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ORIGINAL RESEARCH

Monocytes Reprogrammed by 4-PBA Potently Contribute to the Resolution of Inflammation and Atherosclerosis

Shuo Geng, Ran Lu, Yao Zhang, Yajun Wu, Ling Xie, Blake A. Caldwell, Kisha Pradhan, Ziyue Yi, Jacqueline Hou, Feng Xu, Xian Chen, Liwu Li

BACKGROUND: Chronic inflammation initiated by inflammatory monocytes underlies the pathogenesis of atherosclerosis. However, approaches that can effectively resolve chronic low-grade inflammation targeting monocytes are not readily available. The small chemical compound 4-phenylbutyric acid (4-PBA) exhibits broad anti-inflammatory effects in reducing atherosclerosis. Selective delivery of 4-PBA reprogrammed monocytes may hold novel potential in providing targeted and precision therapeutics for the treatment of atherosclerosis.

METHODS: Systems analyses integrating single-cell RNA sequencing and complementary immunologic approaches characterized key resolving characteristics as well as defining markers of reprogrammed monocytes trained by 4-PBA. Molecular mechanisms responsible for monocyte reprogramming were assessed by integrated biochemical and genetic approaches. The intercellular propagation of homeostasis resolution was evaluated by coculture assays with donor monocytes trained by 4-PBA and recipient naive monocytes. The in vivo effects of monocyte resolution and atherosclerosis prevention by 4-PBA were assessed with the high-fat diet-fed *ApoE*$^{-/-}$ mouse model with IP 4-PBA administration. Furthermore, the selective efficacy of 4-PBA-trained monocytes was examined by IV transfusion of ex vivo trained monocytes by 4-PBA into recipient high-fat diet-fed *ApoE*$^{-/-}$ mice.

RESULTS: In this study, we found that monocytes can be potently reprogrammed by 4-PBA into an immune-resolving state characterized by reduced adhesion and enhanced expression of anti-inflammatory mediator CD24. Mechanistically, 4-PBA reduced the expression of ICAM-1 (intercellular adhesion molecule 1) via reducing peroxisome stress and attenuating SYK (spleen tyrosine kinase)-mTOR (mammalian target of rapamycin) signaling. Concurrently, 4-PBA enhanced the expression of resolving mediator CD24 through promoting PPARγ (peroxisome proliferator-activated receptor γ) neddylation mediated by TOLLIP (toll-interacting protein). 4-PBA-trained monocytes can effectively propagate anti-inflammation activity to neighboring monocytes through CD24. Our data further demonstrated that 4-PBA-trained monocytes effectively reduce atherosclerosis pathogenesis when administered in vivo.

CONCLUSIONS: Our study describes a robust and effective approach to generate resolving monocytes, characterizes novel mechanisms for targeted monocyte reprogramming, and offers a precision therapeutics for atherosclerosis based on delivering reprogrammed resolving monocytes.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ immunity, innate ■ inflammation ■ monocytes ■ therapeutics

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Novelty and Significance

What Is Known?

- Inflammatory monocytes trained by metabolic danger signals such as oxLDL (oxidized low-density lipoprotein) exhibit elevated recruitment and adhesion to vasculature, which can exacerbate atherosclerosis pathogenesis.
- 4-phenylbutyric acid (4-PBA) training can generate monocytes with general anti-inflammatory features.
- Systemic administration of 4-PBA, a derivative compound of butyric acid, can reduce systemic inflammation and alleviate atherosclerosis pathogenesis.

What New Information Does This Article Contribute?

- TRAM (Trif-related adapter molecule) serves as a generic stress sensor for oxLDL in initiating sustained low-grade inflammatory monocyte memory represented by elevated levels of ICAM-1 (intercellular adhesion molecule 1) and chemokine ligand 5 (CCL5), which can be effectively attenuated by the treatment with 4-PBA.
- Anti-inflammatory resolving monocytes trained by 4-PBA are less adhesive, with a salient signature of elevated CD24 expression, and can propagate its anti-inflammatory features to neighboring immune cells. 4-PBA reprograms anti-inflammatory resolving monocytes through Tollip-mediated PPARγ (peroxisome proliferator-activated receptor γ) activation.
- When transfused in vivo, 4-PBA–trained monocytes can alleviate inflammation and propagate immune homeostasis within vasculature tissue niche, and reduce atherosclerosis pathogenesis.

Monocytes have divergent roles in either promoting or reducing the pathogenesis of atherosclerosis, likely dependent upon their polarization states. Exact mechanisms of monocyte polarization triggered by oxLDL, as well as effective intervention of monocyte inflammatory polarization are not well characterized. This study demonstrates that 4-PBA can effectively train resolving monocytes through Tollip-mediated PPARγ activation and CD24 expression. CD24 on resolving monocytes are responsible for propagating anti-inflammatory homeostasis to neighboring immune cells within the vasculature tissues, and reducing atherosclerosis. These findings suggest that monocytes trained by 4-PBA may provide a novel cell-based therapeutic approach for atherosclerosis.

Nonstandard Abbreviations and Acronyms

4-PBA	4-phenylbutyric acid
BMM	bone marrow–derived monocyte
CCL5	chemokine ligand 5
CFSE	carboxyfluorescein succinimidyl ester
DC	dendritic cell
HFD	high-fat diet
ICAM-1	intercellular adhesion molecule 1
IRF5	interferon regulatory factor 5
M-CSF	macrophage colony–stimulating factor
mTOR	mammalian target of rapamycin
NEDD8	neural precursor cell expressed, developmentally downregulated 8
oxLDL	oxidized low-density lipoprotein
PPARγ	peroxisome proliferator-activated receptor γ
SYK	spleen tyrosine kinase
TOLLIP	toll-interacting protein
TRAM	Trif-related adapter molecule

Atherosclerosis is a complex chronic inflammatory disease culminating in the buildup of lipid-laden plaques within arterial vessels. Existing translational efforts are largely focused on developing chemical- or biologic-based therapies that target metabolic and inflammatory processes.[1–3] However, these approaches suffer from inherent drawbacks such as limited delivery precision into inflamed tissues and unintended side effects. As an alternative approach, mobilizing immune cells naturally equipped with effective physiological tropism into inflamed tissues may hold powerful therapeutic potentials in circumventing the drawbacks associated with molecule-based therapies.

To fully harness immune cell-based therapies, fundamental studies navigating the complex dynamics of atherosclerosis-associated immune cells are urgently needed. Emerging studies have identified monocytes as one of the most relevant immune cells involved in both the progression and resolution of atherosclerosis.[4–7] The initial polarization of inflammatory monocyte subsets (eg, Ly6C^{hi} murine monocytes or CD14$^+$; CD16$^+$ intermediate human monocytes) may serve as a key trigger for tissue infiltration, adhesion, and subsequent foamy macrophage formation.[8,9] Strategies that can prevent the initial expansion of inflammatory monocytes would assist in the attenuation of atherosclerosis pathogenesis. Intriguingly, in vivo studies have also revealed that certain subsets of Ly6C^{hi} monocytes with limited proliferation potential can serve as precursors for anti-inflammatory resolving monocytes beneficial for atherosclerosis regression.[10–13] Our recent in vitro studies with single-cell RNA sequencing pseudo-time analyses further validated these in vivo observations

by revealing the presence of distinct subsets of proliferative monocytes adopting either inflammatory or resolving properties.[14,15] Following dedifferentiation into a proliferative Ly6C^{hi} high state,[6,14,15] monocytes bifurcate into either an inflamed state with the sustained stimulation of a low-grade inflammatory signal[6] or remain in an anti-inflammatory proliferative state.[14,15] A clear characterization and derivation of resolving monocytes may facilitate future therapeutic development of monocyte-based precision therapies against atherosclerosis.

We recently reported that monocytes trained with the small chemical compound 4-phenylbutyric acid (4-PBA) are arrested at the proliferative resolving state.[14,15] 4-PBA is a potent peroxisome activator and can induce the expression of a PPAR-mediated peroxisome synthesis program as well as other anti-inflammatory mediators.[16–18] Independent studies reported that the administration of 4-PBA into whole animals can reduce the progression of experimental atherosclerosis.[19–21] To avoid the potential toxic effects of the systemic 4-PBA administration, we tested the approach of employing 4-PBA-trained monocytes in delivering targeted therapeutics against atherosclerosis. Through integrated in vitro and in vivo studies, we defined key resolving characteristics of 4-PBA programmed monocytes, their underlying molecular mechanisms, and their therapeutic potential in attenuating atherosclerosis.

METHODS

Data Availability

All supporting data of this study are available from the corresponding author upon reasonable request. RNA-seq data have been uploaded to the NCBI Gene Expression Omnibus and are accessible under accession number GSE160450. Methylation profiling data have been uploaded to the NCBI Gene Expression Omnibus and are accessible under accession number GSE243358. For detailed experimental methods, materials, and statistical analysis, please see the Supplemental Material and the Major Resource Table.

RESULTS

Monocytes Trained With 4-PBA Exhibit Potent Anti-Inflammatory Resolving Characteristics

Administration of 4-PBA was independently shown to be beneficial in reducing atherosclerosis,[19–21] indicating that 4-PBA may serve as a promising atheroprotective agent. Our in vitro studies reveal that monocytes treated with 4-PBA are arrested in an anti-inflammatory state, suggesting monocytes may be responsible for atheroprotective benefits of 4-PBA in animal models. To better define and harness the therapeutic potential of resolving monocytes programed by 4-PBA, we re-analyzed the gene expression profile of monocytes treated with 4-PBA

and monitored for key signatures indicative of monocyte differentiation and activation. As shown in Figure 1, monocyte subsets trained by 4-PBA express monocytic markers such as *Cd34* and have reduced expression of mature macrophage markers such as *Adgre1* (F4/80), consistent with morphological observation (Figure S1A) demonstrating the monocytic nature of 4-PBA–trained monocytes as compared with control monocyte cultures. Our scRNAseq data also suggest that 4-PBA–trained monocyte subsets express higher levels of genes representative of elevated proliferative potentials such as *Hmmr*, *Mki67,* and *Stmn1* (Figure 1A through 1C). In contrast to traditional culture media supplemented with L929 supernatant which includes additional growth and differentiation factors in addition to M-CSF (macrophage colony-stimulating factor), low-dose pure M-CSF selectively maintains the survival of monocyte-like cells without inducing cellular activation and differentiation.[6,22] We have systematically characterized the 5-day culture model of murine bone marrow monocytes supplemented by low-dose M-CSF, achieving robust maintenance of >99% monocytes by the end of the 5-day culture period.[6] To further validate the culture system, we did comparative flow analyses of culture monocytes with fully differentiated macrophages as well as dendritic cells (DCs). Both PBS- and 4-PBA-trained cells cultured for 5 days supplemented with M-CSF exhibited high levels of the myeloid marker CD11b but were completely negative for the progenitor marker c-kit, indicating an absence of hematopoietic progenitor cell contamination within our culture system (Figure S2A). As compared with isolated peritoneal resident macrophages, both PBS- and 4-PBA-trained cells expressed high levels of monocyte marker CD93 and low levels of macrophage marker CD105,[23] further confirming that our cultured cells were monocyte-like cells rather than fully differentiated macrophages (Figure S2A). Furthermore, compared with bone marrow–derived DCs, PBS- or 4-PBA–trained monocytes were negative for the DC marker CD11c and expressed low levels of MHCII, indicating that DCs were not generated in the culture system (Figure S2B).

We next examined pro- and anti-inflammatory gene signatures of trained monocytes by 4-PBA and observed lower levels of adhesion molecules such as ICAM-1 (intercellular adhesion molecule 1) and drastically elevated anti-inflammatory mediators such as CD24. ICAM-1 plays a pivotal role as a proinflammatory biomarker that facilitates monocyte adhesion to aortic endothelial cells and contributes to the development of atherosclerotic plaques.[24,25] Functionally, we validated that 4-PBA-trained monocytes exhibit significantly reduced adhesion capacity and elevated migratory potential (Figure S1B and S1C; Videos S1 and S2). We further evaluated the foam cell formation potential in monocytes trained by 4-PBA. PBS- and 4-PBA-trained monocytes were incubated with fluorescently conjugated oxLDL (oxidized
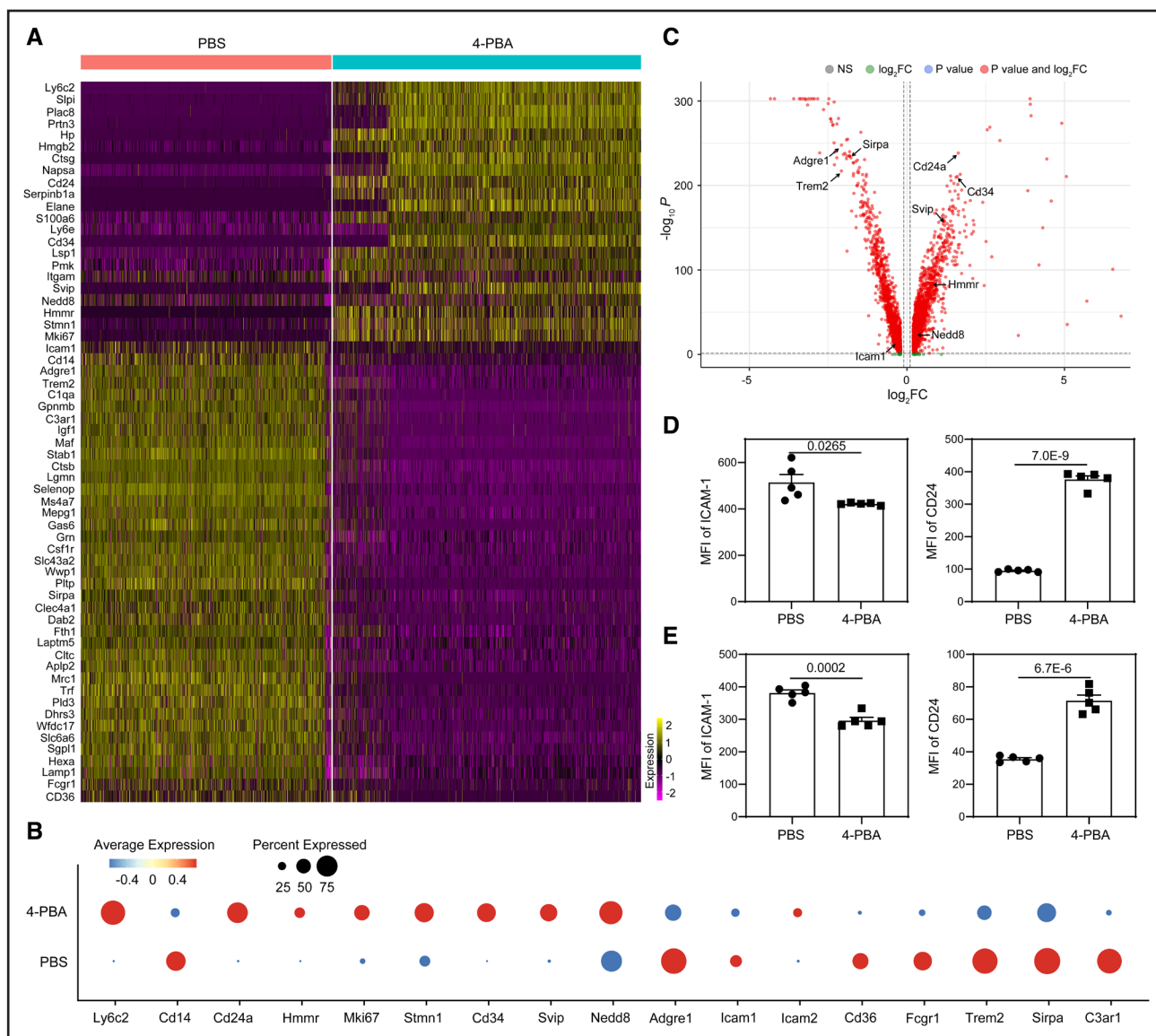
Figure 1. Treatment with 4-phenylbutyric acid (4-PBA) induces anti-inflammatory resolving characteristics of monocytes.
A through **D**, Bone marrow–derived monocytes (BMMs) from wild-type (WT) C57 BL/6 mice were cultured in vitro with macrophage colony-stimulating factor (M-CSF; 10 ng/mL) in the presence 4-PBA (1 mmol/L) or PBS for 5 days. Single-cell RNA sequencing (scRNAseq) was performed, and data sets were processed to compare naive murine monocytes with monocytes trained by 4-PBA. **A,** Heatmaps demonstrating representative genes differentially expressed in different clusters of monocytes challenged with 4-PBA. **B**, Dot plot comparison of representative genes differentially expressed between PBS- vs 4-PBA–trained monocytes. **C**, Volcano plot showing differentially expressed genes between PBS- vs 4-PBA–trained monocytes. **D**, Surface expression of ICAM-1 (intercellular adhesion molecule 1) and CD24 on CD11b+ monocytes cultured was analyzed by flow cytometry. **E**, Peripheral blood mononuclear cells (PBMCs) isolated from healthy human individuals were cultured in vitro with M-CSF (100 ng/mL) in the presence 4-PBA (1 mmol/L) or PBS for 2 days. Surface expression of ICAM-1 and CD24 on total monocytes (including CD14hi, CD16hi, and intermediate monocytes) was analyzed by flow cytometry. Data in **D** and **E** (n=5 for each group, biological replicates) were analyzed with Student t test. Error bars represent means±SEM.

low-density lipoprotein), and the accumulation of intracellular oxLDL was visualized by fluorescence microscopy and quantified by flow cytometry. We observed that 4-PBA treatment significantly reduced the intracellular oxLDL accumulation, suggesting that the form cell formation was remarkably suppressed in 4-PBA-trained monocytes (Figure S3).

The expression of CD24 is found on the surface of various hematopoietic cell populations and thus

CD24 expression is higher in progenitor cells and less-differentiated cells as compared with terminally differentiated cells.[26] CD24 has also been well documented to interact with Siglec-10 to suppress inflammatory responses of innate immune cells to infection, sepsis, and chronic inflammatory diseases.[27] Based on the clues learned from the scRNAseq analyses, we further independently validated the expression of ICAM-1 and CD24 in 4-PBA-treated cells at the protein levels, with

our previously established murine monocyte culture system.[16,28] In line with our single-cell RNA sequencing results, flow cytometry demonstrated that treatment with 4-PBA for 5 days significantly reduced ICAM-1 expression on murine bone marrow–derived monocytes (BMMs) compared with PBS control cells (Figure 1D). In terms of CD24, monocytes trained by 4-PBA were 100% homogenously CD24 $^{++}$ and clearly separated from the populations of control monocytes (Figure S2C).

To validate the translational relevance of these findings, we tested whether 4-PBA can elicit similar prore-solving characteristics in human primary monocytes. We cultured human peripheral blood mononuclear cells with either PBS or 4-PBA and observed a significant reduction of ICAM-1 and elevation of CD24 in 4-PBA-treated cells (Figure 1E). Our data reveal that 4-PBA programmed monocytes adopt key resolving signatures of monocytes with uniformly higher levels of CD24 expression.

4-PBA Injection Reprograms Monocytes In Vivo and Reduces Atherosclerosis

We next tested whether these key signatures of resolving monocytes can be recapitulated in mice treated with 4-PBA in vivo. We initially confirmed that administration of 4-PBA may alleviate atherosclerosis progression in our experimental mouse model. Whereas previous studies administrated 4-PBA through drinking water,[19,20] we elected to treat high-fat diet (HFD)–fed *ApoE*$^{-/-}$ mice with 4-PBA via intraperitoneal injection (100 mg/kg body weight). *ApoE*$^{-/-}$ mice were HFD fed for 4 weeks to induce the development of atherosclerosis and then received IP injections of 4-PBA every 3 days for an additional 4 weeks, during which the mice were continuously HFD fed. In comparison to the mice injected with vehicle control (PBS), those injected with 4-PBA exhibited significantly reduced size of atherosclerotic plaques, as evident from Hematoxylin and Eosin staining (Figure 2A), as well as remarkably diminished lipid deposition in the plaques, as shown by Oil Red O staining (Figure 2B). Moreover, the injection of 4-PBA significantly increased the collagen content within plaques, indicating an improvement in plaque stability (Figure 2C). The plasma levels of total cholesterol, free cholesterol, and triglyceride were also significantly reduced after 4-PBA administration (Figure 2D). Our data validated that long-term IP injection of 4-PBA drastically reduced the atherosclerotic burden in experimental mice.

Consistent with prior independent studies, we also observed proliferating Ly6C^{hi} monocytes in atherosclerotic mice, as evidenced by in vivo Edu incorporation (Figure S4). Notably, administration of 4-PBA substantially increased the frequency of these proliferating monocytes in both the bone marrow and spleen (Figure S4). It is particularly noteworthy that relative to mice receiving vehicle controls, mice injected with 4-PBA exhibited significantly

reduced levels of ICAM-1 and elevated levels of CD24 on Ly6C^{hi} and Ly6C^{low} monocytes harvested from the peripheral blood, bone marrow, and spleen, as well as aorta (Figure 2E through 2H; Figure S5). These data further substantiate that injection of 4-PBA prompts the polarization of monocytes to a resolving state in atherosclerotic mice, which potentially contributes to the amelioration of atherosclerosis pathogenesis.

4-PBA Reduces Monocyte Adhesion by Restoring Pexophagy and Reducing mTOR Signaling

We further examined the molecular and cellular mechanisms responsible for the reprogramming of resolving monocytes by 4-PBA. Previous studies suggest that atherosclerotic stress factors such as oxLDL and/or cholesterol can initiate the inflammatory polarization of monocyte via activation of mTORC1, and that application of mTOR (mammalian target of rapamycin) inhibitor rapamycin effectively reduces atherosclerosis progression.[29] mTOR can be activated on the subcellular platforms of lysosomes or peroxisomes by reactive oxygen species (ROS) resulting from defective pexophagy.[30,31] Consistent with previous reports, we noted that in contrast to high-dose lipopolysaccharide treatment, oxLDL or low-dose lipopolysaccharide did not compromise mitochondria function, but rather improved mitochondria respiration[32] (Figure S6). We, therefore, focused on the effects of oxLDL on inducing the dysfunction of peroxisomes. Flow cytometry analysis revealed a remarkable elevation of intracellular mTOR levels in monocytes after treatment with oxLDL for 5 days (Figure 3A). Intriguingly, we observed that oxLDL drastically increased the subcellular localization of mTOR at PMP70^{+} peroxisomes (Figure 3B). Moreover, oxLDL also increased cellular levels and peroxisomal distribution of SRC kinase SYK (spleen tyrosine kinase; Figure 3C and 3D), which was shown to form a mutually activating positive feedback loop with mTOR with the support of subcellular ROS.[33] These findings corroborate prior studies suggesting that peroxisomes may act as a pivotal platform for sustaining the inflammatory signaling cascade.[34]

We previously reported that membrane-associated adaptor TRAM (Trif-related adapter molecule; also known as TICAM-2) can transmit the signal of super-low dose lipopolysaccharide and is responsible for causing peroxisomal dysfunction,[6,35] as well as inducing key low-grade inflammatory mediators.[6,36] Given that oxLDL or free cholesterol can cause generic membrane stress,[37–39] and that TRAM is one of the few innate membrane adaptors with lipid anchors to stressed membrane region,[40] we next tested the hypothesis that TRAM serves as a general membrane stress sensor capable of mediating the inflammatory effects of oxLDL or free cholesterol in addition to low-dose lipopolysaccharide. As shown
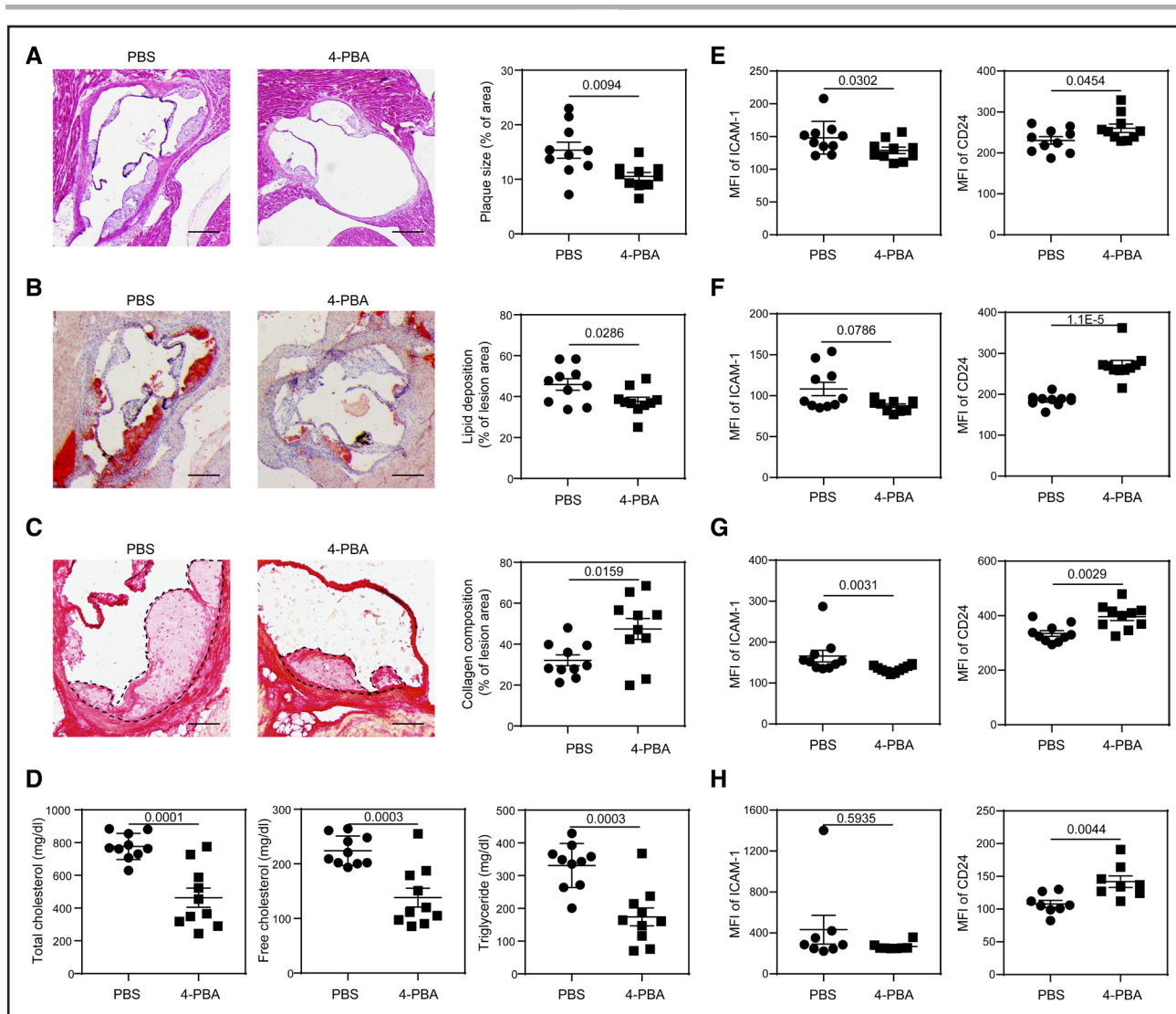
Figure 2. Administration of 4-phenylbutyric acid (4-PBA) alleviates atherosclerotic pathogenesis.
Male *ApoE$^{-/-}$* mice were fed with high-fat diet (HFD) for 4 weeks and intraperitoneally injected with 4-PBA (5 mg/kg body weight) or PBS every 3 days for additional 4 weeks. **A**, Representative images 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. **D**, Detection of total cholesterol, free cholesterol, and triglyceride levels in the plasma. **E** through **H**. Surface expressions of ICAM-1 (intercellular adhesion molecule 1) and CD24 on CD11b$^+$ Ly6G$^-$ Ly6C^{hi} monocytes in the peripheral blood (**E**), bone marrow (BM; **F**), spleen (**G**), and aorta (**H**) were examined by flow cytometry. Data in **A** through **E** were analyzed using Student *t* test, and data in **F** through **H** were analyzed using Mann-Whitney *U* test (n=10 for each group in **A** through **G**; n=8 for each group in **H**; biological replicates). Error bars represent means±SEM.

in Figure 4A, both oxLDL and free cholesterol promoted TRAM clustering on the cell membrane. We further examined cellular activation in wild-type (WT) and TRAM-deficient monocytes challenged with oxLDL. We found that the potent activation of p38 as well as SYK by oxLDL is observed in WT but not *Tram$^{-/-}$* monocytes (Figure 4B and 4C). Functionally, oxLDL or free cholesterol induced ICAM-1 expression and chemokine ligand 5 (CCL5) secretion in WT monocytes but not TRAM-deficient monocytes (Figure 4D). Our data further confirm that oxLDL induces monocyte activation via a generic membrane-associated stress sensor TRAM.

To further validate that low-dose lipopolysaccharide and cholesterol can similarly reprogram monocyte memory, we then examined genome-wide methylation profiles of monocytes trained by varying dosages of lipopolysaccharide or cholesterol. Indeed, principle component analyses showed that exhausted monocytes caused by prolonged challenges with high-dose lipopolysaccharide clustered into a distinct population in terms of their methylation profile. By contrast, monocytes programed by either low-dose lipopolysaccharide or cholesterol clustered together (Figure S7A). We further performed gene ontology (GO) analyses of gene methylation profiles
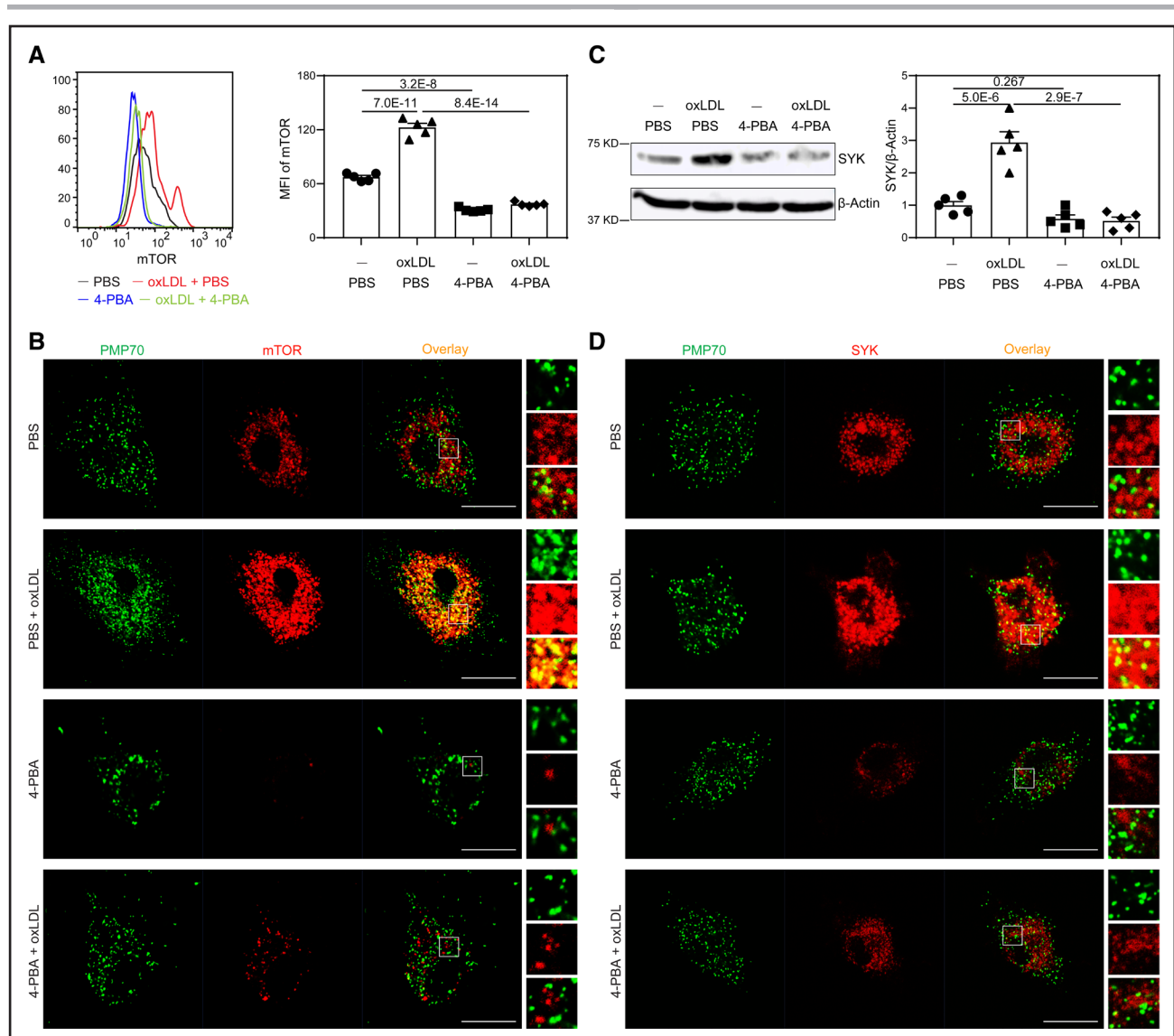
Figure 3. 4-phenylbutyric acid (4-PBA) inhibits mTOR (mammalian target of rapamycin) signaling and restores peroxisome homeostasis in monocytes.
Bone marrow–derived monocytes (BMMs) from wild-type (WT) C57 BL/6 mice were cultured in vitro with M-CSF (macrophage colony-stimulating factor; 10 ng/mL) in the presence of oxLDL (oxidized low-density lipoprotein; 10 µg/mL), 4-PBA (1 mmol/L), or PBS for 5 days. **A**, Representative histogram and quantification of mTOR level in CD11b+ Ly6Chi monocytes as determined by flow cytometry. **B**, Monocytes were stained with anti-PMP70 and anti-mTOR antibodies, and the localization of PMP70+ peroxisomes and mTOR was examined by confocal microcopy. Scale bars, 10 µm. **C**, Protein level of SYK in BMMs was examined by Western blotting, and SYK expression was quantified after normalizing to β-Actin expression. **D**, Monocytes were stained with anti-PMP70 and anti-SYK antibodies, and the localization of peroxisomes and SYK was examined by confocal microcopy. Scale bars, 10 µm. Data in **A** and **C** were analyzed using 2-way ANOVA followed by Šídák post hoc test (n=5 for each group; biological replicates). Error bars represent means±SEM.

and validated the resemblance of monocytes trained by low-dose lipopolysaccharide or cholesterol (Figure S7B; Data Set S1). Mechanistically, previous studies reveal the importance of IRF5 (interferon regulatory factor 5) in initiating and sustaining low-grade inflammatory monocyte polarization as well as in accelerating atherosclerosis progression.[35,41] While prolonged treatment with higher levels of lipopolysaccharide reduces IRF5 levels and causes endotoxin tolerance, low-dose lipopolysaccharide was shown to sustain and activate IRF5.[35] Through targeted pyrosequencing analyses, we observed that the IRF5 enhancer region was significantly methylated in high-dose lipopolysaccharide tolerant monocytes, and remain un-methylated in monocytes trained by either low-dose lipopolysaccharide or cholesterol (Figure S7C). Based on these analyses, we validated the protein levels of IRF5 in trained monocytes. We observed that monocytes trained with oxLDL exhibited significantly elevated levels of IRF5 protein, and in sharp contrast, 4-PBA treatment drastically ablated IRF5 expression (Figure 4E), mechanistically validating the anti-inflammatory effects of 4-PBA on trained monocytes.
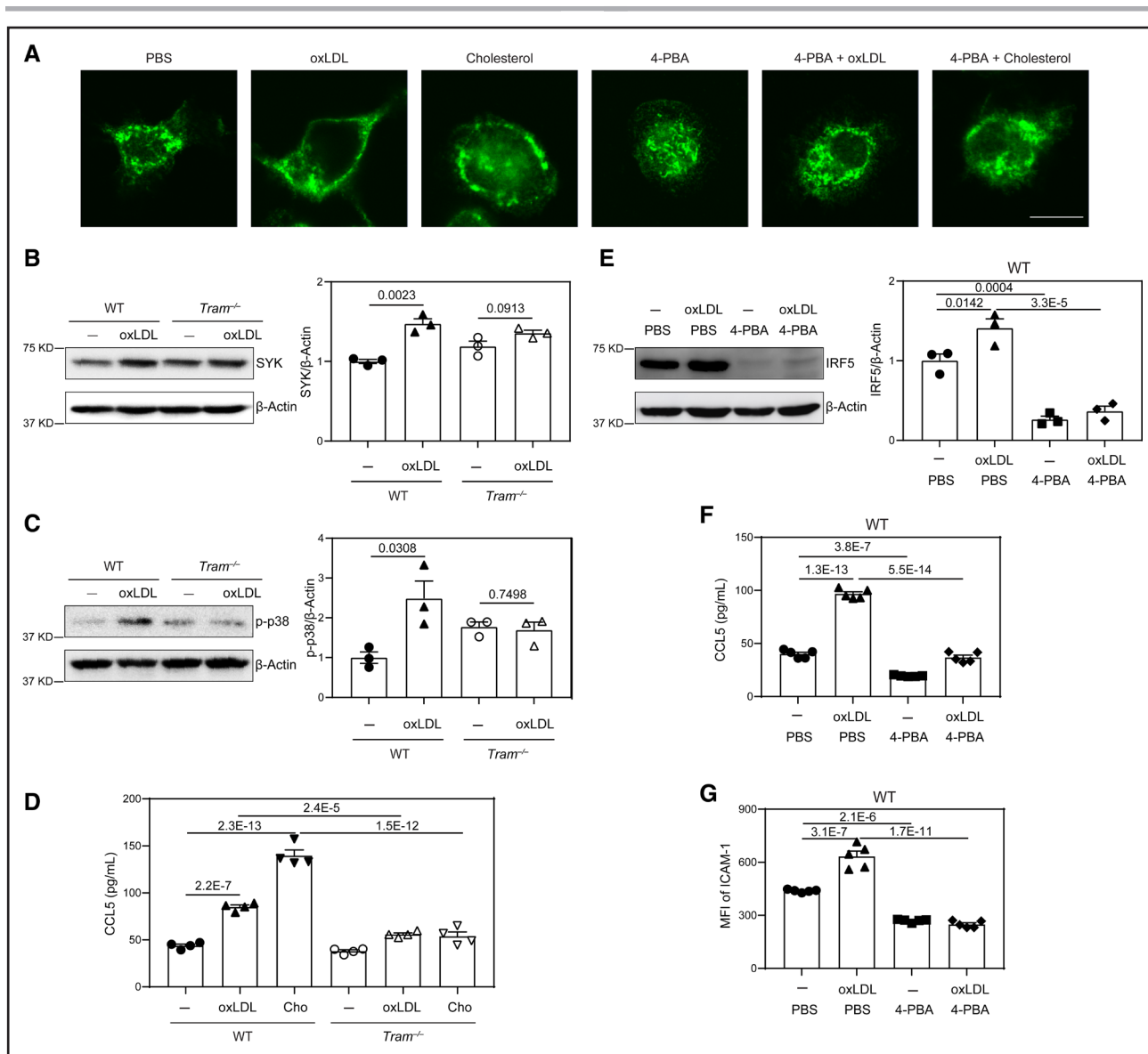
Figure 4. TRAM (Trif-related adapter molecule) serves as a general membrane stress sensor mediating the inflammatory effects of lipids on monocytes.
A, Bone marrow–derived monocytes (BMMs) from wild-type (WT) C57 BL/6 mice were cultured in vitro with M-CSF (macrophage colony-stimulating factor; 10 ng/mL) in the presence of oxLDL (oxidized low-density lipoprotein; 10 μg/mL), cholesterol (10 μg/mL), 4-phenylbutyric acid (4-PBA; 1 mmol/L) or PBS for 5 days. The cells were stained with anti-TRAM antibody, and cellular distribution of TRAM was examined by confocal microcopy. Scale bars, 10 μm. **B** and **C**, BMMs from WT C57 BL/6 mice and *Tram*^{−/−} mice were cultured with M-CSF (10 ng/mL) in the presence of oxLDL (10 μg/mL) or PBS for 5 days. Protein level of SYK (**B**) and phosphorylation of p-38 (**C**) were examined by Western blotting and quantified after normalizing to β-Actin expression. **D**, BMMs from WT C57 BL/6 mice and *Tram*^{−/−} mice were cultured with M-CSF (10 ng/mL) in the presence of oxLDL (10 μg/mL), cholesterol (10 μg/mL) or PBS for 5 days. Production of chemokine ligand 5 (CCL5) was determined by ELISA. **E** through **G**, BMMs from WT C57 BL/6 mice were cultured with M-CSF (10 ng/mL) in the presence of oxLDL (10 μg/mL), 4-PBA (1 mmol/L), or PBS for 5 days. Protein level of IRF5 was examined by Western blotting and quantified after normalizing to β-Actin expression (**E**). Production of CCL5 was determined by ELISA (**F**), and surface expression of ICAM-1 on CD11b^{+} monocytes was determined by flow cytometry (**G**). Data in **B** and **C** were analyzed using Student *t* test, and data in **D** through **G** were analyzed using 2-way ANOVA followed by Šídák post hoc test (n=3 for each group in **B**, **C**, and **E**; n=4 for each group in **D**; n=5 for each group in **F** and **G**; biological replicates. Error bars represent means±SEM.

Next, we tested whether 4-PBA treatment may diffuse the membrane clustering of TRAM via confocal microscopy. We observed that co-incubation of 4-PBA indeed diffused the membrane clustering of TRAM and caused a permissive cytosolic distribution of TRAM (Figure 4A). At the signaling level, we observed that 4-PBA treatment drastically reduced the cellular levels of SYK and mTOR, as well as the co-localization of mTOR and SYK with peroxisome (Figure 3). Functionally, we observed that 4-PBA significantly reduced the

expression of ICAM-1 and CCL5 in oxLDL–treated cells (Figure 4F and 4G). Of note, CCL5 was recently implicated via a comprehensive bioinformatics study as the most relevant inflammatory mediator underlying human atherosclerosis.[42] Collectively, our data reveal that 4-PBA can mitigate the proinflammatory memory of monocytes through the attenuation of TRAM-mediated subcellular inflammatory stress.

4-PBA Promotes the Expression of Anti-Inflammatory Mediator CD24 Through Enhanced PPARγ Activation

4-PBA not only reduced monocyte inflammation and the expression of adhesion molecule ICAM-1 but also uniformly elevated the expression levels of anti-inflammatory mediator CD24. To further characterize the molecular mechanism for elevated CD24 expression, we examined genes induced by 4-PBA in the single-cell RNA sequencing data set and noted the elevated expression of NEDD8 (neural precursor cell expressed, developmentally down-regulated 8; Figure 1A through 1C). NEDD8-mediated PPARγ (peroxisome proliferator-activated receptor γ) neddylation was shown to potently enhance PPARγ activation,[43] which may be responsible for the elevated induction of CD24. We tested the status of PPARγ neddylation by co-immunoprecipitation and observed increased PPARγ neddylation in monocytes trained by 4-PBA (Figure 5A).

To clearly define molecular mechanisms responsible for increased PPARγ neddylation by 4-PBA, we next examined NEDD8-interacting molecule TOLLIP (toll-interacting protein), discovered through an unbiased proteomic analysis of peptides coprecipitated with TOLLIP (Data Set S2). TOLLIP was also independently identified as an important mediator for subcellular organelle fusion and homeostasis.[44,45] Furthermore, *Tollip* deletion results in the expansion of inflammatory monocytes and exacerbates atherosclerosis.[46] We tested whether *Tollip* deficiency ablates the beneficial effects of 4-PBA on monocyte resolution. As shown in Figure 5A, PPARγ neddylation was reduced in *Tollip*-deficient monocytes. Functionally, we observed that *Tollip* deletion significantly dampened the induction of CD24 on monocytes by 4-PBA (Figure 5B; Figure S8). Our data indicate that treatment with 4-PBA promotes PPARγ neddylation and CD24 expression in monocytes in a TOLLIP-dependent manner.

CD24 Potentiates Inflammation Resolution by 4-PBA–Trained Monocytes

CD24 serves as a potent anti-inflammatory mediator and was independently shown to reduce the inflammatory activation of neighboring cells.[47] We next established a coculture system to determine whether 4-PBA programmed monocytes effectively propagate inflammation resolution to neighboring cells through CD24. BMMs from B6.SJL mice (CD45.1⁺) were pretreated with oxLDL to induce inflammatory polarization and then co-incubated with BMMs from WT B6 or *Cd24⁻/⁻* mice
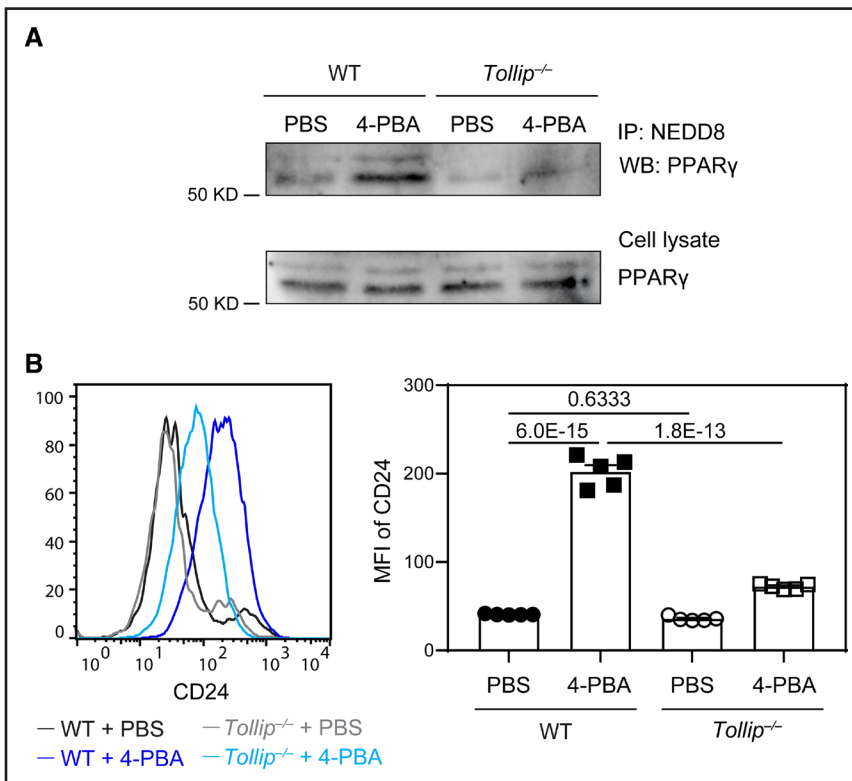

Figure 5. 4-phenylbutyric acid (4-PBA) promotes the expression of CD24 through enhanced PPARγ (peroxisome proliferator-activated receptor γ) activation in a Tollip-dependent manner.

Bone marrow–derived monocytes (BMMs) from wild-type (WT) C57 BL/6 mice and *Tollip⁻/⁻* mice were cultured in vitro with M-CSF (macrophage colony-stimulating factor; 10 ng/mL) in the presence of 4-PBA (1 mmol/L) or PBS for 5 days. **A**, Cell lysate was isolated and subjected to immunoprecipitation with anti-NEDD8 antibodies conjugated to resin. The association of PPARγ with NEDD8 was examined by Western blotting. PPARγ in cell lysate was also examined by Western blotting. **B**, Surface expression of CD24 on CD11b⁺ Ly6G⁻ Ly6Cʰⁱ monocytes was examined by flow cytometry. Mean Fluorescence Intensity (MFI) of CD24 was quantified. Data in **B** were analyzed using 2-way ANOVA followed by Šídák post hoc test (n=5 for each group; biological replicates). Error bars represent means±SEM.

(CD45.2+) that had been treated with PBS or 4-PBA. After coculture for 2 days, the cell surface expression of ICAM-1 and CD24 on CD45.1+ recipient BMMs was examined by flow cytometry. As shown in Figure 6A and 6B, compared with PBS-treated donor monocytes, 4-PBA programmed donor monocytes effectively propagate inflammation resolution to recipient monocytes previously activated by oxLDL as reflected by reduced expression of ICAM-1 and elevated expression of CD24 on recipient monocytes. Importantly, 4-PBA-treated CD24-deficient monocytes failed to impact the expression of ICAM-1 nor CD24 on neighboring recipient monocytes (Figure 6A and 6B), suggesting that CD24 is required for the propagation of inflammation resolution.

Given that we observed TOLLIP is required for PPARγ neddylation and CD24 induction by 4-PBA, we then tested whether *Tollip* deletion may similarly abolish the inflammatory resolution by 4-PBA. Indeed, neither expression of ICAM-1 nor CD24 on neighboring recipient monocytes was significantly impacted by *Tollip*-deficient monocytes trained with 4-PBA (Figure 6C and 6D).

4-PBA–Trained Monocytes Effectively Reduce Atherosclerosis Pathogenesis

Although systemic administration of 4-PBA can alleviate atherosclerosis progression, it may also interfere with the normal differentiation of monocytes to macrophages, subsequently leading to a decreased number of resident macrophages across various tissues. To enhance the therapeutic specificity of 4-PBA, it is critical to refine its efficacy for targeted purposes. Having defined the phenotypic and mechanistic aspects of resolving monocytes trained by 4-PBA, we then tested whether 4-PBA–trained monocytes are sufficient to render atheroprotection when transfused into recipient experimental animals. Both male and female *ApoE*−/− mice were subjected to HFD for 4 weeks and then transfused weekly for an additional 4 weeks with BMMs trained with either PBS or 4-PBA. The recipient mice were supplemented with HFD during this process, allowing the development of atherosclerosis. We observed that both male and female recipient mice transfused with 4-PBA–programmed monocytes exhibited a significant reduction in plaque sizes and decreased plaque lipid content (Figure 7A and 7B; Figure S9A and S9B), as
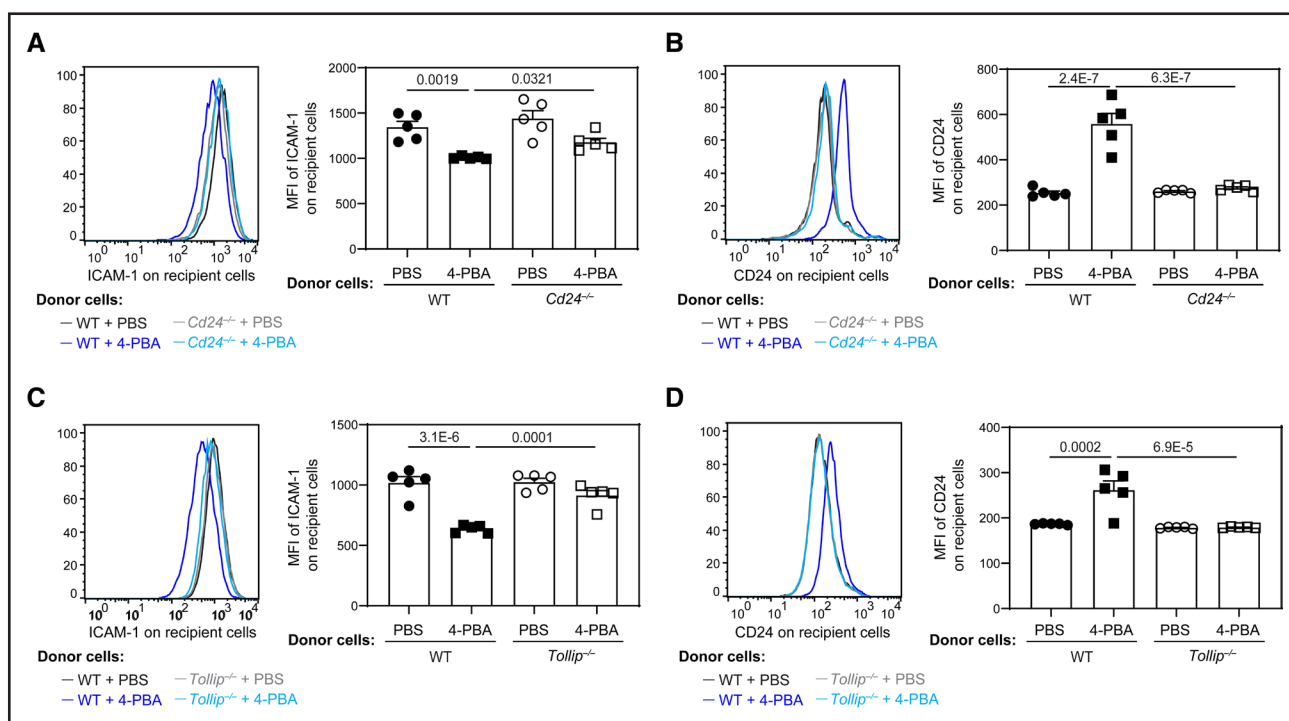

Figure 6. 4-phenylbutyric acid (4-PBA) trained monocytes propagate resolving nature to neighboring monocytes through CD24 and Tollip.
A and **B**, Bone marrow–derived monocytes (BMMs) from wild-type (WT) C57 BL/6 mice and *Cd24*−/− mice, which are both CD45.2+, were cultured in vitro with M-CSF (macrophage colony-stimulating factor; 10 ng/mL) in the presence of 4-PBA (1 mmol/L) or PBS for 5 days. Recipient BMMs prepared from B6 SJL mice, which are CD45.1+, were treated with oxLDL (oxidized low-density lipoprotein; 10 μg/mL) for 3 days and then cocultured in vitro with CD45.2+ donor cells for 2 days. Surface expressions of ICAM-1 (intercellular adhesion molecule 1; **A**) and CD24 (**B**) on CD45.1+ recipient BMMs were examined by flow cytometry. **C** and **D**, BMMs from WT C57 BL/6 mice and *Tollip*−/− mice, which are both CD45.2+, were cultured with M-CSF (10 ng/mL) in the presence of 4-PBA (1 mmol/L) or PBS for 5 days. Recipient BMMs prepared from B6 SJL mice, which are CD45.1+, were treated with oxLDL (10 μg/mL) for 3 days and then cocultured with CD45.2+ donor cells in vitro for 2 days. Surface expression of ICAM-1 (**C**) and CD24 (**D**) on CD45.1+ recipient BMMs was examined by flow cytometry. Data were analyzed using 2-way ANOVA followed by Šídák post hoc test (n=5 for each group; biological replicates). Error bars represent means±SEM.
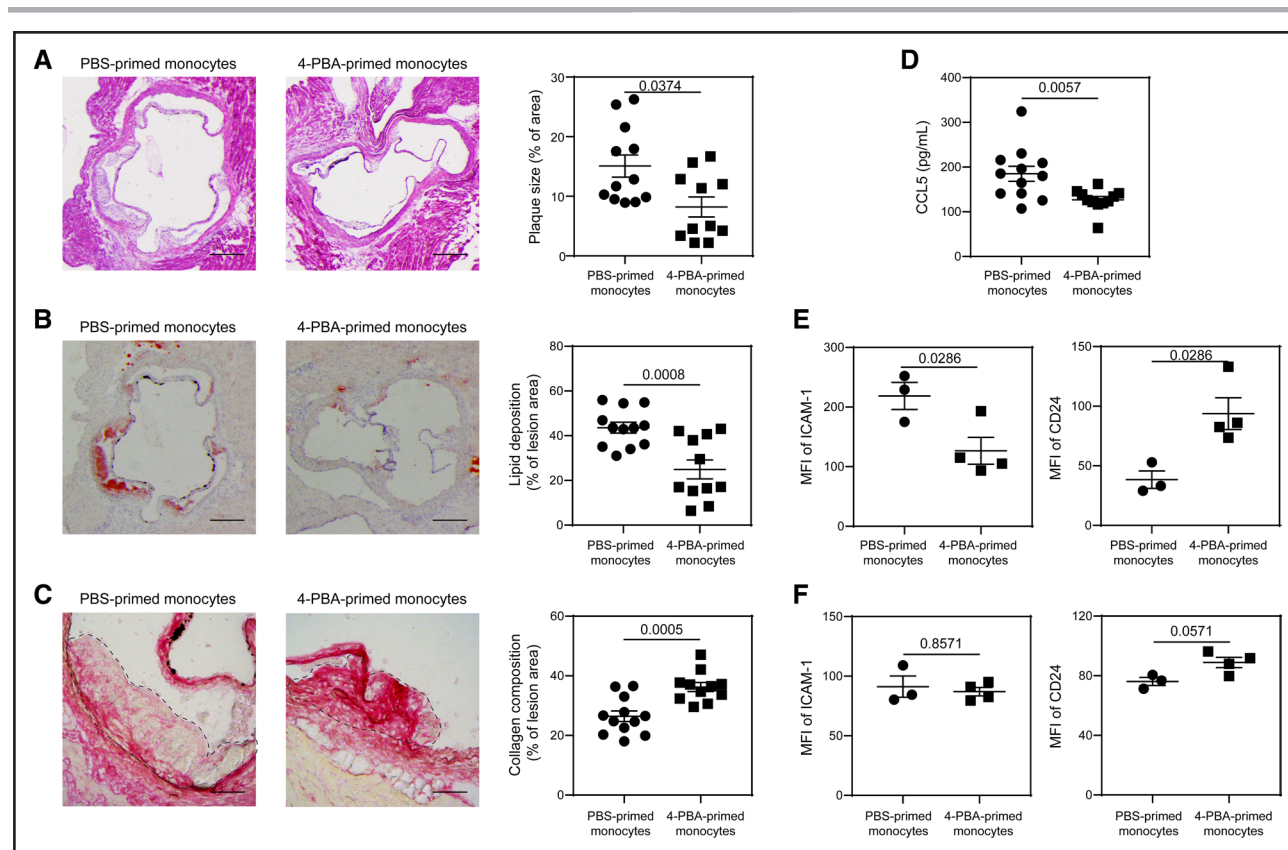
Figure 7. Adoptive transfer of monocytes polarized by 4-phenylbutyric acid (4-PBA) alleviates atherosclerosis.
A through **D**, Male *ApoE*−/− mice, serving as recipients, were fed with high-fat diet (HFD) for 4 weeks. Bone marrow−derived monocytes (BMMs) from *ApoE*−/− mice were treated with PBS or 4-PBA (1 mmol/L) for 5 days. PBS- or 4-PBA-polarized monocytes (3×10⁶ 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. **D**, Detection of chemokine ligand 5 (CCL5) level in the plasma by ELISA. **E** and **F**, PBS- or 4-PBA-polarized monocytes were labeled with CFSE immediately before adoptive transfer, and tissues were harvested 1 week after the last monocyte transfer. Surface expressions of ICAM-1 and CD24 on host CFSE⁻ CD11b⁺ Ly6G⁻ Ly6Cʰⁱ monocytes in the aorta (**E**) and bone marrow (BM; **F**) were determined by flow cytometry. Data in **A**, **E**, and **F** were analyzed using Mann-Whitney *U* test, and data in **B** through **D** were analyzed using Student *t* test (n=12 for PBS-trained monocytes group and n=11 for 4-PBA−trained monocytes group in **A** through **D**; n=3 for PBS-trained monocytes group and n=4 for 4-PBA-trained monocytes group in **E** and **F**; biological replicates). Error bars represent means±SEM.

well as dramatically higher plaque collagen content relative to mice transfused with control monocytes (Figure 7C; Figure S9C). Moreover, mice transfused with 4-PBA−programmed monocytes had significantly lower plasma levels of CCL5 (Figure 7D).

Given that our in vitro data demonstrated 4-PBA−trained monocytes effectively propagate anti-inflammatory activity to neighboring monocytes (Figure 6), we hypothesized that injection of 4-PBA-trained monocytes would induce a similar phenotypic change in monocytes in vivo. To test this, we labeled transfused monocytes with carboxyfluorescein succinimidyl ester (CFSE) before injection and then analyzed the surface expression of ICAM-1 and CD24 on CFSE-negative monocytes in recipient mice. We observed elevated CD24 expression on host resident monocytes in the aorta and bone marrow following the adoptive transfer of 4-PBA-trained monocytes

when compared with those receiving PBS-trained monocytes (Figure 7E and 7F). Injection of 4-PBA-trained monocytes significantly reduced ICAM-1 expression on host monocytes in the aorta but not in the bone marrow (Figure 7E and 7F), indicating that aortic monocytes of atherosclerotic mice became less adhesive after receiving 4-PBA-programmed monocytes.

We further tested whether transfused monocytes trained by 4-PBA may also propagate their anti-inflammatory effects to other resident immune cells such as neutrophils, B cells, and T cells. We observed that recipient mice transfused with 4-PBA−trained monocytes exhibit anti-inflammatory neutrophils expressing higher levels of CD24 and CD200R (Figure S10A); B regulatory cells expressing higher levels of CD24 (Figure S10B); and CD8 T regulatory cells expressing higher levels of CD122 (Figure S10C), in multiple immune niches

such as peripheral blood, bone marrow, spleen in addition to the aortic tissue. CD24hi B cells represent a regulatory B cell subset with potent immunosuppressive capacity.[48] CD122+ CD8 T cells are well-characterized T regulatory cells suppressing cytotoxic function of T cells.[49] CD200R neutrophil subset was shown to be anti-inflammatory with reduced swarming and elastase release.[50] Together, our data suggest that the transfusion of 4-PBA-trained monocytes may propagate inflammation resolution to both innate and adaptive immune niches to propagate their atheroprotective effects.

We performed additional experiments to further validate that the transfused monocytes can indeed infiltrate into the inflamed plaque area and modulate the immune environment of recipient mice. To this regard, we cotransfused an equal amount of PBS-or 4-PBA-trained monocytes labeled by CFSE together with separately cultured and purified Ly6C+ proinflammatory monocyte subsets (independently trained by super-low-dose lipopolysaccharide and purified in vitro) labeled by CellTrace Far Red into atherosclerotic mice to address 2 questions. First, we sought to determine whether the transfused monocytes can effectively traffic into the inflamed aortic plaque area. Second, we aimed to test whether the transfusion of 4-PBA–trained monocytes may influence the aortic infiltration of cotransfused Ly6C+ proinflammatory monocytes (which were well known to potently traffic into the inflamed aortic tissues[5,51]). Indeed, we observed that 24 hours upon transfusion, CFSE-labeled monocytes trained by either 4-PBA or PBS control can effectively traffic into the aorta (Figure S11A). Transfused CFSE+ monocytes were also detected in the bone marrow (Figure S11B) and spleen (Figure S11C), while a few transfused monocytes migrated to the lymph nodes (Figure S11D). Second, we observed that the numbers of infiltrated Far Red+ proinflammatory monocytes within the aortic tissue were significantly reduced in the group of mice cotransfused with 4-PBA–trained monocytes, as compared with the mice cotransfused with PBS control trained monocytes (Figure S11A). Our data suggest that the aortic infiltration of 4-PBA-trained monocytes may effectively attenuate the recruitment of proinflammatory monocytes to the inflamed plaque area of atherosclerotic mice.

These data collectively highlight the efficacy of the adoptive transfer of 4-PBA–programmed monocytes in mitigating the progression of atherosclerosis. These results also indicate a promising approach to immune cell therapy through utilizing resolving monocytes for the treatment of atherosclerosis.

CD24 Is Required for the Resolving Efficacy of 4-PBA-Trained Monocytes In Vivo

Given the striking effect of 4-PBA training in converting all monocytes into CD24-expressing anti-inflammatory monocytes, we further tested whether the therapeutic effect of 4-PBA–trained monocytes was indeed mediated by CD24. To test this, we used an independent atherosclerosis model with WT C57BL/6 mice intravenously injected with a single dose of AAV8-mPCSK9-D377Y, followed by feeding with HFD for 1 month. Experimental mice were then divided into 4 groups receiving equal amounts of weekly transfusion with the following monocytes: (1) PBS-trained WT monocytes; (2) 4-PBA-trained WT monocytes; (3) PBS-trained Cd24−/− monocytes; and (4) 4-PBA-trained Cd24−/− monocytes, for an additional month on HFD.

We observed that recipient mice transfused with 4-PBA-trained WT monocytes developed significantly reduced atherosclerosis, as compared with recipient mice transfused with PBS-trained control WT monocytes, evidenced by reduced plaque size (Figure 8A); reduced lipid deposition (Figure 8B); and elevated collagen content within the plaques (Figure 8C). In sharp contrast, recipient mice transfused with 4-PBA–trained Cd24−/− monocytes exhibited similar atherosclerosis pathogenesis as compared with mice transfused with PBS-trained Cd24−/− monocytes (Figure 8). Our data suggest that CD24 was indispensable for the resolving efficacy of 4-PBA-trained monocytes, and the induction of CD24 expression and functionality on monocytes by 4-PBA may be crucial for the success of this monocyte-based treatment.

DISCUSSION

Our work identified a robust approach for reprogramming resolving monocytes capable of effectively propagating inflammation resolution and treating atherosclerosis. Monocytes arrested by 4-PBA persist in a less-differentiated, proresolving state without further differentiation into the mature and inflammatory macrophages typically involved in exacerbating atherosclerosis. 4-PBA–trained monocytes exhibit reduced levels of adhesion molecule ICAM-1, immune cell chemokine CCL5, and mature macrophage marker F4/80 while also possessing drastically elevated levels of proresolving mediators such as CD24. 4-PBA programmed monocytes can potently propagate inflammation resolution through CD24-mediated intercellular communication with neighboring cells. Mechanistically, we demonstrated that 4-PBA effectively reduces monocyte inflammatory signaling by reducing TRAM-mediated cellular stress, as indicated by reduced peroxisome assembly of mTOR and SYK activation. Concurrently, 4-PBA robustly promotes the expression of anti-inflammatory CD24 by facilitating TOLLIP-mediated PPARγ-neddylation and activation.

Our study outlined a novel immune cell-based therapeutic alternative for the future treatment of atherosclerosis. Previously, 4-PBA was shown to be effective in treating experimental atherosclerosis.[19–21] However, systemic administration of chemical compounds such
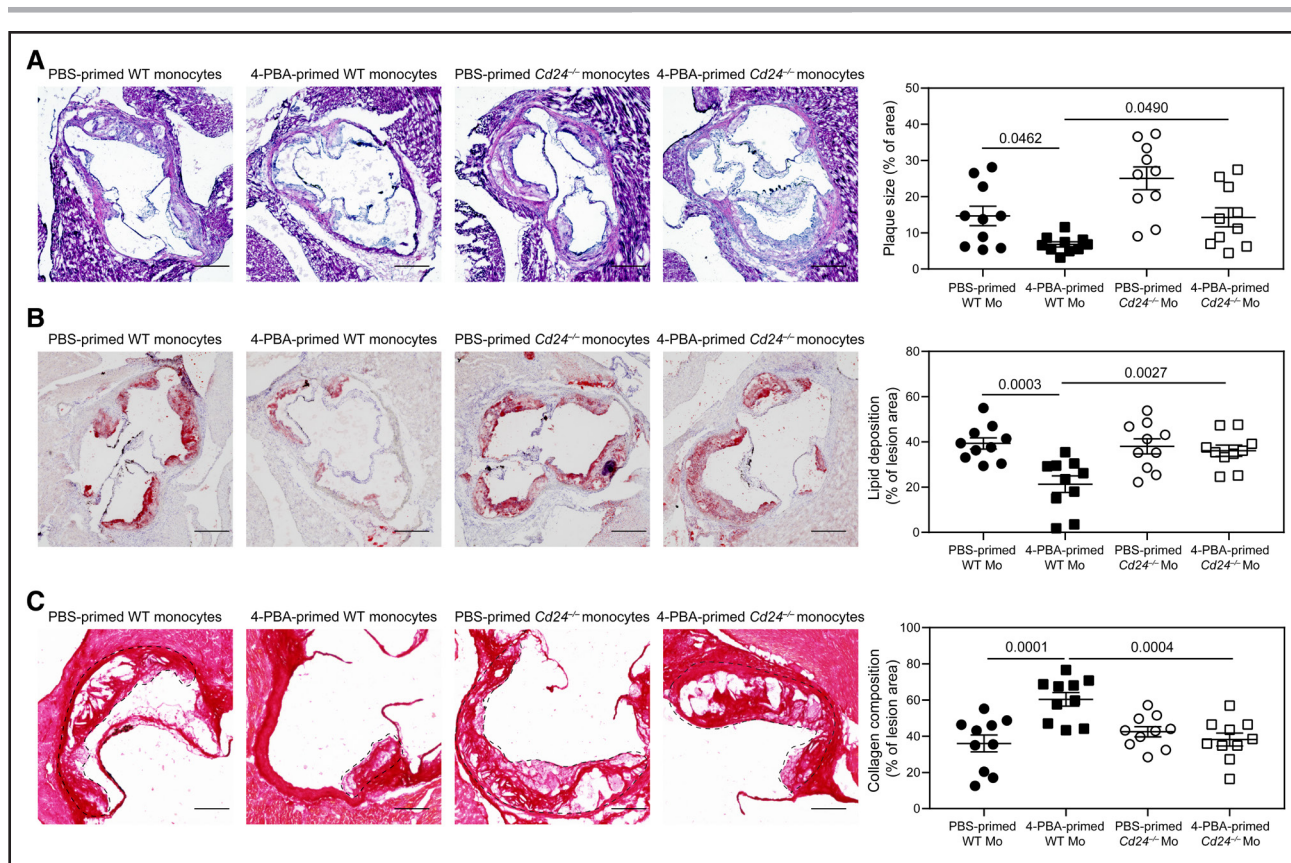
Figure 8. CD24 mediates the resolving efficacy of 4-phenylbutyric acid (4-PBA)-trained monocytes in atherosclerotic mice.
A through **C**, Male wild-type (WT) C57 BL/6 mice, serving as recipients, were intravenously injected with a single dose of AAV8-mPCSK9-D377Y (5×10^{11} vector genomes per mouse) and fed with high-fat diet (HFD) for 4 weeks. Bone marrow−derived monocytes (BMMs) from WT C57 BL/6 and $Cd24^{-/-}$ mice were treated with PBS or 4-PBA (1 mmol/L) for 5 days. PBS- or 4-PBA-polarized monocytes (3×10^{6} cells per mouse) were then adoptively transferred by intravenous injection to HFD-fed recipient 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 2-way ANOVA followed by Šídák post hoc test (n=10 for each group; biological replicates). Error bars represent means±SEM.

as 4-PBA introduces a plethora of side effects, potentially altering host defense, cellular development, and other vital tissue functions. Our ex vivo characterization demonstrated that monocytes treated with 4-PBA are arrested at a less-mature monocytic state, which suggests that long-term systemic injection of 4-PBA would likely impact systemic macrophage development and host anti-microbial defense. By contrast, the administration of 4-PBA-reprogrammed monocytes would enable clinicians to selectively harness their anti-inflammatory and therapeutic potential while alleviating the side effects of systemic 4-PBA application.

Through integrated single-cell RNA sequencing and relevant functional analyses, our current work not only presents a systematic characterization of 4-PBA programmed resolving monocytes but also reveals key mechanistic insights into monocyte reprogramming dynamics. We determined that 4-PBA reprograms resolving monocytes by reducing peroxisome-mediated mTOR and SYK signaling circuitry. Our findings validate previous reports

that mTOR inhibitors such as rapamycin serve as effective agents in reducing atherosclerosis.[52] Nanoparticle-mediated delivery of mTOR inhibitors was reported to be effective in treating experimental atherosclerosis.[53,54] Our mechanistic studies further defined the TRAM adaptor as a membrane-associated stress sensor of inflamed monocytes and demonstrated that 4-PBA can effectively reduce TRAM-mediated membrane stress. TRAM is one of the few innate signaling adaptors with covalently associated lipid motifs allowing them to be anchored onto the membrane lipid leaflet.[55,56] Lipid-modifications such as palmitoylation or myristoylation not only serve to anchor signaling molecules to the cell membrane[57–59] but also can facilitate the sensing of membrane stress signals independent of cell surface receptors.[60,61] Previous studies revealed that increased membrane stress and/or rigidity can facilitate the assembly of lipid rafts, where lipid-conjugated protein adaptors such as TRAM can dock and undergo activation.[62–65] Independent reports demonstrate that cholesterol and oxLDL can generically

increase membrane rigidity and lipid raft formation.[37–39] Complementing these studies, our current work revealed that oxLDL indeed can cause membrane clustering of the TRAM adaptor in activated monocytes. Our functional data further validated that TRAM may serve as a general membrane stress sensor for monocyte activation given that the induction of ICAM-1 and CCL5 by oxLDL is ablated in TRAM-deficient monocytes. Our data can also reconcile previous independent findings that implicate TRAM as a key signaling adaptor for oxidized phospholipid-induced monocyte activation.[66,67] Our data demonstrating the role of 4-PBA in alleviating TRAM clustering and reducing monocyte inflammatory polarization suggest that additional compounds that relieve membrane stress may be similarly effective in dampening monocyte low-grade inflammatory memory.

Our data reveal the key role of CD24 plays in 4-PBA–trained monocytes during the propagation of anti-inflammatory resolution. This is consistent with emerging studies reporting the beneficial roles of CD24 in reducing tissue inflammation.[27,68–70] We observed that 4-PBA can potently and uniformly elevate CD24 levels on all monocytes, regardless of their subsets based on the conventional Ly6C levels. Independent studies reveal that CD24 can inhibit inflammatory activation of neighboring cells through ligating its cognate inhibitory receptor Siglec-10.[47,68] A recombinant CD24-immunoglobulin protein has been shown to exert anti-inflammatory effects for the treatment of chronic inflammatory diseases such as diabetes.[27,68] Our analyses of the aortic local immune environment suggest that 4-PBA–trained monocytes can indeed propagate anti-inflammatory attributes to not only neighboring monocytes and neutrophils but also adaptive T and B cells. The propagation of inflammation resolution may likely be important for the therapeutic efficacy of transfused monocytes. However, our current work only serves as an initial attempt to address the fundamental principle of resolving monocytes in treating atherosclerosis. Future pharmacodynamics and kinetics characterization of trained resolving monocytes are clearly needed to further define their therapeutic potential, which should include their detailed trafficking to various tissues, half-lives, as well as dynamic interactions with systemic and local immune cells. We collected compelling data demonstrating the role of CD24 uniformly elevated on resolving monocytes. However, there are likely other unexplored mechanisms that might also contribute to the resolving phenotype of 4-PBA–trained monocytes. Refinement and purification of monocyte subsets trained by 4-PBA are needed to examine additional mediators involved in propagating homeostasis besides CD24. In addition, 4-PBA–trained monocytes likely would not fully ablate all aspects of atherosclerotic inflammation. Nevertheless, our current study reveals a proof of principle for reprogramming monocytes into a homeostatic resolution state with enhanced cellular expression of CD24, thereby providing a targeted and effective immune cell-based anti-inflammatory therapy capable of treating atherosclerosis and perhaps other chronic inflammatory conditions.

Taken together, our study demonstrates the potential feasibility of 4-PBA–trained monocytes for promoting homeostatic resolution in vitro and in vivo by programming monocytes into a less-differentiated, noninflammatory, and CD24-expressing homeostatic resolving state. We further demonstrate the presence as well as the expansion of CD24-expressing intermediate monocytes by 4-PBA treatment ex vivo, suggesting the feasibility of generating resolving primary human monocytes. Further refinement of innate monocyte-based approaches harbors tremendous promise for generating immune cell-based precision therapies for atherosclerosis.

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