorta, spleen, and blood from control-diet-fed *Apoe*-KO and *Apoe/Ccl1-DKO* mice. n=number of mice.



Supplementary Figure S2: Immunohistopathological analysis of atherosclerotic lesions in fat-fed mice. Mice of the indicated genotypes were challenged for 2 months with a high-fat diet and aortic root and ascending aorta cross-sections were examined. Quantitative results for both regions are shown in Fig. 3 and in Supplementary Table S1. Representative images of the aortic root are shown here. **(A)** anti-Mac3 immunohistochemistry to detect macrophages (arrows). Sections were counterstained with hematoxylin. **(B)** anti-SMα-actin immunohistochemistry to detect VSMCs (arrows). **(C)** Masson's trichrome stain to detect collagen (blue staining, black arrows). Red arrows show necrotic cores. **(D)** anti-CCR8 immunohistochemistry. Arrows show CCR8+ areas. **(E)** anti-Ki67 immunohistochemistry to detect proliferating cells (arrows). **(F)** anti-cleaved caspase3 immunohistochemistry to detect apoptotic cells (arrows). **(G)** anti-CD4 immunofluorescence counterstained with Topro-3. Arrows show CD4+ cells. Red lines in A-F mark atherosclerotic lesion boundaries, and the black boxes in the left images are shown at a higher magnification in the right images. White lines in all immunofluorescence images mark atherosclerotic lesion boundaries.



Supplementary Figure S3: Representative examples of gating strategies for Th1, Th2 and Treg populations. Flow cytometry-determined content of $CD4^+$ T cells and Th1 ($CD4^+$ $IFN\gamma^+$), Th2 ($CD4^+IL4^+$) and Tregs ($CD4^+$ $CD25^{high}$ $FOXP3^+$) in spleen from 11-month-old mice fed control diet. **(A)** For Treg determination, samples were fixed, permeabilized and stained with anti-CD4, anti-Foxp3 and anti-CD25 fluorescent antibodies. Expression of Foxp3 and CD25 was analyzed in the CD4-positive cell population. **(B)** For Th1 and Th2 determination, cells were cultured for 4 hours with PMA and ionomycin in the presence of brefeldin A before fixation, permeabilization and staining with anti-CD4, anti-IFNγ and anti-IL-4 fluorescent antibodies. Expression of IFNγ and IL-4 was analyzed in the CD4-positive cell population.

Supplementary Table S1: Quantitative immunohistopathological analysis of atherosclerotic lesions in fat-fed *Apoe-KO* and *Apoe/Ccl1-DKO* mice

	AORTIC ROOT			ASCENDING AORTA		
	Apoe-KO	***Apoe/Ccl1-DKO***	**p-value**	***Apoe-KO***	***Apoe/Ccl1-DKO***	**p-value**
CCR8	0.2192 ± 0.0459 n=6	0.1730 ± 0.0314 n=6	0.43	0.2359 ± 0.0467 n=6	0.2835 ± 0.0326 n=6	0.42
Mac3	0.1339 ± 0.0098 n=10	0.1518 ± 0.0152 n=14	0.38	0.1052 ± 0.0158 n=10	0.1692 ± 0.0269 n=14	0.08
SM α-actin	0.0059 ± 0.0024 n=10	0.0050 ± 0.0007 n=14	0.68	0.0038 ± 0.0007 n=10	0.0089 ± 0.0025 n=14	0.10
Caspase 3	0.0026 ± 0.0007 n=10	0.0024 ± 0.0006 n=11	0.84	0.0039 ± 0.0017 n=11	0.0041 ± 0.0013 n=11	0.92
Necrotic core	0.0151 ± 0.0036 n=10	0.335 ± 0.0093 n=14	0.12	0.0020 ± 0.0006 n=9	0.0205 ± 0.007 n=14	0.05
Collagen	0.1034 ± 0.0218 n=8	0.1331 ± 0.0191 n=13	0.33	0.0424 ± 0.0144 n=10	0.0650 ± 0.0177 n=14	0.36

Mice of the indicated genotypes were challenged for 2 months with a high-fat diet and cross-sections through the aortic root and ascending aorta were examined to quantify the area of the atherosclerotic lesions with immunoreactivity for CCR8, Mac3 (macrophages), SMα-actin (VSMCs), and caspase 3 (apoptosis), or occupied by necrotic cores and collagen (all in mm^2). Results are expressed as mean ± SEM. The results of some parameters are expressed as percentage of atherosclerotic area in Figure 2 (Mac3) and Figure 3 (CCR8, necrotic core, SM α-actin, collagen, and caspase 3).