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Genetic Deletion of Chemokine Receptor Ccr6 Decreases Atherogenesis in *ApoE*-deficient Mice

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SUPPLEMENTAL MATERIAL

Detailed Methods

Animals

Ccr6$^{-/-}$ mice were generated as previously described.[1] *Ccr6*$^{-/-}$*ApoE*$^{-/-}$ mice were obtained by crossing *ApoE*$^{-/-}$ mice on a C57BL/6J background (Jackson Labs, Bar Harbor, ME) with *Ccr6*$^{-/-}$ mice on a C57BL/6N background (Division of Cancer Therapy, NCI). Female littermate *Ccr6*$^{+/+}$*ApoE*$^{-/-}$ and *Ccr6*$^{-/-}$ *ApoE*$^{-/-}$ mice were weaned at 6 weeks, fed a Western diet (TD88137; Harlan Teklad, Madison, WI) for an additional 10 or 18 weeks, and then sacrificed for analysis. Female mice sacrificed at 16 weeks of age were subjected to all the analyses detailed below. Female mice sacrificed at 24 weeks of age and male littermates sacrificed at 19 weeks of age were subjected only to analysis of lesion area in the aorta. All mice were kept in pathogen-free conditions and animal study protocols were approved by the Animal Care and Use Committee of the NIAID, NIH. Blood counts were analyzed by the Department of Laboratory Medicine, Clinical Center (NIH, Bethesda, MD) using an automated hematology analyzer (Cell Dyn 3700, Abbott Laboratories).

Atherosclerotic Lesion Analysis

The extent of atherosclerosis was assessed by quantifying lesion size in whole aortas and aortic roots, as previously described.[2] Briefly, mice were anesthetized and perfused with PBS/0.2 mM EDTA prior to the removal of hearts for sectioning. Aortas were stained with Sudan IV or frozen for RNA extraction. Hearts were bisected transversely and snap frozen in OCT with the cut side facing down. Relevant sections were captured by cutting the frozen blocks at 100 μm increments until the valves appeared. The sections were cut at 10 μm thickness starting from the appearance of the first aortic sinus valve leaflet. Six consecutive sections 50 μm apart (with 3 leaflets of the aortic valve) were stained with 0.5% Oil Red O solution and counterstained with hematoxylin (Histoserv Inc., Germantown, MD). Images of the entire aorta were captured with Leica AF6000 LX microscope (Mannheim, Germany) and analyzed by Image J (NIH), whereas images of the aortic root were captured with a Zeiss light microscope (Jena, Germany) and analyzed by IVision software (Biovision Inc., Exton, PA).

Lipid Analysis

Serum samples were obtained after mice had fasted for four hours. Total cholesterol and HDL, LDL/VLDL levels were analyzed by EnzyChrom Kit (BioAssay Systems, Hayward, CA), and triglyceride level was determined by Stanbio Triglyceride LiquiColor assay (Stanbio Lab., Boerne, TX).

Immunohistochemistry

Frozen aortic root sections were stained with MOMA-2 (MCA519G; Serotec, Raleigh, NC) as primary antibody and goat anti-rat Alexa Fluor 488 as secondary antibody (Molecular Probes, Carlsbad, CA) for macrophage quantification, as described previously.[2] For Ccl20 staining, sections were fixed in acetone for 10 minutes and then incubated with blocking buffer (2% goat serum in 1% BSA/PBS) for 30 minutes. After incubating with primary mAb against Ccl20 (R&D, Minneapolis, MN) for 60 minutes, goat anti-rat Alexa Fluor 568 (Molecular Probes, Carlsbad, CA) was applied for visualization. Masson's trichrome staining for collagen was carried out by Histoserv Inc. (Germantown, MD). Images were captured using a Zeiss microscope (Jena, Germany) and analyzed by IVision software (Biovision Inc., Exton, PA).

Cell Isolation and Flow Cytometry

Primary leukocytes were harvested from mouse peripheral blood, spleen and bone marrow. Anti-coagulated peripheral blood was treated with lysing buffer (BD Biosciences, San Jose, CA) to remove erythrocytes. Splenocytes were released into HBSS by gentle homogenization or liberase digestion (Roche Applied Science, Indianapolis, IN) and filtered through 100 μm nylon mesh (BD Biosciences, San Jose, CA). Bone marrow was flushed from tibia and femur with HBSS/1% FCS/10 mM HEPES, and then cultured in RPMI1640 with 40 ng/ml M-CSF to obtain bone marrow-derived macrophages. Cells

were first stained with a Live/Dead marker (Invitrogen, Carlsbad, CA) in PBS for 20 minutes and then washed with FACS buffer (PBS, 1% BSA, 0.1% sodium azide) and incubated with rat anti-mouse CD16/32 for 15 minutes to block Fc receptors. Then cells were incubated at 4ºC for 30 minutes with the following mouse-specific fluorochrome-conjugated antibodies: CD45-PerCP (BD Biosciences, Cat: 557235), CD45-APCCy7 (BD Biosciences, Cat: 557659), CD3-FITC (BD Biosciences, Cat: 555274), CD4-APC Cy7 (eBioscience, Cat: 47-0042-82), CD11b-APC (BD Biosciences, Cat: 553312), CD11b-PerCP-Cy5.5 (BD Biosciences, Cat: 550993), Gr1-APC (eBioscience, Cat: 17-5931-82), Ly6C-FITC (BD Biosciences, Cat: 553942), Ly6G-APC-Cy7 (BD Biosciences, Cat: 560600), 7/4-Alexa Fluor 647 (AbD Serotec., Cat: MCA771A647), MHCII-Pacific Blue (Biolegend, Cat: 116422), CD11b-PE (BD Biosciences, Cat: 553311), F4/80-APC (AbD Serotec, Cat: MCA497APC) Ccr6-PE (R&D Systems, Cat: FAB590P), CD4-APC (eBioscience, Cat: 17-0041-83), CD8α-APC (eBioscience, Cat: 17-0081-82), CD19-APC (eBioscience, Cat: 17-0193-82), CD117-APC (eBioscience, Cat: 17-1171-82), CD11c-APC (eBioscience, Cat: 17-0114-82), NK1.1-APC (eBioscience, Cat: 17-5941-82), CD115-biotinylated (eBioscience, Cat: 13-1152-85), SA-PE (eBioscience, Cat: 12-4317-87), CD11b-APCCy7 (eBioscience, Cat: 47-0112-82), and F4/80-PECy7 (eBioscience, Cat: 25-4801-82). Cells were analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo software (version 7.5.5; Treestar, Ashland, OR). Mouse inflammatory monocytes were defined as Lin^- $CD115biot/SA^+CD11b^+$ $F4/80^+Ly6C^{high}$ (Lin: CD4/CD8α/CD19/CD117/CD11c/NK1.1) cells and sorted using a FACSAria-blue instrument (BD Biosciences, San Jose, CA). Antibodies used for sorting included CD4-APC, CD8α-APC, CD19-APC, CD117-APC, CD11c-APC, NK1.1-APC, CD115-biotinylated, SA-PE, CD11b-APCCy7, F4/80-PECy7 (eBioscience, San Diego, CA) and Ly6C-FITC (BD Biosciences, San Jose, CA).

Bone Marrow Transplantation

Female *ApoE*$^{-/-}$ mice (8 weeks old) were irradiated with a dose of 900 rads and transplanted with 5×10^6 bone marrow cells from either wild-type or *Ccr6*$^{-/-}$ mice. The bone marrow cells were obtained by flushing the femurs and tibias of female donor mice with PBS and 2 mM EDTA. After bone marrow reconstitution, mice were maintained on a chow diet for 4 weeks to recover and then switched to a Western Diet for an additional 10 weeks, followed by the analysis of atherosclerotic lesions in the whole aorta.

Intravenous Injection of Ccl20

Sterile PBS or mouse recombinant Ccl20 (2 µg or 20 µg, Pepro Tech, Rocky Hill, NJ) were injected into the tail vein of *ApoE*$^{-/-}$ mice (female, 9 weeks old, Jackson Labs, Bar Harbor, ME) in a volume of 100 µl. Mice were killed after 15 hours and blood counts were analyzed using an automated hematology analyzer (Cell Dyn 3700, Abbott Laboratories).

Chemotaxis Assay

Chemotaxis was measured using a Boyden Chamber assay.[3] Briefly, purified mouse monocytes or RAW 264.7 cell suspensions (30 µl, 3×10^6 cells/ml) were added to the upper chamber and mouse Ccl20 was added to the lower chamber separated by a membrane containing 8 µm diameter pores (Neuro Probe Inc., Gaithersburg, MD). Following a 5 h incubation in a 37°C, 5% CO_2 incubator, chemotaxis was assessed by counting the number of migrated cells in three random fields per well (400× magnification). Chemotaxis index was calculated by comparing the number of cells migrating toward Ccl20 and the number of cells migrating toward control media.

mRNA Expression Analysis

Total aorta RNA was isolated by homogenization in Trizol (Invitrogen, Carlsbad, CA), and total RAW 264.7 cell RNA was prepared using RNeasy kit (Qiagen, Valencia, CA). One microgram of total RNA was converted into cDNA using reagents from Promega Corp. (Madison, WI). Real- time PCR (ABI Prism 7900HT, Applied Biosystems) was used to determine mRNA levels using either SYBR Green

(Supplemental Table II) or Taqman primers (Applied Biosystems, Carlsbad, CA). All samples were normalized to GAPDH (Ccr6 knockout did not affect GAPDH expression in *ApoE*$^{-/-}$ mice) and relative changes in expression levels were determined by the ΔΔCT methods, displayed as either gene amplicons or mRNA fold change.

Cytokine Assays

Mouse serum was frozen at -80ºC and thawed prior to measurement. Ccl20 protein levels were determined using murine Quantikine ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Statistical Analysis

The data were analyzed using unpaired t tests (two-tailed) or Two-way ANOVA tests with Prism 5.0 software (GraphPad Software, San Diego, CA) and data are presented as the mean ± SEM. The cutoff for statistical significance was defined as $P<0.05$.

1. Hedrick MN, Lonsdorf AS, Shirakawa AK, Richard Lee CC, Liao F, Singh SP, Zhang HH, Grinberg A, Love PE, Hwang ST, Farber JM. CCR6 is required for IL-23-induced psoriasis-like inflammation in mice. *J Clin Invest.* 2009;119:2317-2329.
2. Saederup N, Chan L, Lira SA, Charo IF. Fractalkine deficiency markedly reduces macrophage accumulation and atherosclerotic lesion formation in CCR2-/- mice: evidence for independent chemokine functions in atherogenesis. *Circulation.* 2008;117:1642-1648.
3. Wan W, Zou H, Sun R, Liu Y, Wang J, Ma D, Zhang N. Investigate the role of PTEN in chemotaxis of human breast cancer cells. *Cell Signal.* 2007;19:2227-2236.

Supplemental Tables

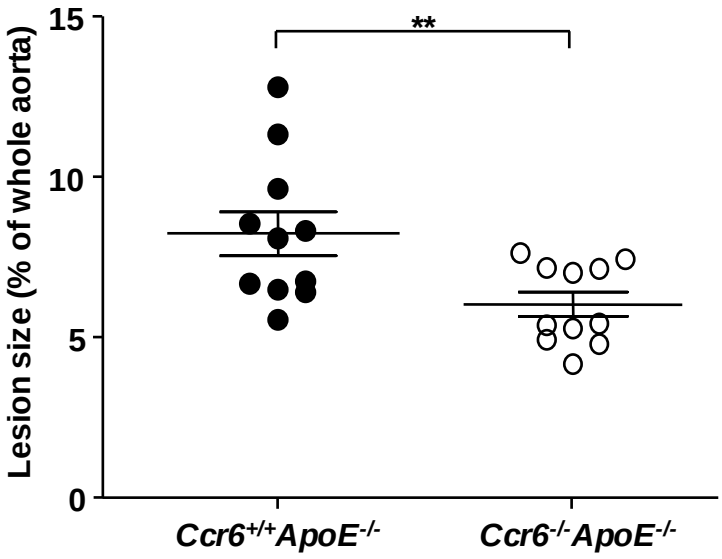
Supplemental Table I. Ccr6 deficiency does not affect lipid profiles or body weight in the *ApoE*$^{-/-}$ mouse model of atherosclerosis. Mice (n=7 - 10) were 16 weeks old and had been on a Western Diet for 10 weeks at the time of euthanasia. Data are the mean ± SEM. Lipid values are in mg/dL.

Genotype	Total cholesterol	HDL cholesterol	LDL/VLDL cholesterol	Triglyceride	Body weight (g)
Ccr6$^{+/+}$*ApoE*$^{-/-}$	1492 ± 50	82 ± 22	1375 ± 63	145 ± 3	22.7 ± 1.9
Ccr6$^{-/-}$*ApoE*$^{-/-}$	1609 ± 24	84 ± 5	1281 ± 28	137 ± 5	24.1 ± 2.4

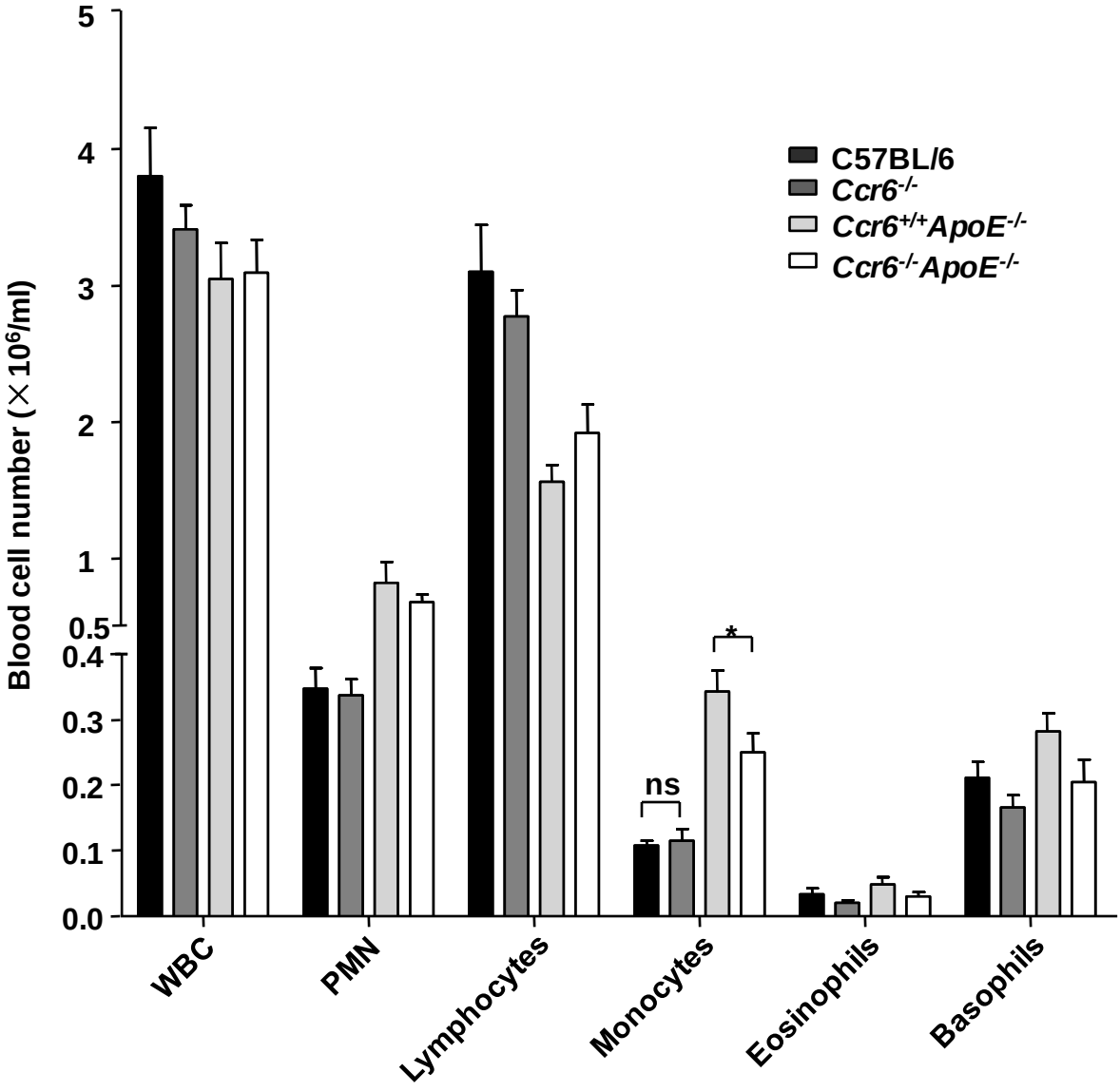
Supplemental Table II. Sequences of the primers used for qPCR with SYBR Green.

Gene product	**Primer name***	**Primer sequence (5' → 3')**	**Amplicon length (bp)**
GAPDH	GAPDH F	aactttggcattgtggaagg	223
	GAPDH R	acacattgggggtaggaaca	
Ccr2	Ccr2 F	agagagctgcagcaaaaagg	185
	Ccr2 R	ggaaagaggcagttgcaaag	
Ccr5	Ccr5 F	cgaaaacacatggtcaaacg	176
	Ccr5 R	ttcctactcccaagctgcat	
Ccr6	Ccr6 F	ttgtcctcaccctaccgttc	239
	Ccr6 R	gatgaaccacactgccacac	
Cx3cr1	Cx3cr1 F	ggagactggagccaacagag	196
	Cx3cr1 R	tcttgtctggctgtgtcctg	
Ccl20	Ccl20 F	cgactgttgcctctcgtaca	177
	Ccl20 R	aggaggttcacagccctttt	
Cx3cl1	Cx3cl1 F	ggaaagaaacgtggtccaga	165
	Cx3cl1 R	gccctcagaatcacagggta	

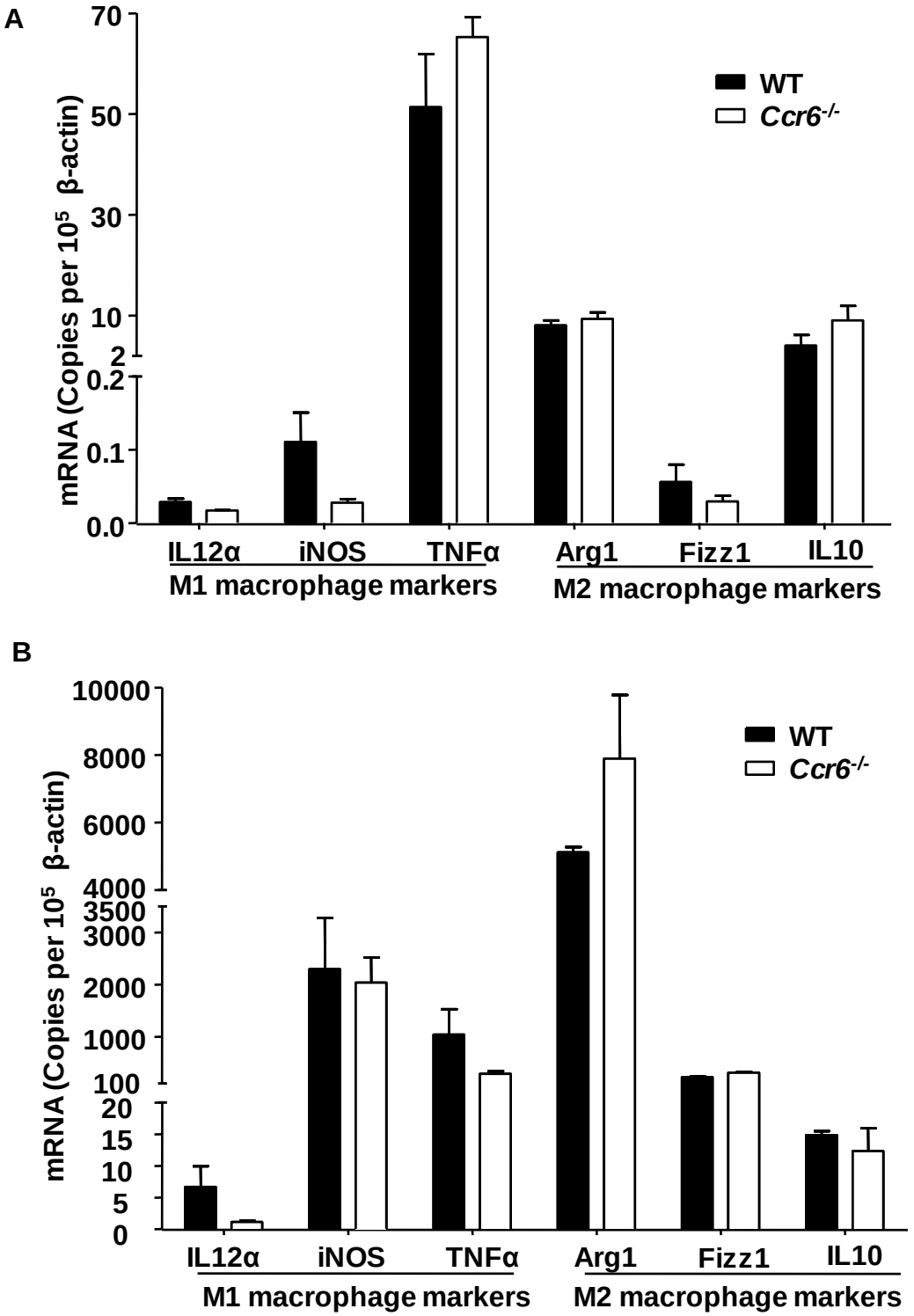
* F, forward; R, reverse.



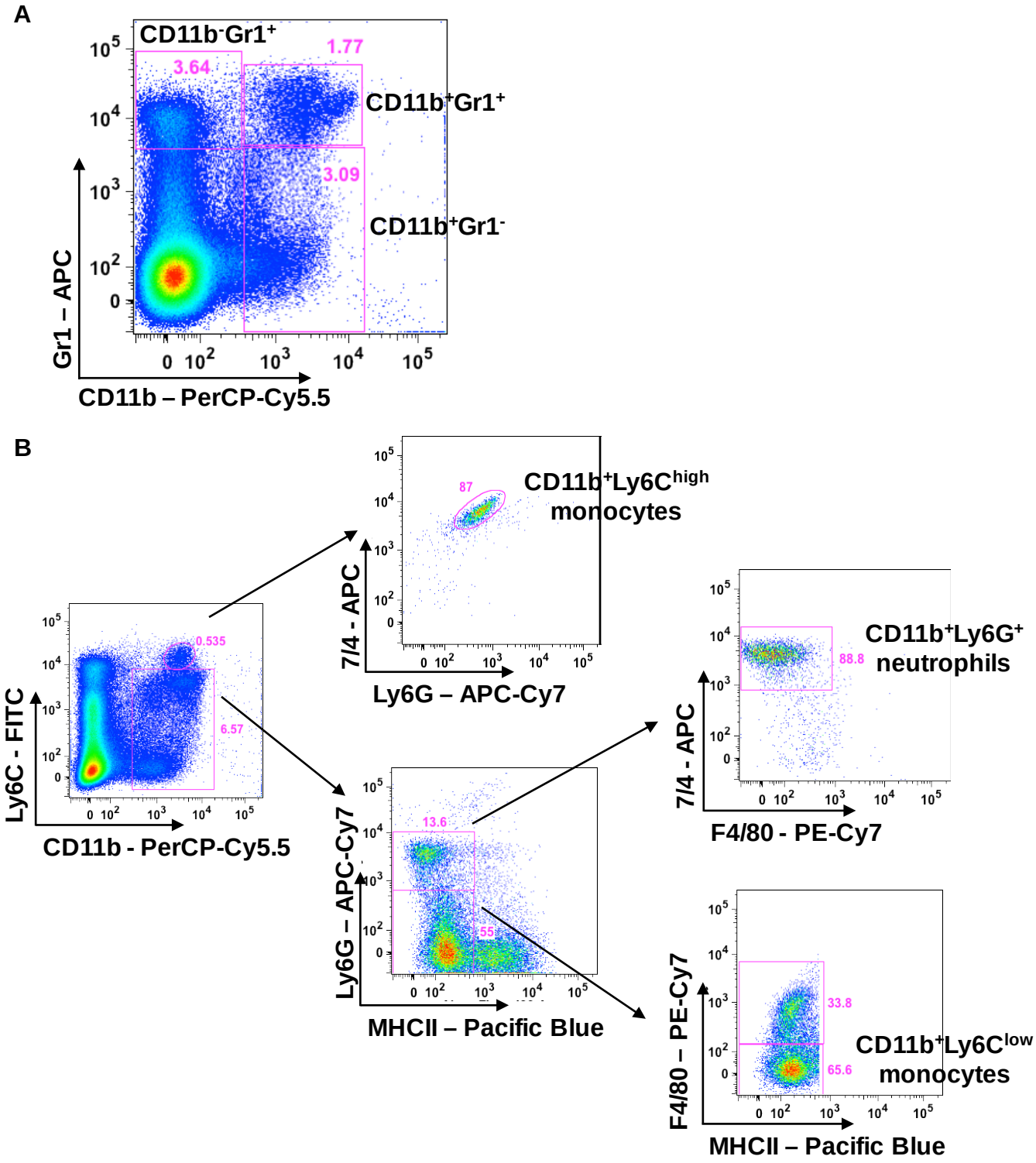
Supplemental Figure I: Ccr6 knockout decreases atherosclerosis for males in the *ApoE*$^{-/-}$ mouse model of atherosclerosis. Male *Ccr6*$^{+/+}$*ApoE*$^{-/-}$ and *Ccr6*$^{-/-}$*ApoE*$^{-/-}$ mice were fed a high-fat Western diet for 13 weeks (19 weeks of age) and then analyzed for atherosclerosis development in whole aorta. Quantification of the atherosclerotic lesions was reported as percentage of the whole aorta. Eleven mice were used in each group (***P*=0.009). Each symbol represents data from a single mouse, and each data set is summarized as mean ± SEM.



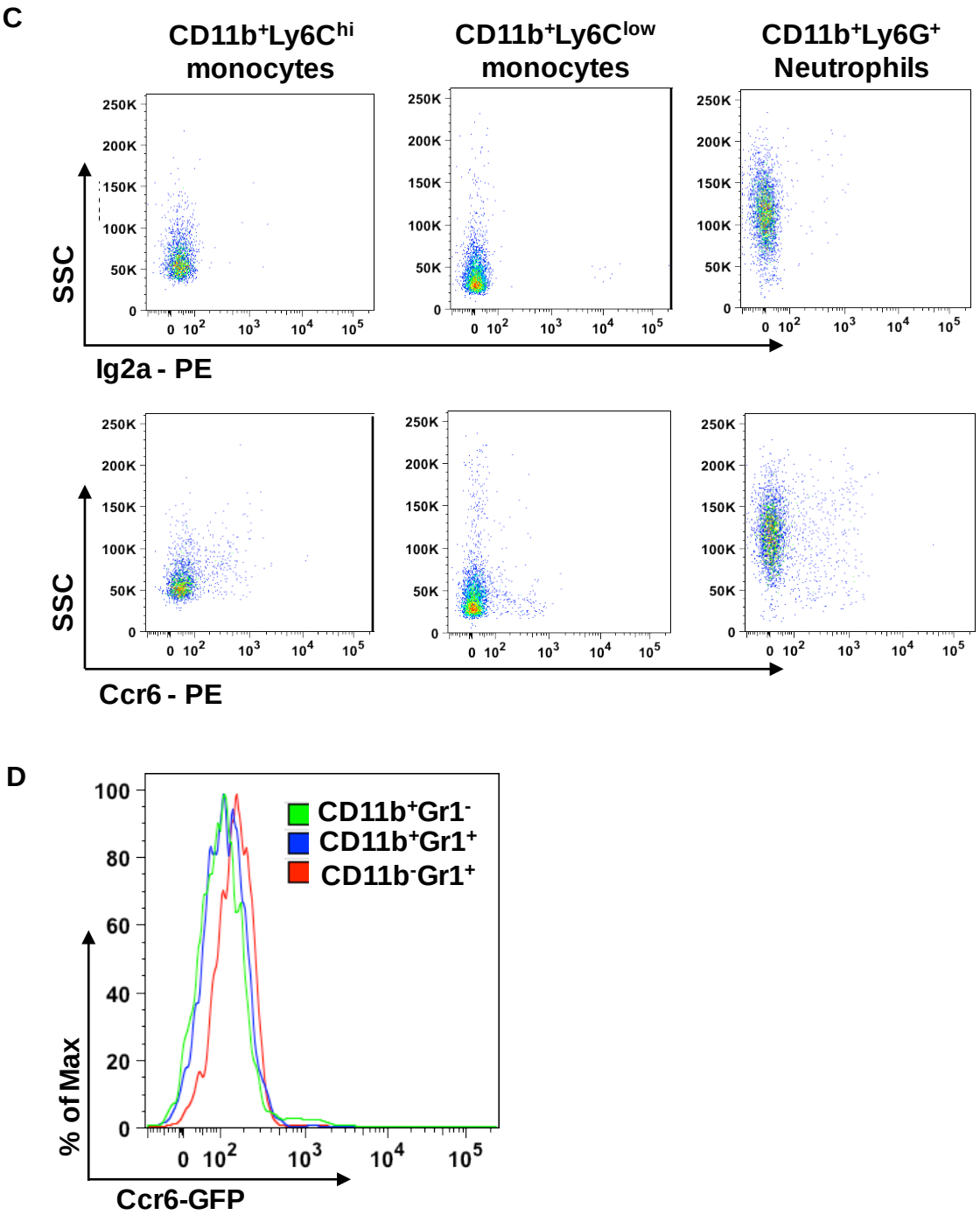
Supplemental Figure II: Ccr6 deficiency does not change the number of total white blood cells, peripheral blood lymphocytes, neutrophils, eosinophils or basophils in either wild type C57BL/6 mice or *ApoE*$^{-/-}$ mice (female, 16 weeks old). Absolute numbers of different blood cell subsets in C57BL/6 mice, *Ccr6*$^{-/-}$ mice, *Ccr6*$^{+/+}$*ApoE*$^{-/-}$ mice and *Ccr6*$^{-/-}$*ApoE*$^{-/-}$ mice were determined by complete blood count (CBC), as described in Methods. Data are summarized as mean ± SEM (n=9 mice in each group, **P*=0.011 for monocytes).



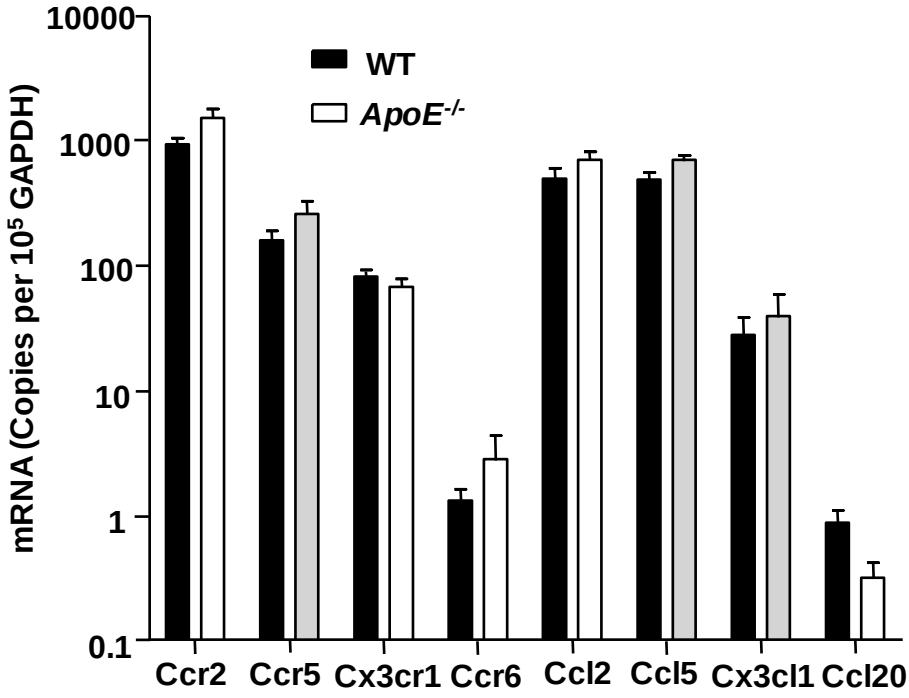
Supplemental Figure III: Ccr6 deficiency does not affect the differentiation of bone marrow derived monocytes/macrophages. Bone marrow derived macrophages from wild-type and *Ccr6*$^{-/-}$ mice were stimulated 18 hrs with either 25 ng/ml IFNγ and 100 ng/ml LPS or 10 ng/ml IL-4 to derive M1/M2 macrophages. Real-time PCR was used to analyze the mRNA levels of IL12α, iNOS, TNFα (M1 markers) and Arg1, Fizz1, IL-10 (M2 markers) both before (A) and after (B) stimulation. All samples were normalized to β-actin.



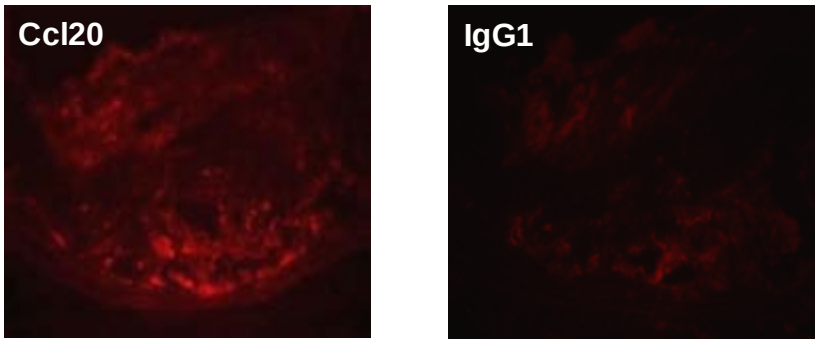
Supplemental Figure IV: Ccr6 is expressed on primary mouse splenic monocytes. A, Leukocytes from the spleen of Ccr6 EGFP knock-in mice were stained with antibodies to CD11b and Gr1 and gated on $CD11b^+Gr1^-$, $CD11b^+Gr1^+$ and $CD11b^-Gr1^+$ cells. The percentage of the total gated population is indicated within the box. B, Representative flow cytometry staining of $CD11b^+Ly6C^{high}$ monocytes ($CD11b^+Ly6C^{hi}7/4^{bri}Ly6G^-$ cells), $CD11b^+Ly6C^{low}$ monocytes ($CD11b^+Ly6C^{low}Ly6G^-MHCII^-F4/80^-$ cells) and $CD11b^+Ly6G^+$ neutrophils ($CD11b^+Ly6G^+Ly6C^{low}MHCII^-7/4^{bri}F4/80^-$ cells) from the spleen of *ApoE*$^{-/-}$ mice.



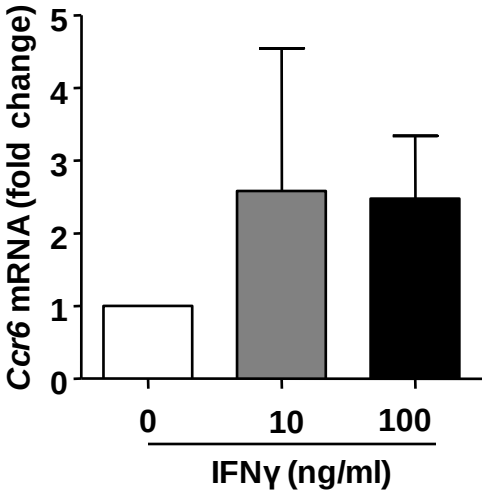
Supplemental Figure IV (cont.): Ccr6 is expressed on primary mouse splenic monocytes. C, Representative flow cytometry staining of either isotype control (upper panel) or Ccr6 specific antibody (lower panel) on CD11b^{+}Ly6C^{high} monocytes (left column), CD11b^{+}Ly6C^{low} monocytes (middle column) and CD11b^{+}Ly6G^{+} neutrophils (right column) from the spleen of *ApoE*$^{-/-}$ mice. D, Ccr6 expression on blood CD11b^{+}Gr1^{-}, CD11b^{+}Gr1^{+}, CD11b^{-}Gr1^{+} cells was examined by GFP expression and cell gates were made as in A.



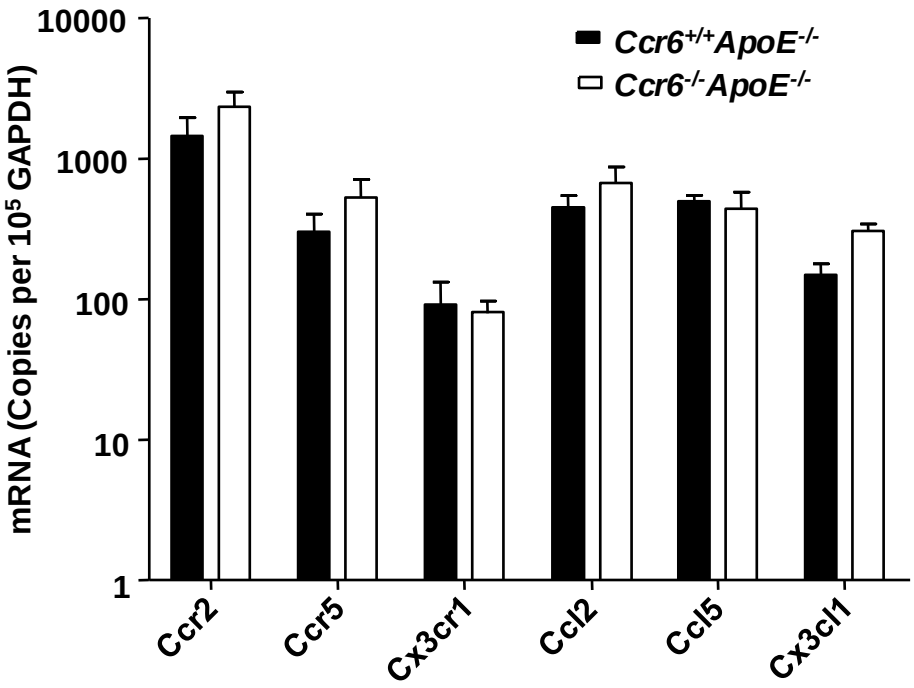
Supplemental Figure V: Ccl20 and Ccr6 are expressed in the aorta of *ApoE*$^{-/-}$ mice. Real-time PCR analysis of chemokines and chemokine receptors involved in atherosclerosis in aortas from wild type C57BL/6 mice and *ApoE*$^{-/-}$ mice were shown (n=4 mice in each group).



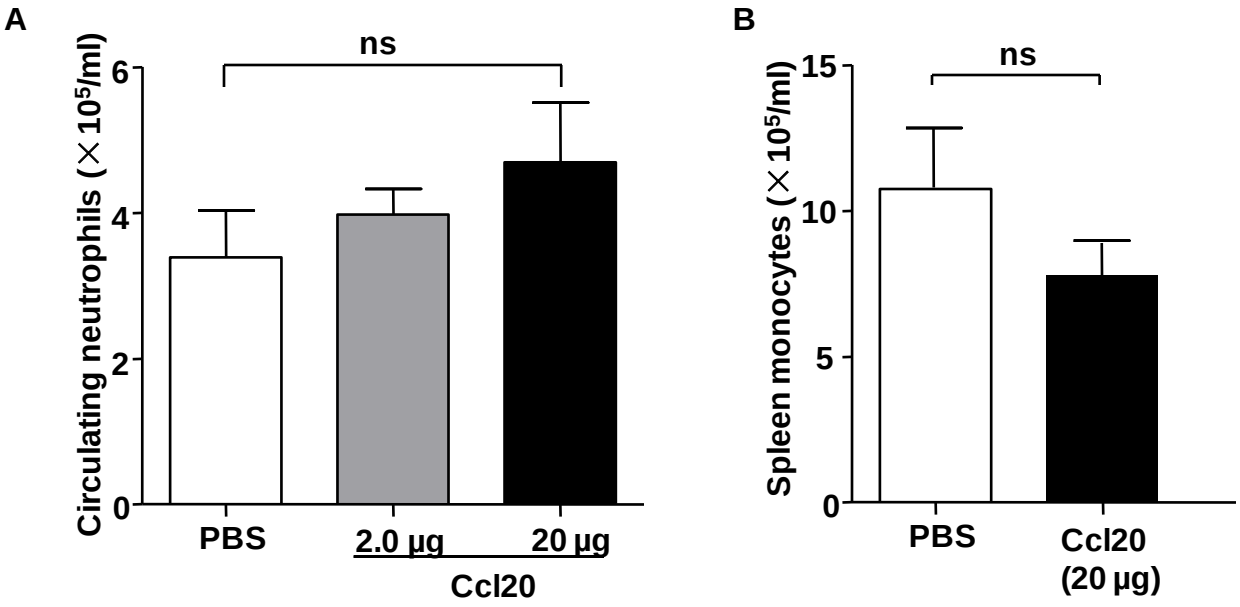
Supplemental Figure VI: Representative immunofluorescence photomicrographs of aortic root sections from *Ccr6*$^{+/+}$*ApoE*$^{-/-}$ mice stained with rat anti-mouse Ccl20 monoclonal antibody and rat IgG1 isotype control. Goat anti-rat Alexa Fluor 568 was used as secondary antibody for visualization.



Supplemental Figure VII: IFNγ induced production of Ccr6 mRNA in primary monocytes. Purified primary mouse monocytes from the bone marrow of C57BL/6 mice were stimulated with different doses of IFNγ for 24 hrs and then mRNA levels of Ccr6 were examined by real-time PCR.



Supplemental Figure VIII: Ccr6 deficiency does not affect the expression of other chemokine receptors and chemokines (Ccr2, Ccr5, Cx3cr1 and Ccl2, Ccl5, Cx3cl1) involved in atherosclerosis. Real-time PCR was used to analyze the RNA expression in aortas from *Ccr6*$^{+/+}$*ApoE*$^{-/-}$ and *Ccr6*$^{-/-}$*ApoE*$^{-/-}$ mice (n=3 mice in each group).



Supplemental Figure IX: Circulating neutrophil levels and total number of spleen monocytes do not change after tail vein injection of Ccl20 in *ApoE*$^{-/-}$ mice. Blood neutrophil levels and spleen monocyte numbers were quantified 15 hours post injection (n=3-4 in each group). The experiment was repeated two times and representative data are shown as mean ± SEM.