# Supplemental Materials

## Expanded Materials & Methods

### scRNA-seq data re-analysis

We performed re-analyses of publically deposited scRNAseq datasets comparing naïve murine monocytes with monocytes trained by 4-PBA. These single cell datasets were analyzed using the standard Seurat pipeline (v4.3.0) in R as described [15]. For quality filtering, cells that have fewer than 200 unique genes were removed. The data were then normalized and scaled followed by dimensionality reduction by principal component analysis (PCA). The samples were then clustered and visualized with UMAP. With this result, differentially expressed genes were analyzed using the non-parametric Wilcoxon rank sum test with the R package as we described [15]. Genes demonstrating significant difference in expression levels were quantified with the standard Seurat R package and represented in the volcano plot. The dot plot was generated with the dot size representing the percentage of cell within the cell population expressing the gene of interest and the color intensity representing the normalized gene expression levels as we previously described [6]. Gene expression levels of specific genes in PBS-primed murine monocytes and monocytes trained with 4-PBA datasets were calculated in R. These expression levels were then ranked in ascending order in Microsoft Excel. The percentile of the sample data was then calculated. The mean and variance of the sample quantiles were used to calculate the Z-score. This Z-score was then plotted against the gene expression levels to get the quantile-quantile (q-q) plot. scRNA-seq data were deposited to the NCBI Gene Expression Omnibus and are accessible under accession number GSE160450.

### Experimental animals

WT C57BL/6 mice, *ApoE*$^{-/-}$ mice, and B6 SJL mice were purchased from the Jackson Laboratory. *Tram*$^{-/-}$ mouse colony on C57BL/6 background was provided by Dr. Holger Eltzschig (University of Texas Houston Medical School, USA). *Tollip*$^{-/-}$ mouse colony on C57BL/6 background was provided by Dr. Jürg Tschopp (University of Lausanne, Switzerland). *Cd24*$^{-/-}$ mouse colony on C57BL/6 background was provided by Dr. Yang Liu (University of Maryland, USA). The mice were bred and maintained under pathogen–free conditions in the animal facility at Virginia Tech. All animal procedures were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Intuitional Animal Care and Use Committee of Virginia Tech. Both male and female mice between 7 and 10 weeks of age were used for experiments, and no sex-specific effects were observed.

Based on our previous publications [6, 71] and the calculations performed by others with similar assessment [72, 73], we assessed that a minimum sample size of 5 mice per group was required to achieve 80% power to detect a 50% change in atherosclerotic burden at a significance level of 0.05. For ex vivo measurement of biochemical markers on harvested cells, at least three animals per group were used according to published studies [74-77]. Animals that showed health concerns unrelated to the experimental conditions (for example, fight wounds and dermatitis) were excluded. For all the *in vivo* animal studies (Figures 2, 7, 8, S4, S5, S9, S10, and S11), the samples were collected and analyzed by different investigators, ensuring that investigators who analyzed the data were blinded to the treatment groups.

### *In vitro* priming of mouse monocytes and flow cytometry analyses

Mouse BMM cultures were prepared as described before [15, 28]. BM cells isolated from WT C57BL/6 mice and *Tollip*$^{-/-}$ mice were cultured in complete RPMI 1640 medium supplemented with 10 ng/mL mouse M-CSF (Peprotech, no. 315-02) in the presence of 1 mM 4-PBA (MilliporeSigma, no. SML0309), 10 μg/mL oxLDL (Kalen Biomedical, no. 770202), 10 μg/mL cholesterol (MilliporeSigma, no. C4951), or PBS. Fresh 4-PBA, oxLDL, cholesterol or PBS was added to the cell cultures every 2 days. After 5 days, BMMs were harvested, filtered through 70 μm cell strainer, and incubated with anti-CD16/CD32 antibodies (1:100 dilution, BD Biosciences, no. 553141) to block Fc receptors. To determine surface expression of ICAM-1 and CD24, the BMMs were stained with fluorochrome-conjugated anti–CD11b (1:200 dilution, BioLegend, no. 101226), anti–Ly6C (1:200 dilution, BioLegend, no. 128018), anti–ICAM-1 (1:200 dilution, BioLegend, no. 116120), and anti–CD24 (1:200 dilution, BioLegend, no. 138504 or no. 101806) antibodies. Propidium iodide (MilliporeSigma, no. P4170) was added before flow cytometry to label dead cells. To compare the surface phenotype of in vitro cultured BMMs sustained by low-dose M-CSF, peritoneal resident macrophages, and bone marrow-derived dendritic cells (DCs), macrophages were isolated from the peritoneal cavity from WT C57BL/6 mice following a published protocol [78], and bone marrow-derived DCs were obtained from the murine bone marrow cultures supplemented with GM-CSF as previously described [79]. The cells were filtered through 70 μm cell strainer, blocked with anti-CD16/CD32 antibodies (1:100 dilution, BD Biosciences, no. 553141), and stained with fluorochrome-conjugated anti–CD11b (1:200 dilution, BioLegend, no. 101208), anti–c-kit (1:200 dilution, BioLegend, no. 105806), anti–CD93 (1:200 dilution, BioLegend, no. 136509), anti–CD105 (1:200 dilution, BioLegend, no. 120413), anti–CD11c (1:200 dilution, BioLegend, no. 117310), and anti–MHCII (1:200 dilution, BioLegend, no. 107606) antibodies. Propidium iodide was added before flow cytometry to label dead cells. To detect intracellular mTOR expression, BMMs were first stained

with fluorochrome-conjugated anti–CD11b (1:200 dilution, BioLegend, no. 101208), and anti–Ly6C (1:200 dilution, BioLegend, no. 128016) antibodies, fixed and permeabilized using Cyto-Fast™ Fix/Perm Buffer Set (BioLegend, no. 426803), and then stained with fluorochrome-conjugated anti–mTOR antibody (1:200 dilution, Cell Signaling Technology, no. 5043S). The samples were examined using FACSCanto II (BD Biosciences), and data were analyzed using FlowJo V10 (Tree Star).

**Detection of foam cell formation**

Mouse BMMs were trained with 4-PBA or PBS as described above, and foam cell formation was examined with an oxLDL uptake assay kit (Cayman Chemical, no. 601180) according to the manufacturer's instructions. Briefly, trained BMMs were incubated with DyLight 488 conjugated oxLDL for 24 hours. After washing with PBS, the cells were stained with Hoechst 33342 and observed under a fluorescence microscope. The cells were then harvested and labeled with propidium iodide. The level of intracellular oxLDL in the living cells was quantified using flow cytometry.

**HFD feeding, 4-PBA injection, and adoptive transfer of *in vitro* trained monocytes**

Age- and sex-matched *ApoE*$^{-/-}$ mice were fed with HFD (Harlan Teklad 94059) for 4 weeks to induce atherosclerosis development, and then the mice were intraperitoneally injected with PBS or 4-PBA (5 mg/kg body weight) every 3 days for 4 weeks. The mice were continuously fed with HFD during the regimen. One day after the last injection, the mice were euthanized, and tissues were harvested for subsequent analyses.

Adoptive transfer of *in vitro* trained monocytes was conducted as reported previously [6, 28]. BM cells isolated from *ApoE*$^{-/-}$ mice were cultured in complete RPMI 1640 medium supplemented with M-CSF (10 ng/mL) in the presence of 4-PBA (1 mM), or PBS for 5 days as described above. Age- and sex-matched recipient *ApoE*$^{-/-}$ mice were first fed with HFD for 4 weeks to induce atherosclerosis development, and were then transfused with $3 \times 10^6$ in vitro trained BMMs through intravenous injection once a week for a total of 4 weeks. The recipient mice were continuously maintained on HFD. One week after the last cell transfer, the mice were euthanized, and tissues were harvested for subsequent analyses.

The AAV-PCSK9 murine model of atherosclerosis was established as reported [80] and employed to test whether CD24 was required for the therapeutic efficacy of 4-PBA-trained monocytes. AAV8-mPCSK9-D377Y vectors were produced by the Center for Cardiovascular Diseases and Sciences of Louisiana State University. Age- and sex-matched WT C57BL/6 mice were intravenously injected with a single dose of AAV8-mPCSK9-D377Y ($5 \times 10^{11}$ vector genomes per mouse) and fed with HFD for 4 weeks. BMMs

from WT C57 BL/6 and *Cd24*$^{-/-}$ mice were treated with PBS or 4-PBA (1 mM) for 5 days. PBS- or 4-PBA-trained monocytes ($3 \times 10^6$ cells per mouse) were then adoptively transferred by intravenous injection to HFD-fed recipient mice once a week for 4 weeks. The recipient mice were continuously maintained on HFD. One week after the last cell transfer, the mice were euthanized, and tissues were harvested for subsequent analyses.

**Histological analyses of atherosclerotic lesions**

Histological analyses of atherosclerotic lesions were performed following established protocols [6, 28]. Briefly, 10 μm-thick sections of the proximal aorta, which had been freshly frozen and embedded in optimal cutting temperature (OCT) compound, were fixed in 4% neutral buffered formalin and subsequently subjected to Hematoxylin and Eosin (H&E) staining. Oil Red O staining was performed using a kit (Newcomer Supply, no. 9119A), and collagen staining was performed using a Picrosirius Red Stain Kit (Polysciences, no. 24901) according to the manufacturers' instructions. The samples were observed under BZ-X810 microscope (KEYENCE). The percentages of total lesion area in the aortic root area, lipid deposition in the atherosclerotic plaques, and collagen composition in the plaques were analyzed using Image J 1.53t (NIH).

**Determination of plasma lipids**

Plasma samples were collected from the mice treated as described above. Total and free cholesterol levels were quantified with a kit purchased from MilliporeSigma (no. MAK043), and triglyceride level was quantified with a kit purchased from BioVision (no. K622). All assays were performed according to the manufacturers' instructions.

**Flow cytometry analyses of monocyte phenotype *in vivo***

*ApoE*$^{-/-}$ mice were fed with HFD and injected with 4-PBA as described above. Peripheral blood, BM, spleen, and aorta were harvested, and single-cell suspensions were prepared for flow cytometry as reported previously [6]. Briefly, blood, BM, and spleen samples were disassociated with mechanical processes followed by lysis of red blood cells with ACK buffer (Quality Biological, no. 118-156-101). Aorta samples were cut into small pieces and then transferred into an enzyme cocktail containing 450 U/mL collagenase type I (Worthington, no. LS004196), 250 U/mL collagenase type XI (MilliporeSigma, no. C7657), 120 U/mL hyaluronidase (MilliporeSigma, no. H6254), and 120 U/mL DNAse (MilliporeSigma, no. DN25), and 20 mM HEPES (MilliporeSigma, no. H0887). The samples were incubated at 37°C in an automated

tissue dissociator (Miltenyi Biotec) for 60 minutes, and red blood cells were lysed with ACK buffer. The processed samples of all tissues were filtered through 70 μm cell strainers to obtain single-cell suspensions. The samples were incubated with anti-CD16/-CD32 antibodies (1:100 dilution, BD Biosciences, no. 553141) to block Fc receptors followed by staining with fluorochrome-conjugated anti–CD11b (1:200 dilution, BioLegend, no. 101226), anti–Ly6C (1:200 dilution, BioLegend, no. 128018), anti–Ly6G (1:200 dilution, BioLegend, no. 127616, 127608 or 127624), anti–ICAM-1 (1:200 dilution, BioLegend, no. 116120 or 116108), and anti–CD24 (1:200 dilution, BioLegend, no. 101806 or 101808) antibodies. Propidium iodide was added before flow cytometry to label dead cells. The samples were then examined using FACSCanto II (BD Biosciences), and data were analyzed using FlowJo V10 (Tree Star).

**Assessing the propagation effects of transfused monocytes to resident immune cells**

BM cells isolated from *ApoE*$^{-/-}$ mice were cultured in complete RPMI 1640 medium supplemented with M-CSF (10 ng/mL) in the presence of 4-PBA (1 mM), or PBS for 5 days as described above. Age- and sex-matched recipient *ApoE*$^{-/-}$ mice were first fed with HFD for 4 weeks to induce atherosclerosis development, and were then transfused with $3 \times 10^6$ in vitro trained BMMs through intravenous injection once a week for a total of 4 weeks. In the experiments to test the influence of transferred monocytes on host monocytes, the *in vitro* trained monocytes were firstly labeled with CFSE (3 μM) before the adoptive transfer. The recipient mice were continuously maintained on HFD. One week after the last cell transfer, the blood, BM, spleen, and aorta single cell suspensions were prepared for flow cytometry as described above. The samples were blocked with anti-CD16/-CD32 antibodies (1:100 dilution, BD Biosciences, no. 553141), followed by staining with fluorochrome-conjugated anti–CD11b (1:200 dilution, BioLegend, no. 101226), anti–Ly6C (1:200 dilution, BioLegend, no. 128018), anti–Ly6G (1:200 dilution, BioLegend, no. 127616, 127608 or 127624), anti–ICAM-1 (1:200 dilution, BioLegend, no. 116120 or 116108), anti–CD24 (1:200 dilution, BioLegend, no. 101806 or 101808), anti–CD200R (1:200 dilution, BioLegend, no. 123916), anti–CD19 (1:200 dilution, BioLegend, no. 115520), anti–CD3 (1:200 dilution, BioLegend, no. 100205), anti–CD8 (1:200 dilution, BioLegend, no. 100705), and anti–CD122 (1:200 dilution, BioLegend, no. 123214) antibodies. Propidium iodide was added before flow cytometry to label dead cells. The samples were then examined using FACSCanto II (BD Biosciences), and data were analyzed using FlowJo V10 (Tree Star).

**Detecting the distribution of transfused monocytes in atherosclerotic mice**

BM cells isolated from *ApoE*$^{-/-}$ mice were cultured in complete RPMI 1640 medium supplemented with M-CSF (10 ng/mL) in the presence of 4-PBA (1 mM), or PBS for 5 days as described above. PBS- and 4-PBA primed monocytes were labeled with CFSE (3 μM, Thermo Fisher Scientific, no. C34572). BM cells isolated from *ApoE*$^{-/-}$ mice were cultured in complete RPMI 1640 medium supplemented with M-CSF (10 ng/mL) in the presence of low-dose LPS (100 pg/mL) for 5 days as described before [6]. Ly6C$^{+}$ monocytes in the culture were purified by flow cytometry sorting and labeled with CellTrace™ Far Red (FR, 1.5 μM, Thermo Fisher Scientific, no. C34572). Equal numbers of CFSE-labeled monocytes and CellTrace™ FR-labeled Ly6C$^{+}$ monocytes were mixed and intravenously injected to age- and sex-matched recipient *ApoE*$^{-/-}$ mice, which had been fed with HFD for 4 weeks. Each recipient mouse was injected with $1 \times 10^6$ CFSE$^{+}$ monocytes and $1 \times 10^6$ FR$^{+}$ monocytes. One hour after monocyte transfusion, the aorta, BM, spleen, and lymph nodes (the cervical, brachial, mesenteric and inguinal lymph nodes were pooled together) were harvested and single cell suspensions were prepared for flow cytometry. Propidium iodide was added before flow cytometry to label dead cells. The numbers of transfused monocytes in each tissue were analyzed using FACSCanto II (BD Biosciences).

**Western blotting**

BMMs from WT C57BL/6 and *Tram*$^{-/-}$ mice were treated with 4-PBA (1 mM), oxLDL (10 μg/mL), or PBS for 5 days as described above. Total protein was extracted with RIPA buffer (Thermo Fisher Scientific, no. 89900) containing a protease inhibitor cocktail (MilliporeSigma, no. P8340) and a phosphatase inhibitor cocktail (MilliporeSigma, no. P5726). Protein samples were subjected to SDS-PAGE (10% acrylamide) and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with blocker (Bio-Rad, no. 1706404) at room temperature for 1 hour and then incubated overnight at 4°C with primary anti–SYK (1:1000 dilution, Cell Signaling Technology, no. 2712S), anti–p-p38 (1:1000 dilution, Cell Signaling Technology, no. 4511S), anti-IRF5 (1:1000 dilution, Cell Signaling Technology, no. 4950S), or anti–β-actin antibody (1:1000 dilution, Cell Signaling Technology, no. 5125), followed by incubation with HRP-conjugated anti–rabbit IgG (1:1000 dilution, Cell Signaling Technology, no. 7074) for 1 hour at room temperature. Blots were developed by an enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific, no. 32106) and detected with Amersham Imager 600 (GE HealthCare).

**Confocal microscopy**

To detect lysosomal distribution of mTOR and SYK, WT BMMs were treated with 4-PBA (1 mM), oxLDL (10 μg/mL), or PBS for 5 days as described above. The cells were rinsed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After blocking with 10% goat serum, the samples were stained with primary rabbit anti–mouse PMP70 antibody (1:1000 dilution), followed by staining with Alexa Fluor 488 goat anti–rabbit secondary antibody (1:1000 dilution). Paraformaldehyde, Triton X-100, goat serum, rabbit anti–mouse PMP70 antibody, and Alexa Fluor 488 goat anti–rabbit secondary antibody were all supplied in the SelectFX Alexa Fluor 488 peroxisome labeling kit (Thermo Fisher Scientific, no. S34201). After washing with PBS, the cells were stained with CoraLite®594 conjugated anti–mTOR antibody (1:300 dilution; Proteintech, no. CL594-66888) or PE conjugated anti–SYK antibody (1:500 dilution; BioLegend, no. 646004). After washing with PBS, the samples were mounted with antifade mountant (Invitrogen). To detect the distribution of TRAM, WT BMMs were treated with 4-PBA (1 mM), oxLDL (10 μg/mL), cholesterol (10 μg/mL) or PBS for 5 days as described above. The cells were rinsed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After blocking with 10% goat serum, the samples were stained with biotin conjugated anti–mouse TRAM antibody (1:500 dilution, Biorbyt, no. orb452158), followed by staining with Alexa Fluor 488 conjugated streptavidin (1:1000 dilution, Thermo Fisher Scientific, no. S32354) After washing with PBS, the samples were mounted with antifade mountant (Invitrogen, no. P36930). The specificity of the primary antibodies was validated by staining the samples with isotype controls, including rabbit IgG isotype control (Thermo Fisher Scientific, no. 31235), PE conjugated mouse IgG (BioLegend, no. 400112), CoraLite®594 conjugated mouse IgG2a isotype control (Proteintech, no. CL594-65208), and biotin conjugated rabbit IgG isotype control (Abcam, no. ab200208). All the samples were observed under an LSM 900 confocal microscope (ZEISS). Images were processed with ZEN lite (ZEISS).

**Immunoprecipitation**

Immunoprecipitation was conducted using Pierce™ Co-Immunoprecipitation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. To screen the proteins associated with Tollip, antibodies against Tollip (NOVUS, no. H00054472-M01) were conjugated to the resin supplied in the kit. WT BMMs were cultured for 5 days, and cell lysate was isolated and then incubated with antibody-conjugated resin overnight at 4°C. Immunoprecipitated products were eluted and subjected to mass spectrometry analysis. To determine the neddylation of PPARγ, antibodies against NEDD8 (Thermo Fisher Scientific, no. MA5-32552) were conjugated to the resin. BMMs from WT and *Tollip*$^{-/-}$ mice were treated with 4-PBA (1 mM) or PBS for 5 days as described above. Equal amounts of cell lysate samples

were incubated with antibody-conjugated resin overnight at 4°C. Immunoprecipitated products were eluted and subjected to Western blotting with primary anti- PPARγ antibody (1:1000 dilution, Thermo Fisher Scientific, no. PA3-821A).

**Mass spectrometry analysis**

Tollip immunoprecipitates were eluted with 8 M urea. The eluate was reduced with 5 mM Dithiothreitol (DTT) and alkylated with 15 mM Iodoacetamide (IAA) followed by overnight trypsin digestion. The peptides were cleaned with home-made C18 stage-tips. The clean peptides were dissolved in 0.1% formic acid and analyzed on a Q-Exactive Orbitrap mass spectrometer coupled with an Easy nanoLC 1000 (Thermo Fisher Scientific, San Jose, CA). Peptides were loaded on to a 15 cm C18 RP analytical column (75 μm inner diameter, 2 μm, 100-Å particle, Acclaim Pepmap RSLC, Thermo-Fisher). Analytical separation of all peptides was achieved with 60-min gradient. A linear gradient of 2 to 30% buffer B over 40 min and 30% to 80% buffer B over 5 min was executed at a 300 nl/min flow rate followed 15-min wash with 100%B, where buffer A was aqueous 0.1% formic acid, and buffer B was 100% acetonitrile and 0.1% formic acid.

LC-MS experiments were performed in a data-dependent mode with full MS at a resolution of 70,000 at m/z 200 followed by high energy collision-activated dissociation-MS/MS of the top 20 most intense ions with a resolution of 17,500 at m/z 200. High energy collision-activated dissociation-MS/MS was used to dissociate peptides at a normalized collision energy of 27 eV in the presence of nitrogen bath gas atoms. Dynamic exclusion was 15.0 seconds. Mass spectra were processed, and peptide identification was performed using the MaxQuant software version 1.5.3.17 (Max Planck Institute, Germany). Protein database searches were performed against the UniProt human protein sequence database. A false discovery rate (FDR) for peptide-spectrum match (PSM) was set at 1% and FDR for protein assignment was set at 5%. Search parameters included up to two missed cleavages at Lys/Arg on the sequence, oxidation of methionine, and protein N-terminal acetylation as a dynamic modification. Carbamidomethylation of cysteine residues was considered as a static modification. Peptide identifications are reported by filtering of reverse and contaminant entries and assigning to their leading razor protein.

**Co-culture assay**

BMMs from B6 SJL mice ($CD45.1^+$) were used as recipient cells; BMMs from WT C57BL/6 mice, *Cd24*$^{-/-}$ mice, and *Tollip*$^{-/-}$ mice ($CD45.2^+$) were used as donor cells. Co-culture assay was performed

using previously established protocol with modifications [6]. Briefly, donor monocytes were treated with 4-PBA (1 mM) or PBS for 5 days, and an equal number of trained donor cells were added to recipient monocytes that had been treated with oxLDL (10 μg/mL) for 3 days. The donor and recipient cells were subsequently co-cultured for additional 2 days in the presence of M-CSF (10 ng/mL). The cells were harvested and stained with anti–CD45.1 (1:200 dilution, BioLegend, no. 110730), anti–CD24 (1:200 dilution, BioLegend, no. 101806), and anti–ICAM-1 (1:200 dilution, BioLegend, no. 116108) antibodies. The surface phenotype of $CD45.1^+$ recipient BMMs was determined with flow cytometry.

**ELISA**

For the detection of CCL5 produced by monocytes *in vitro*, WT and *Tram*$^{-/-}$ BMMs were treated with 4-PBA (1 mM), oxLDL (10 μg/mL), cholesterol (10 μg/mL) or PBS for 5 days as described above, and supernatant was collected. For the detection of CCL5 level *in vivo*, plasma samples were collected from the atherosclerotic mice that received monocyte transfusion as described above. The concentration of CCL5 in the supernatant and plasma samples was assessed with an ELISA kit (R&D Systems, no. MMR00).

**Adhesion assay**

Adhesiveness of monocytes was measured with Vybrant™ Cell Adhesion Assay Kit (Thermo Fisher Scientific, no. V13181) according to published method [81]. WT BMMs were treated with 4-PBA (1 mM) or PBS for 5 days. The cells were harvested and labeled with calcein AM (5 μM) at 37°C for 30 minutes. An equal number of calcein-labeled monocytes were added to each well of a 96-well plate followed by incubation at 37°C for 2 hours. Non-adherent cells were removed by careful washing, and fluorescence from adherent cells was measured.

**Detection of monocyte migratory capacity**

WT BMMs were treated with 4-PBA (1 mM) or PBS as described above. After 5 days, the monocytes were harvested, and the migratory capacity was examined with the method reported previously [82]. Monocytes were suspended in complete RPMI containing M-CSF (10 ng/mL) and loaded into polystyrene crosshatched nanofiber scaffolds. The samples were placed in an incubation chamber set at 37°C with 5% $CO_2$ and observed under Axio Observer Z1 microscope with digitally controlled three-axis stage (ZEISS). The movement of monocytes during 10 hours was recorded with 5-minute intervals between images. Instantaneous velocity of monocytes was analyzed with ImageJ.

**Assessment of monocyte proliferation *in vivo***

Age- and sex-matched *ApoE*$^{-/-}$ mice were fed with HFD and intraperitoneally injected with PBS or 4-PBA (5 mg/kg body weight) as described above. Proliferation of monocytes was analyzed with In Vivo EdU Flow Cytometry 50 Kit 488 (MilliporeSigma, no. BCK488-IV-FC-S) following the manufacturers' instructions. Briefly, EdU (50 mg/kg body weight) was intraperitoneally injected to the mice 4 hours before euthanasia, and BM and spleen were harvested to prepare single cell suspensions. The cells were first stained with anti–CD11b (1:200 dilution, BioLegend, no. 101212), anti–Ly6C (1:200 dilution, BioLegend, no. 128026), and anti–Ly6G (1:200 dilution, BioLegend, no. 127616) antibodies against surface antigens. After fixation and permeabilization, the cells were incubated with Click-iT® reaction cocktail supplied in the kit. EdU$^{+}$ population within Ly6C$^{hi}$ monocytes was examined by flow cytometry.

**Seahorse assay**

Seahorse assay was conducted using a kit (Agilent, no. 103015-100). WT BMMs were seeded the Seahorse Bioscience XF24 (Agilent) cell culture plates and treated with oxLDL (10 μg/mL), low dose LPS (100 pg/mL) or PBS for 5 days. The test was conducted by sequentially exposing the cells to 0.5 μM oligomycin, 0.6 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), and 0.1 μM rotenone/antimycin A, in accordance with the manufacturer's instructions.

**Genomic methylation analysis**

WT BMMs were treated with cholesterol (10 μg/mL), low dose LPS (100 pg/mL), high dose LPS (1 μg/Ml) or PBS for 5 days. Genomic DNA was prepared from cultured monocytes using a DNeasy Blood & Tissue Kit (QIAGEN, no. 69504), bisulfite-treated using an EpiTect Bisulfite Kit (QIAGEN, no. 59104), and processed and hybridized to individual array wells of an Infinium Mouse Methylation BeadChip (Illumina), as previously described [83]. All arrays were processed and run at the Children's Hospital of Philadelphia Center for Applied Genomics. Raw Infinium IDAT files were processed into corrected beta values using the openSesame pipeline (1.14.2; default parameters), and differentially methylated regions (DMRs) were identified using the sesame DML function [84]. PBS- and LPS-treated cultured bone marrow monocyte array values were previously published, but were collected and sequenced at the same time as low dose LPS- and cholesterol-stimulated samples. Infinium BeadChip probe annotations were obtained using the sesame KYCG function [84]. Principal components analysis was performed using the R stats package. Methylation profiling data have been uploaded to the NCBI Gene

Expression Omnibus and are accessible under accession number GSE243358. Gene enrichment GO analyses were performed as described [83].

**Bisulfite pyrosequencing**

We employed the pyrosequencing reaction with bisulfite-treated genomic DNA as previously described [83]. A PyroMark PCR Kit (QIAGEN, no. 978705) was used to amplify the regions of interest with selective primers (Data Set 3), which were subsequently sequenced on a PyroMark Q48 Autoprep instrument.

***In vitro* priming of human monocytes and flow cytometry analyses**

Peripheral blood collected from healthy individuals was purchased from Research Blood Components, LLC (Boston, MA). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque™ (MilliporeSigma, no. GE17-5442-02), and then cultured with 100 ng/ml human M-CSF (Peprotech, no. 300-25) in the presence of 4-PBA (1 mM) or PBS. After 2 days, cells were harvested, blocked with TruStain FcX™ (BioLegend, no. 422302) and stained with fluorochrome-conjugated anti–CD14 (1:50 dilution, BioLegend, no. 325618), anti–CD16 (1:50 dilution, BioLegend, no. 360710), anti–ICAM-1 (1:50 dilution, BioLegend, no. 353114), or anti–CD24 (1:50 dilution, BioLegend, no. 311106) antibodies. Propidium iodide was added before flow cytometry to label dead cells. The samples were then examined using FACSCanto II, and data were analyzed using FlowJo V10.

**Statistics**

Statistical analyses were performed using GraphPad Prism 10.1.2 (GraphPad). All data are expressed as means ± SEM, and the sample number for each data set is provided in figure legends. Normal distributions were examined using the Shapiro-Wilk test. When a normal distribution was confirmed, comparisons between 2 groups were performed by using 2-tailed Student's t test, and comparisons among multiple groups were carried out with 1-way ANOVA followed by Tukey's post hoc test or 2-way ANOVA followed by Šídák's post hoc test. For data that were not normally distributed or data collected from in vivo or ex vivo animal studies with a smaller sample size ($n<10$), comparisons between 2 groups were performed using Mann-Whitney *U* test, and comparisons among multiple groups were performed with Kruskal-Wallis test followed by Dunn's post hoc test. For in vitro cell culture experiments under well-controlled conditions, at least 3 biological replicates were performed assuming normal distribution, according to studies commonly carried out by independent groups [85-94]. In addition, previous studies with larger sample sizes have shown normality in the expression of ICAM-1 [95], CD24 [96], mTOR [97], SYK [98],

IRF5 [99], CCL5 [100], CD93 [101], CD105 [102], CD11c [103], MHCII [103, 104], CD200R [105], and CD122 [106]. $P < 0.05$ was considered statistically significant.

**Online Figures S1-S11**

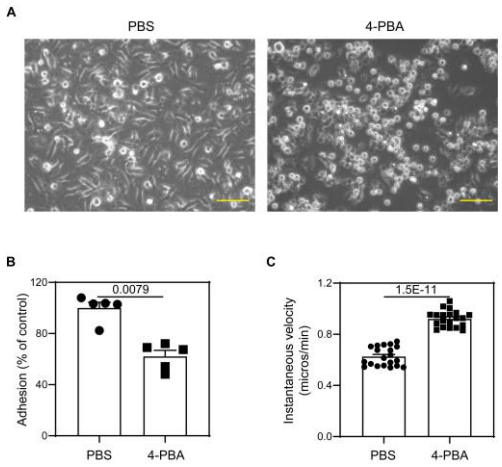


**Figure S1. Treatment with 4-PBA induces morphological and behavioral changes in monocytes.** BMMs from WT C57 BL/6 mice were cultured with M-CSF (10 ng/mL) in the presence 4-PBA (1 mM) or PBS for 5 days. **A,** Morphology of monocytes was observed under a conventional bright field microscope. Scale bars, 50 μm. Adhesiveness (**B**) and migratory capacity (**C**) of monocytes were evaluated. Data in **B** and **C** were analyzed using Mann-Whitney *U* test (n = 5 for each group in **B**; n = 20 for each group in **C**; biological replicates). Error bars represent means ± SEM.

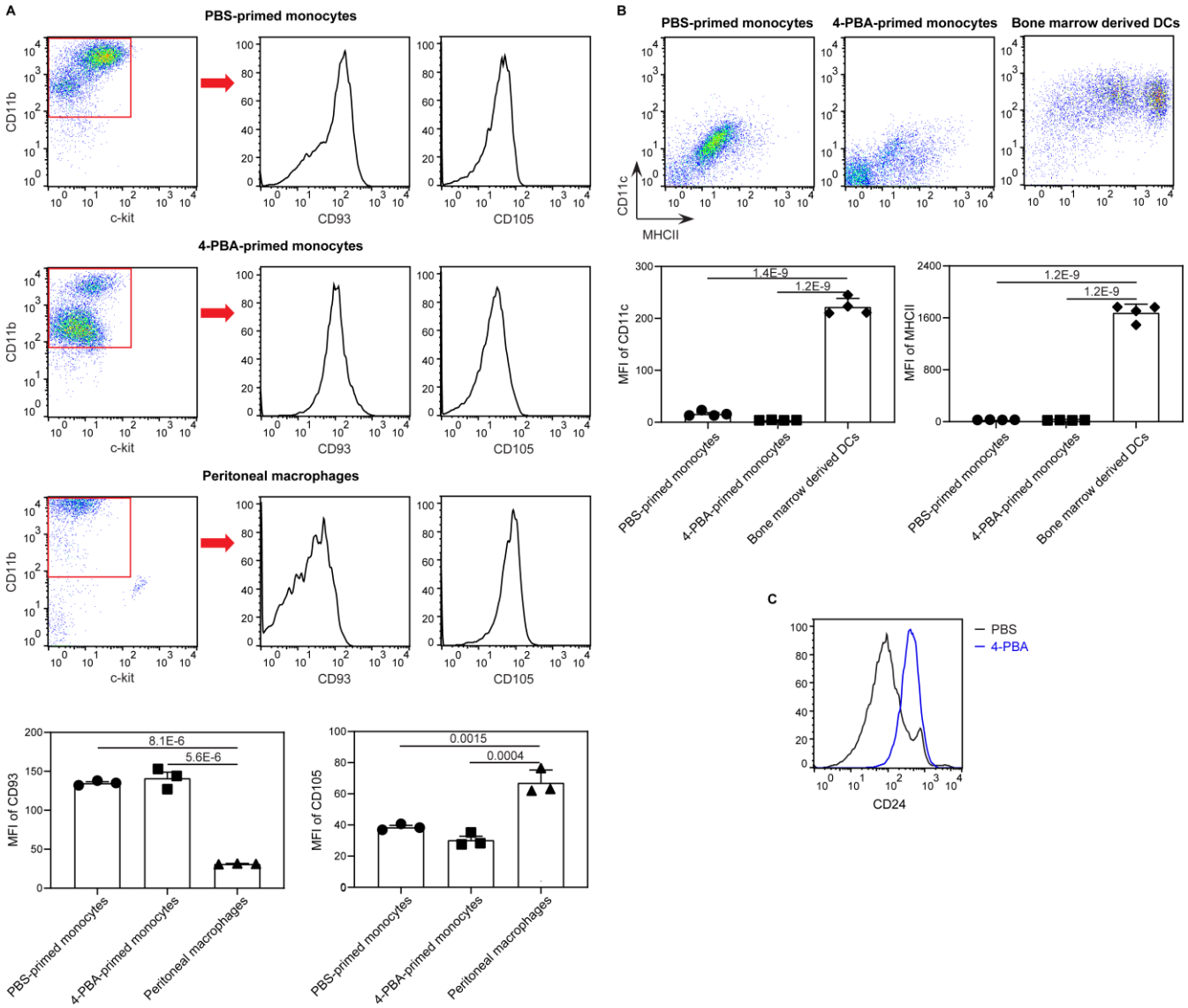


**Figure S2. Characterization of *in vitro* trained monocytes as compared to mature macrophages and dendritic cells.**

**A,** BMMs from WT C57 BL/6 mice were trained in vitro with M-CSF (10 ng/mL) in the presence 4-PBA (1 mM) or PBS for 5 days. As a comparison, resident peritoneal macrophages were isolated from the peritoneal cavity of WT C57 BL/6 mice. Key markers for monocytes (CD11b, CD93), macrophages (CD11b, CD105), DC (CD11b, CD11c, MHCII) as well as hematopoietic stem cells (c-kit) were examined by flow cytometry. **B,** As another comparison, bone marrow derived DCs were prepared and analyzed with flow cytometry. **C,** Representative histograms demonstrating uniformly high expression of CD24 on all 4-PBA-trained monocytes. Data were analyzed using 1-way ANOVA followed by Tukey's post hoc test (n = 3 for each group in **A**; n = 4 for each group in **B**; biological replicates). Error bars represent means ± SEM.

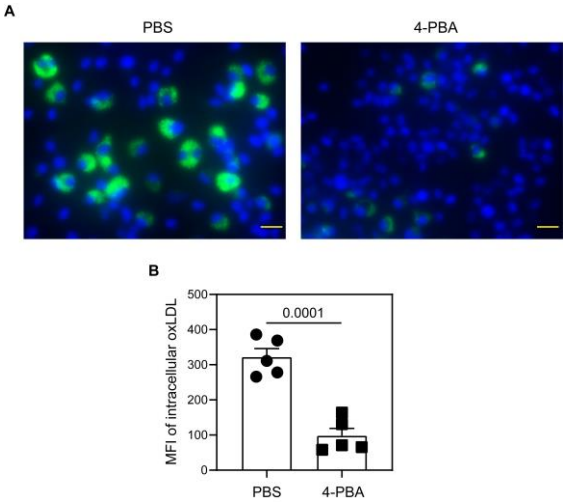


**Figure S3. Foam cells formation is inhibited in 4-PBA-trained monocytes.**

BMMs from WT C57 BL/6 mice were cultured *in vitro* with M-CSF (10 ng/mL) in the presence 4-PBA (1 mM) or PBS for 5 days. The cells were then incubated with DyLight 488 conjugated oxLDL for 24 hours, followed by staining with Hoechst 33342. **A,** The accumulation of oxLDL in monocytes was observed with fluorescence microscopy. Scale bars, 20 μm. **B,** The intracellular level of oxLDL was quantified by flow cytometry. Data were analyzed using Student's t test (n = 5 for each group in **B**; biological replicates). Error bars represent means ± SEM.

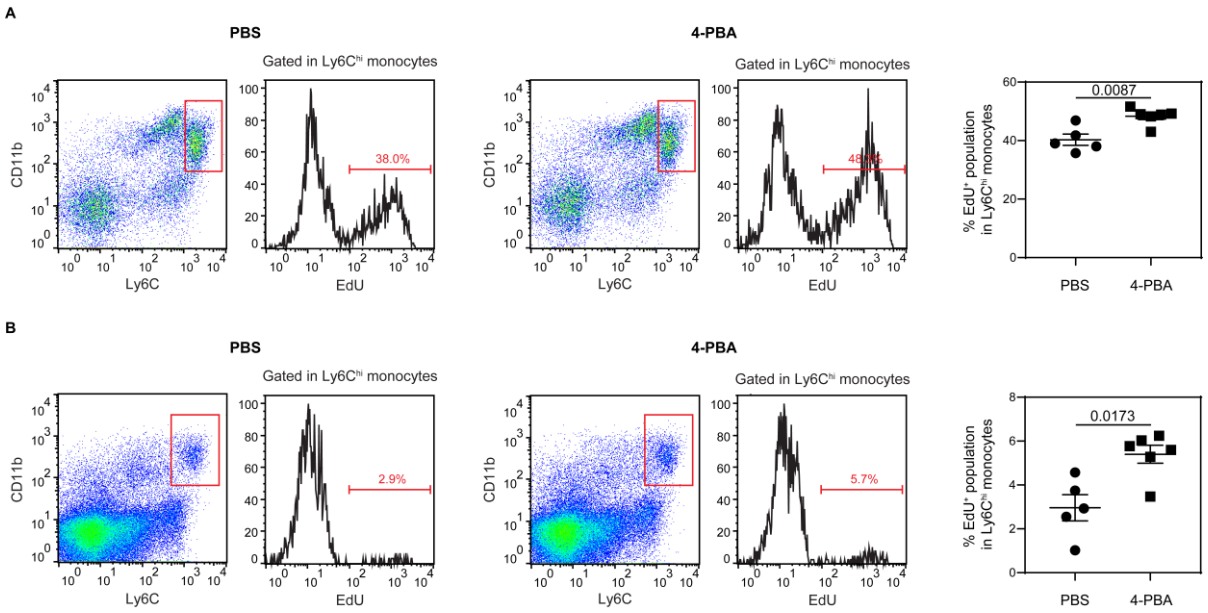


**Figure S4. Injection of 4-PBA promotes monocyte proliferation in atherosclerotic mice.**

Male *ApoE*$^{-/-}$ mice were fed with HFD for 4 weeks and intraperitoneally injected with 4-PBA (5 mg/kg body weight) or PBS every 3 days for additional 4 weeks. EdU (50 mg/kg body weight) was intraperitoneally injected to the mice 4 hours before euthanasia. EdU$^{+}$ proliferating population in CD11b$^{+}$ Ly6G$^{-}$ Ly6C$^{hi}$ monocytes in the BM (**A**) and spleen (**B**) was detected by flow cytometry. Data were analyzed using Mann-Whitney *U* test (n = 5 for PBS group and n = 6 for 4-PBA group; biological replicates). Error bars represent means ± SEM.

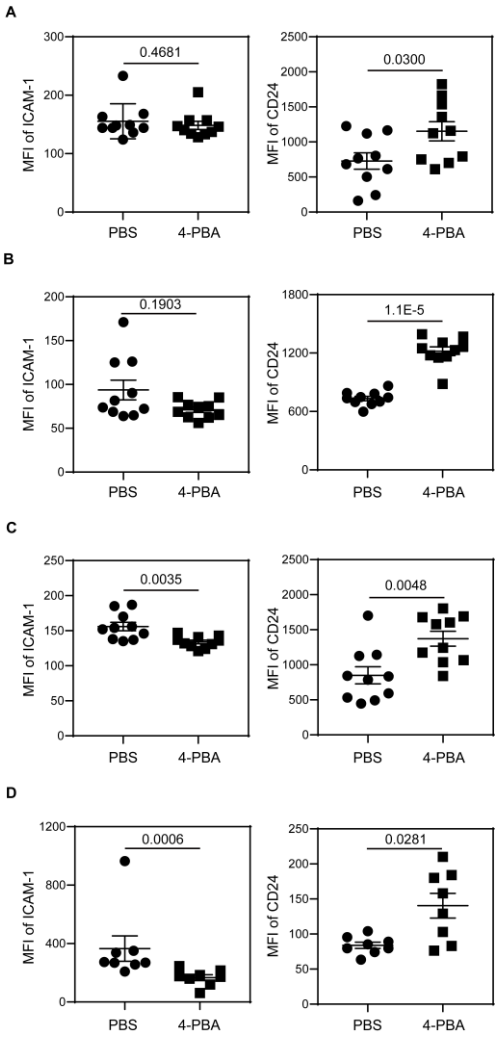


**Figure S5. Administration of 4-PBA reprograms monocytes in vivo.**

Male *ApoE*$^{-/-}$ mice were fed with HFD for 4 weeks and intraperitoneally injected with 4-PBA (5 mg/kg body weight) or PBS every 3 days for additional 4 weeks. **A-D,** Surface expressions of ICAM-1 and CD24 on CD11b$^{+}$ Ly6G$^{-}$ Ly6C$^{low}$ monocytes in the peripheral blood (**A**), BM (**B**), spleen (**C**), and aorta (**D**) were examined by flow cytometry. Data of ICAM-1 in **A** were analyzed using Mann-Whitney U test; data of CD24 in **A** were analyzed using Student's t test; data in **B** and **D** were analyzed using Mann-Whitney U test; data in **C** were analyzed using Student's t test (n = 10 for each group in **A-C**; n = 8 for each group in **D**; biological replicates). Error bars represent means ± SEM.

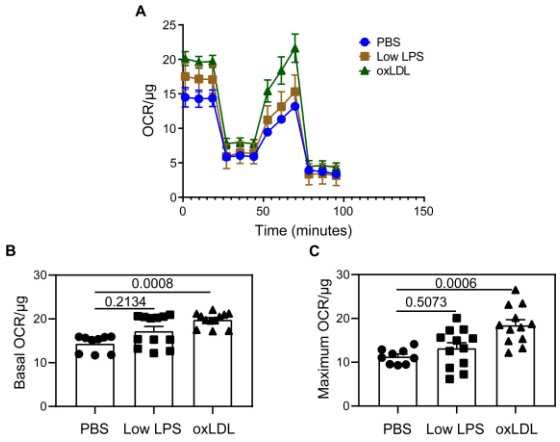


**Figure S6. Low dose LPS and oxLDL exert similar effects in enhancing the mitochondrial respiration of monocytes.**

BMMs from WT C57 BL/6 mice were cultured *in vitro* with M-CSF (10 ng/mL) in the presence of low dose LPS (100 pg/mL), oxLDL (10 µg/mL), or PBS for 5 days. **A,** Oxygen consumption rate (OCR) of each group was determined by Seahorse assay. **B-C,** The basal and maximum OCR/ug levels were quantified. Data in **B** were analyzed using Kruskal-Wallis test followed by Dunn's post hoc test, and data in **C** were analyzed using 1-way ANOVA followed by Tukey's post hoc test (n = 9 for PBS group, n = 12 for Low LPS group and oxLDL group; biological replicates). Error bars represent means ± SEM.

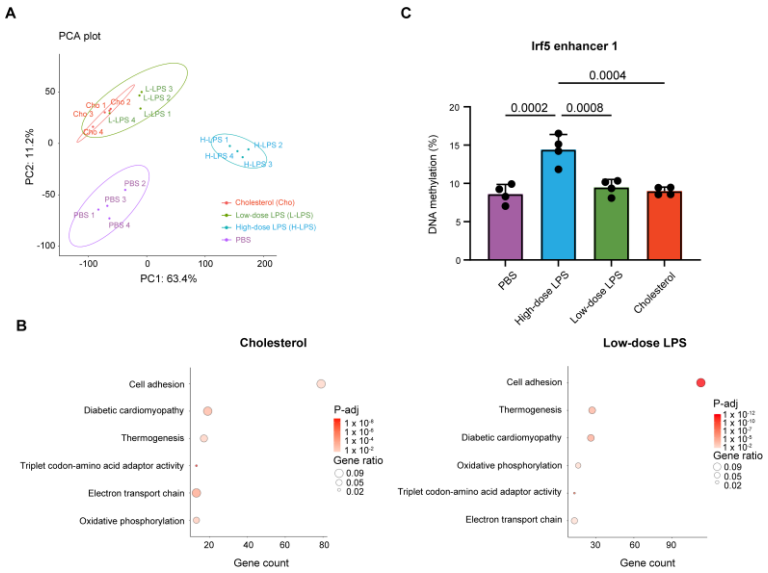


**Figure S7. Low dose LPS and cholesterol stimulation elicits similar DNA methylation reprogramming in monocytes.**

BMMs from WT C57 BL/6 mice were cultured *in vitro* with M-CSF (10 ng/mL) in the presence of low dose LPS (100 pg/mL), high dose LPS (1 µg/mL), cholesterol (10 µg/mL) or PBS for 5 days. DNA methylation profiles were analyzed. **A,** Principal component analysis of differentially methylated CpG probes in monocytes was conducted. Ovals indicate the normal distribution for each treatment. **B,** Dot plots for shared enriched gene ontology (GO) terms at hypomethylated regions in cholesterol- and low-dose LPS-treated cultured BMMs. Dot size indicates the gene ratio associated with each term, and dot color indicates adjusted P values (Holm-Bonferroni corrected). **C,** Bisulfite pyrosequencing DNA methylation levels at Irf5-linked enhancers in PBS control or high-dose LPS-, low-dose LPS-, or cholesterol-stimulated BMMs. Data in **C** were analyzed using 1-way ANOVA followed by Tukey's post hoc test (n = 4 for each group; biological replicates). Error bars represent means ± SEM.

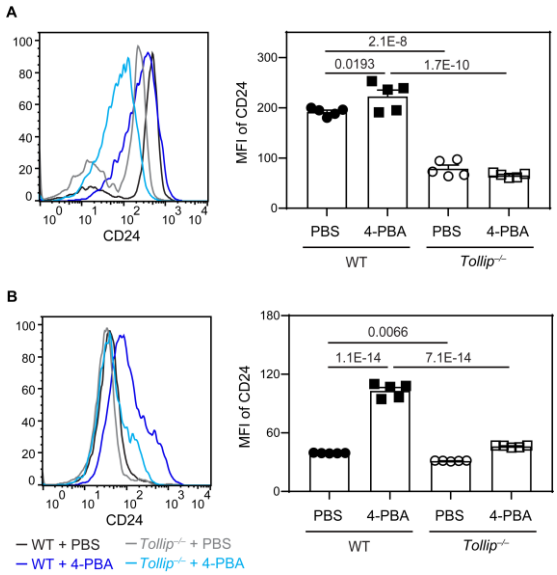


**Figure S8. 4-PBA promotes the expression of CD24 in a Tollip dependent manner.**

BMMs from WT C57 BL/6 mice and *Tollip*$^{-/-}$ mice were cultured *in vitro* with M-CSF (10 ng/mL) in the presence of 4-PBA (1 mM) or PBS for 5 days. Surface expression of CD24 on CD11b$^{+}$ Ly6C$^{low}$ monocytes (**A**) and CD11b$^{+}$ Ly6C$^{-}$ monocytes (**B**) was examined by flow cytometry. Data were analyzed using 2-way ANOVA followed by Šídák's post hoc test (n = 5 for each group; biological replicates). Error bars represent means ± SEM.

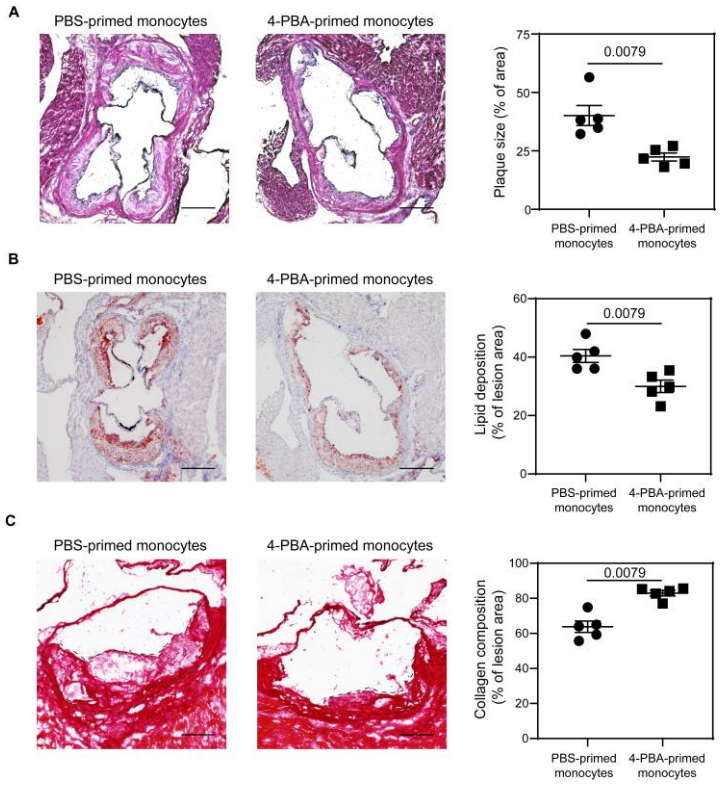


**Figure S9. Adoptive transfer of monocytes polarized by 4-PBA alleviates atherosclerosis.**

Female *ApoE*$^{-/-}$ mice, serving as recipients, were fed with HFD for 4 weeks. BMMs from *ApoE*$^{-/-}$ mice were treated with PBS or 4-PBA (1 mM) for 5 days. PBS- or 4-PBA-polarized monocytes ($3 \times 10^6$ cells per mouse) were then adoptively transferred by intravenous injection to HFD-fed *ApoE*$^{-/-}$ mice once a week for 4 weeks. Tissues were harvested 1 week after the last monocyte transfer. **A,** Representative images of H&E-stained atherosclerotic lesions and quantification of plaque size demonstrated as the percentage of lesion area within aortic root area. Scale bars, 300 μm. **B,** Representative images of Oil Red O–stained atherosclerotic plaques and quantification of lipid deposition within lesion area. Scale bars, 300 μm. **C,** Representative images of Picrosirius red–stained atherosclerotic plaques and quantification of collagen content within lesion area. Scale bars, 100 μm. Data were analyzed using Mann-Whitney *U* test (n = 5 for each group; biological replicates). Error bars represent means ± SEM.

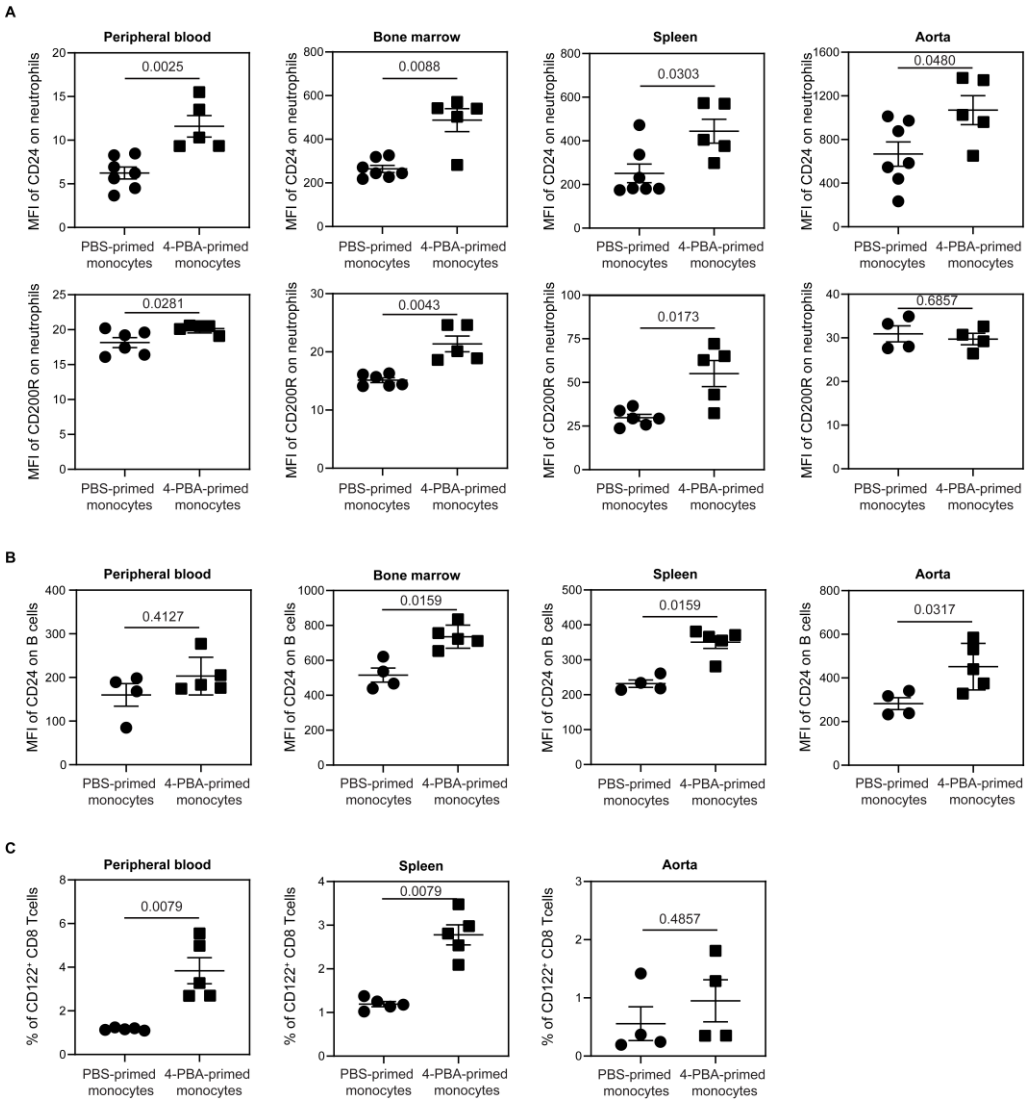


**Figure S10. 4-PBA-trained monocytes propagate anti-inflammatory effects to other resident immune cells.**

Male *ApoE*$^{-/-}$ mice, serving as recipients, were fed with HFD for 4 weeks. BMMs from *ApoE*$^{-/-}$ mice were treated with PBS or 4-PBA (1 mM) for 5 days. PBS- or 4-PBA-trained monocytes ($3 \times 10^6$ cells per mouse) were then adoptively transferred by intravenous injection to HFD-fed *ApoE*$^{-/-}$ mice once a week for 4 weeks. Tissues were harvested 1 week after the last monocyte transfer. **A**, The expressions of CD24 and CD200R on the surface of $Ly6G^+$ neutrophils in the peripheral blood, bone marrow, spleen, and aorta were analyzed by flow cytometry (n = 7 for PBS-primed monocytes group and n = 5 for 4-PBA-primed monocytes group in the analysis of CD24; n = 6 for PBS-primed monocytes group in the analysis of CD200R in the peripheral blood, bone marrow and spleen, n = 4 for PBS-primed monocytes group in the analysis of CD200R in the aorta, n = 5 for 4-PBA-primed monocytes group in the analysis of CD200R in the peripheral blood, bone marrow and spleen, and n = 4 for 4-PBA-primed monocytes group in the

analysis of CD200R in the aorta; biological replicates). **B**, CD24 expressions on the surface of $CD19^+$ B cells in the peripheral blood, bone marrow, spleen, and aorta were analyzed by flow cytometry (n = 4 for PBS-treated monocytes group and n = 5 for 4-PBA-trained monocytes group; biological replicates). **C**, The frequencies of $CD3^+$ $CD8^+$ $CD122^+$ T cells in the peripheral blood, spleen, and aorta were determined by flow cytometry (n = 5 for each group in the analysis of peripheral blood and spleen samples, and n = 4 for each group in the analysis of aorta samples; biological replicates). Data were analyzed using Mann-Whitney *U* test. Error bars represent means ± SEM.

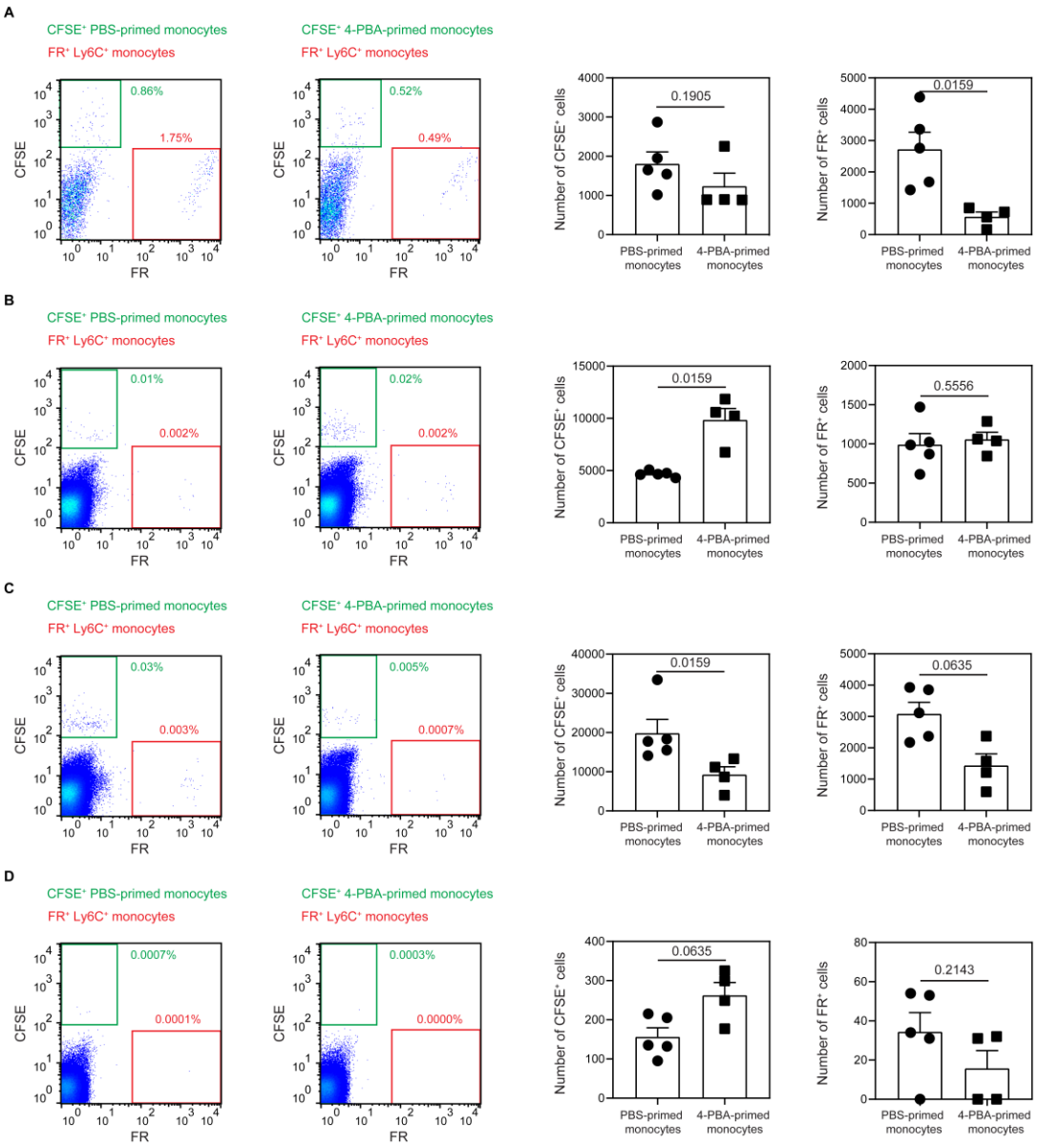


**Figure S11. Transfused monocytes infiltrate the inflamed tissues and modulate the immune environment of atherosclerotic mice.**

Male *ApoE*$^{-/-}$ mice, serving as recipients, were fed with HFD for 4 weeks. BMMs from *ApoE*$^{-/-}$ mice were treated with PBS or 4-PBA (1 mM) for 5 days and then labeled with CFSE. In a separate cell culture system, BMMs from *ApoE*$^{-/-}$ mice were treated with low dose LPS (100 pg/mL) for 5 days, and Ly6C$^{+}$ monocytes in the culture were sorted with flow cytometry, followed by staining with CellTrace™ FR. Equal amount of CFSE$^{+}$ monocytes and FR$^{+}$ monocytes were mixed and then adoptively transferred by intravenous injection to recipient mice ($1 \times 10^6$ CFSE$^{+}$ monocytes + $1 \times 10^6$ FR$^{+}$ monocytes per mouse). Tissues were harvested 24 hours after monocyte transfer, and the distribution of injected monocytes in the aorta (**A**), bone marrow (**B**), spleen (**C**), and lymph nodes (**D**) was analyzed with flow cytometry. Data

were analyzed using Mann-Whitney *U* test (n = 5 for PBS-primed monocytes group and n = 4 for 4-PBA-primed monocytes group; biological replicates). Error bars represent means ± SEM.

**Online Video 1. Migratory capacity of PBS-trained monocytes**

**Online Video 2. Migratory capacity of 4-PBA-trained monocytes**

**Data Set 1. GO terms associated with different gene methylation**

**Data Set 2. Result of mass spectrometry analysis**

**Data Set 3. Primers used for bisulfite pyrosequencing**