

**INTRODUCTION**

Atherosclerosis and related cardiovascular complications are leading causes of morbidity and mortality worldwide with serious economic and health tolls. One of the key risk factors for atherosclerosis is the establishment of nonresolving inflammation. However, the limited understanding of underlying mechanisms presents a major road block for effective prevention and treatment (1, 2).

Emerging studies suggest that the lack of inflammation resolution during atherosclerosis may occur because of reprogramming and polarization of innate leukocytes under persistent low-grade inflammatory challenges (3, 4). Some of the well-studied aspects of polarized “memory” macrophages during atherosclerosis favor foam cell formation, with reduced efferocytosis and elevated necrosis, which collectively contribute to the pathogenesis of atherosclerosis (5, 6). In addition to the well-studied macrophages during atherosclerosis, neutrophils may also be involved through less-characterized mechanisms (7). Neutrophils constitute 50 to 70% of circulating white blood cells and have been shown to be elevated in circulating blood and atherosclerotic plaques from human patients and experimental animals with unstable plaques (8–11). Although the correlation of higher neutrophil populations with increased risks of unstable atherosclerotic plaques has been increasingly appreciated, the role of differentially polarized neutrophils in the pathogenesis of atherosclerosis under chronic inflammatory conditions is still poorly understood.

Using a previously developed murine model of low-grade inflammation and atherosclerosis that we developed through repetitive injections of subclinical-dose lipopolysaccharide (LPS), we tested the hypothesis that the differentially reprogrammed neutrophils under chronic inflammatory conditions may play a critical role during the pathogenesis of atherosclerosis. To test this hypothesis, we characterized the unique polarization of neutrophils by subclinical low-dose LPS both in vitro and in vivo, as well as the underlying molecular and cellular mechanisms. Through transfusing uniquely programmed neutrophils into recipient mice, we directly examined the role of reprogrammed neutrophils during the pathogenesis of atherosclerosis and plaque stability. We identified that disruption of peroxisome homeostasis by subclinical low-dose LPS is uniquely responsible for the inflammatory polarization of neutrophils. We further examined the efficacy of restoring neutrophil peroxisome homeostasis with 4-phenylbutyrate (4-PBA) and the therapeutic potential of 4-PBA–programmed neutrophil transfusion as a treatment for experimental atherosclerosis.

**RESULTS**

**Reduced plaque stability in mice challenged with subclinical-dose endotoxin**

We first confirmed our previous report that subclinical low-dose LPS exacerbates atherosclerosis progression through Oil-Red O and hematoxylin and eosin (H&E) staining (4). High-fat diet (HFD)–fed ApoE−/− mice injected weekly with subclinical low-dose LPS (5 ng/kg body weight) for 4 weeks developed significantly larger atherosclerotic plaques as compared to mice injected with phosphate-buffered saline (PBS) (Fig. 1, A and B). It is most noteworthy that chronic injection of subclinical low-dose LPS significantly reduced plaque stability as reflected in significantly reduced collagen content within the plaque (Fig. 1C). Matrix metalloproteinase 9 (MMP9), responsible for degrading collagen, and inflammatory lipid mediators such as leukotriene B4 (LTB4) have been closely associated with reduced plaque stability (12, 13). We then tested the levels of MMP9 and observed a significant elevation of plasma MMP9 and LTB4 levels in mice injected with LPS as compared to mice injected with PBS (Fig. 1D). LPS administration also induced remarkable elevation of circulating myeloperoxidase (MPO) (fig. S1), another proinflammatory mediator promoting plaque instability (14). On the other hand, the circulating levels of the anti-inflammatory mediator transforming growth factor–β (TGFβ) were significantly reduced in mice injected with low-dose LPS (Fig. 1D). Similar effects were observed in regular chow diet (RD)–fed mice injected with low-dose LPS (fig. S2).
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CD62L (Fig. 2), another classical indicator of neutrophil activation, these neutrophils had a significant reduction of surface-attached LPS-injected mice as compared to PBS-injected mice (Fig. 2). In addi-

markers such as CD11b and Dectin-1 were significantly elevated in face activation markers using flow cytometry. Select inflammatory mice injected with subclinical low-dose LPS by examining key sur-

Therefore, we decided to test the activation status of neutrophils in this process has not been studied. 15, the primary producers of MMP9 and LTB4 (20). Both MMP9 and LTB4 are strongly associated with unstable atherosclerotic plaques, and among innate leukocytes, neutrophils are

Inflammatory polarization of neutrophils during atherosclerosis

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ersclerotic plaques, and among innate leukocytes, neutrophils are the primary producers of MMP9 and LTB4 (15, 16). However, the potential role of neutrophils in this process has not been studied. Therefore, we decided to test the activation status of neutrophils in mice injected with subclinical low-dose LPS by examining key sur-

face activation markers using flow cytometry. Select inflammatory markers such as CD11b and Dectin-1 were significantly elevated in LPS-injected mice as compared to PBS-injected mice (Fig. 2). In addition, these neutrophils had a significant reduction of surface-attached CD62L (Fig. 2), another classical indicator of neutrophil activation (17). We observed a similar trend in neutrophils harvested from cir-

culating blood (Fig. 2A), spleen (Fig. 2B), and bone marrow (BM) (Fig. 2C). In addition to increased activation, we also observed higher percentages of neutrophils in the blood, spleens, and atherosclerotic plaques of mice chronically injected with LPS (Fig. S3). Along with markers of neutrophil activation, we also examined the expression of key homeostatic molecules such as leucine-rich repeat containing 32 (LRRC32) and ferroportin (FPN) in neutrophils from LPS-treated mice. LRRC32 is a cell surface–conjugated molecule involved in the processing of latent TGFβ to its soluble and active form (18), while FPN is involved in the polarization of innate leukocytes into an anti-

inflammatory state through the modulation of intracellular iron content (19). We observed that neutrophils from mice chronically injected with LPS had significantly reduced surface levels of both LLRC32 and FPN (Fig. 2, A to C). Instead of mean fluorescence intensity (MFI), we also used geometric MFI as a parameter to analyze the expressions of tested molecules, demonstrating a similar modula-

tion of neutrophil phenotype after LPS treatment (Fig. S4). To-

gether, our data reveal an in vivo proinflammatory polarization of neutrophils in mice chronically injected with low-dose LPS.

Mechanisms underlying the inflammatory polarization of neutrophils

After characterizing the polarization of neutrophils in vivo by subclinical-dose LPS challenge, we further examined whether low-

dose LPS could directly polarize neutrophils in vitro. BM-derived neutrophils were cultured with granulocyte colony-stimulating fac-

tor (G-CSF), together with or without LPS overnight. The activation status of the neutrophils was determined through measurement of secreted inflammatory mediators by enzyme-linked immunosorbent assay (ELISA) and examination of key cell surface markers by flow cytometry. We observed significantly higher levels of MMP9, LTB4, and MPO in the supernatant of neutrophils cultured with low-dose LPS as compared to those cultured with G-CSF alone (Fig. 3A). We also observed significantly elevated cell surface levels of CD11b and Dectin-1 and significantly reduced levels of CD62L, LRRC32, and FPN on neutrophils cultured with low-dose LPS (Fig. 3B). Coculture of neutrophils with low-dose LPS and oxidized low-density lipopro-

tein (oxLDL) further synergized the induction of MMP9, LTB4, and MPO (Fig. 3A). In addition to inflammatory lipid mediators, we also examined the expression of selected microRNAs (miRNAs) expressed by neutrophils. Our previous study revealed that subclinical- 

dose LPS could potently induce the expression of miR-24 in mono-

cytes, a critical miRNA involved in the propagation of nonresolving inflammation (4). We observed that neutrophils challenged with subclinical-dose LPS also express miR-24 (Fig. 3C). Subclinical dose LPS not only induced the inflammatory miR-24 but also potently suppressed the expression of miR-126 (Fig. 3C), a key miRNA in-

volved in tissue homeostasis and vascular integrity (20).

Given our finding of polarized inflammatory neutrophils both in vitro and in vivo by subclinical-dose LPS, we next examined the po-

tential underlying mechanism. Since the activation of 5-lipoxygenase (5-LOX) mediated by oxidized calmodulin–dependent protein ki-

nase II (oxCAMKII) has been shown to be important for the expres-

sion of inflammatory mediators such as LTB4, we tested the activation status of oxCAMKII and 5-LOX in neutrophils challenged with subclinical-dose LPS. As shown in Fig. 3D, we observed that neu-

trophils cultured with subclinical-dose LPS had increased levels of oxCAMKII and 5-LOX. Through in situ immunohistochemical staining, we further confirmed that the levels of oxCAMKII were
Fig. 2. Subclinical endotoxin primes neutrophils into a proinflammatory state in atherosclerotic mice. ApoE−/− mice were administrated with PBS or superlow-dose LPS, together with HFD for 4 weeks. The surface phenotypes of Ly6G+ neutrophils in the peripheral blood (A), spleen (B), and BM (C) were analyzed with flow cytometry. Data are representative of two independent experiments, and error bars represent means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, Student's t test (n = 5 for each group). ns, not significant.
Fig. 3. Superlow-dose LPS induces inflammatory polarization of neutrophils in vitro. Neutrophils were purified from the BM of wild-type C57BL/6 mice and treated with PBS, superlow-dose LPS (100 pg/ml), and/or oxLDL (10 μg/ml) for 2 days. (A) Levels of MMP9, LTB4, and MPO were determined by ELISA (n = 3 for each group). (B) The surface phenotype of neutrophils was analyzed by flow cytometry (n = 3 for each group). (C) Levels of miR-24 and miR-126 were determined by real-time reverse transcription polymerase chain reaction (RT-PCR) (n = 4 for each group). (D) The expressions of oxCaMKII and 5-LOX were determined by Western blot. (E) Representative histogram and quantification of p-STAT1 level as determined by flow cytometry (n = 3 for each group). (F) Representative histograms and quantification of ATF4 and KLF2 levels as determined by flow cytometry (n = 5 for each group). Data are representative of three independent experiments, and error bars represent means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, (A to C and E) one-way analysis of variance (ANOVA) and (F) Student’s t test.
significantly elevated in either RD-fed or HFD-fed ApoE$^{-/-}$ mice chronically injected with low-dose LPS as compared to corresponding control mice with PBS injection (fig. S5).

OxCAMKII-mediated signal transducer and activator of transcription 1 (STAT1) activation is responsible for the expression of Dectin-1 (21, 22). We therefore measured the activation status of STAT1 through detecting the levels of phosphorylated STAT1 (p-STAT1) by flow cytometry and observed significantly elevated levels of p-STAT1 in neutrophils challenged with subclinical-dose LPS (Fig. 3E). On the other hand, homeostatic transcription factors such as Krüppel-like factor-2 (KLF2) and activating transcription factor 4 (ATF4) are involved in transcribing homeostatic molecules such as FPN and LRRC32 and reducing reactive oxygen species (ROS)–mediated activation of oxCAMKII (23–26). We observed that subclinical-dose LPS significantly reduced the cellular levels of KLF2 and ATF4 in neutrophils (Fig. 3F). Collectively, the activation of oxCAMKII and the reduction of homeostatic KLF2 and ATF4 may be responsible for polarizing neutrophils into a nonresolving inflammatory state conducive to atherosclerosis.

**In vitro polarized nonresolving inflammatory neutrophils are sufficient to confer plaque instability**

To test whether polarized neutrophils by subclinical-dose LPS are directly responsible for exacerbated atherosclerosis and plaque instability, we performed experiments by transfusing in vitro polarized neutrophils into recipient animals. BM neutrophils were polarized through an in vitro culture with G-CSF and either low-dose LPS or PBS overnight. HFD-fed ApoE$^{-/-}$ mice were injected intravenously with neutrophils cultured in vitro with either PBS or LPS on a weekly basis for 1 month. The viability of transferred neutrophils was determined by annexin V/PI (propidium iodide) staining, and the results demonstrated that >95% of cultured neutrophils remained viable after in vitro polarization for 24 hours (fig. S6, A and B) and ~90% viable for 48 hours (fig. S6, C and D), consistent with the literature reports showing that murine BM neutrophils have a longer life span than circulating neutrophils and G-CSF delays neutrophil apoptosis (27, 28). Moreover, we tested the surface phenotype of the neutrophils before transfer. The neutrophils polarized by low-dose LPS for 24 hours exhibited elevated expressions of CD11b and Dectin-1 and reduced expressions of LRRC32, FPN, and CD62L (fig. S6E). One week after the final injection, mice were sacrificed and examined. We observed that mice injected with LPS-polarized neutrophils had significantly larger plaque sizes and higher lipid deposition within the plaques (Fig. 4, A and B). The collagen content within the plaques from mice injected with LPS-polarized neutrophils was significantly lower compared to that of mice injected with PBS-treated neutrophils (Fig. 4C). The mice receiving LPS-polarized neutrophils demonstrated significantly higher levels of plasma cholesterol and triglycerides than their counterparts receiving PBS-treated neutrophils (fig. S7A). We further tested the status of key enzymes such as oxCAMKII responsible for generating inflammatory mediators within the plaques through immunohistochemical staining and observed a significantly elevated signal of oxCAMKII within the plaques of mice transfused with LPS-polarized neutrophils as compared to mice transfused with PBS-treated control neutrophils (Fig. 4D). Adoptive transfer of LPS-primed neutrophils also resulted in the elevation of total macrophage load and the frequency of SR-A$^+$ macrophages in atherosclerotic plaques, suggesting that primed neutrophils may communicate with monocyte/macrophage

![Image](http://advances.sciencemag.org/)

**Fig. 4. Neutrophils polarized by superlow-dose LPS aggravate atherosclerosis.** Neutrophils purified from ApoE$^{-/-}$ mice were treated with PBS or superlow-dose LPS (100 pg/ml) for 24 hours. PBS- or LPS-polarized neutrophils ($2 \times 10^6$ cells per mouse) were then adoptively transferred by intravenous injection to HFD-fed ApoE$^{-/-}$ mice once a week for 4 weeks. Samples were collected 1 week after the last neutrophil transfer. (A) Representative images of H&E-stained atherosclerotic lesions and quantification of plaque size exhibited 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, 300 μm. (D) Representative images of oxCAMKII levels by confocal microscopy. Scale bars, 100 μm. (E) Determination of circulating MPO, MMP9, and LTBA4 levels by ELISA. Data are representative of two independent experiments, and error bars represent means ± SEM. *P < 0.05 and **P < 0.01, Student’s t test (n = 5 to 6 for each group).
Polarized inflammatory neutrophils exhibit disrupted peroxisome homeostasis and elevated ROS

Our data reveal a novel aspect of neutrophil polarization that is conducive for exacerbated atherosclerosis. Our findings complement emerging studies that suggest that neutrophils may be differentially programmed into distinct functional states with significant pathological implications (29). However, the cellular and molecular mechanisms underlying neutrophil polarization remain less studied. Given our above observation that neutrophils programmed by subclinical-dose LPS exhibit elevated oxCAMKII, a critical signaling molecule involved in the expression of inflammatory mediators (30), we further tested whether ROS were involved. We measured the levels of intracellular ROS in neutrophils cultured with PBS and LPS through staining with a ROS-specific fluorescent probe followed by flow cytometry. We observed that neutrophils programmed with subclinical-dose LPS or oxLDL exhibited significantly higher levels of ROS as compared to control neutrophils cultured with PBS (fig. S8 and Fig. 5A). Because altered peroxisome homeostasis is critically important for ROS generation, we examined peroxisome homeostasis with particular focus on its ability to communicate with the lysosome in neutrophils treated with subclinical-dose LPS. As shown in Fig. 5C, neutrophils cultured with subclinical-dose LPS exhibited a disruption of proper fusion between the peroxisome and the lysosome. In contrast, PBS-cultured control neutrophils exhibited efficient fusion of the peroxisome with the lysosome.

To test whether the disruption of peroxisome homeostasis is responsible for LPS-mediated neutrophil polarization, we used a selective compound, 4-PBA, which is known to induce effective peroxisome homeostasis (31). As shown in Fig. 5C, the application of 4-PBA effectively restored the fusion of the peroxisome and lysosome in cells treated with LPS. Furthermore, 4-PBA treatment effectively ameliorated the induction of ROS by LPS in neutrophils (Fig. 5, A and B). The activation of oxCAMKII and the induction of 5-LOX by LPS were also effectively ameliorated by the addition of 4-PBA (Fig. 5D). Incubation with 4-PBA also led to a reduction of p-STAT1 and restoration of KLF2/ATF4 in neutrophils challenged with LPS (Fig. 5E).

Our data indicate that altered peroxisome homeostasis in neutrophils programmed by subclinical-dose LPS is responsible for elevated ROS and inflammatory polarization and that restoration of peroxisome homeostasis may hold promise for maintaining proper neutrophil function and alleviating atherosclerosis progression.

Enhanced neutrophil homeostasis alleviates atherosclerosis

On the basis of our finding that 4-PBA could potentially enhance neutrophil homeostasis, we next tested whether these neutrophils with enhanced homeostasis could alleviate atherosclerosis. To test this, ApoE<sup>−/−</sup> mice fed with HFD were transfused weekly for 4 weeks with neutrophils cultured with either PBS or 4-PBA. The neutrophils polarized with 4-PBA for 24 hours demonstrated reduced CD11b and Dectin-1 expression and elevated LRRC32, FPN, and CD62L expression (fig. S6E), exhibiting a similar phenotype as the neutrophils primed with 4-PBA for 48 hours. One week after the final transfusion, mice were sacrificed for analysis.

We observed that mice transfused with 4-PBA–programmed neutrophils had a threefold reduction in plaque sizes and a twofold reduction of the plaque lipid content (Fig. 6, A and B). Collagen staining also revealed that mice transfused with 4-PBA–programmed neutrophils had significantly higher levels of plaque collagen as compared to mice transfused with control neutrophils (Fig. 6C). Mice transferred with 4-PBA–polarized neutrophils exhibited a significant reduction of plasma cholesterol and triglycerides as compared to mice transfused with PBS-treated neutrophils (fig. S10A). Furthermore, we observed a decline in activated SR-A<sup>+</sup> macrophages within the plaques of mice transfused with 4-PBA–polarized neutrophils (fig. S10B).

In addition to significantly reduced plaque sizes, we further measured inflammatory markers in the experimental mice. We observed that mice transfused with 4-PBA–programmed neutrophils had significantly lower plasma levels of MPO, LTB4, and MMP9 and significantly elevated levels of TGFβ (Fig. 6D). As compared to mice transfused with control neutrophils, mice transfused with 4-PBA–programmed neutrophils had significantly lower proinflammatory miR-24 and higher homeostatic miR-126 in circulation (Fig. 6E). Our data indicate that 4-PBA–programmed neutrophils can be effectively used to enhance tissue homeostasis in vivo and hold promising potential to treat atherosclerosis.

**DISCUSSION**

Our study reveals a novel programming dynamic of neutrophils involved in the generation of unstable atherosclerotic plaques. We showed that pathologically relevant superlow-dose endotoxin can program neutrophils into a distinct state, with altered balance of nonresolving inflammation that is conducive to the development of unstable atherosclerotic plaques. The nature of nonresolving inflammatory neutrophils is reflected in elevated ROS due to disrupted peroxisome homeostasis, resulting in the skewed activation of oxCAMKII and downstream expression of LTBP, MMP9, and miR24 and reduced ATP4/KLF2-mediated expression of resolving mediators such as LRRC32 and miR-126. We further demonstrated that the restoration of neutrophil peroxisome homeostasis through the application of 4-PBA can effectively resolve neutrophil inflammation and that neutrophils reprogrammed by 4-PBA can potentially attenuate atherosclerosis progression.
Fig. 5. 4-PBA enhances peroxisome homeostasis in neutrophils. Neutrophils were purified from the BM of wild-type C57BL/6 mice and treated with PBS, superlow-dose LPS (100 pg/ml), and/or 4-PBA (1 mM) for 2 days. (A) Representative histogram of ROS level determined by CellROX labeling. (B) Quantification of ROS levels in neutrophils (n = 3 for each group). (C) Representative confocal microscopy images of the neutrophils stained with anti-PMP70 (peroxisomal membrane protein 70) and anti-LAMP1 (lysosomal-associated membrane protein 1) antibodies to demonstrate the localization and fusion of peroxisomes and lysosomes. Scale bars, 5 μm. (D) Western blot data of oxCaMKII and 5-LOX expression. (E) Representative histograms and quantification of p-STAT1, ATF4, and KLF2 levels as determined by flow cytometry. (F) Surface phenotype of neutrophils was analyzed by flow cytometry (n = 3 for each group). (G) The levels of MPO, MMP9, and LTB4 were determined by ELISA (n = 3 for each group). Data are representative of three independent experiments, and error bars represent means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, one-way ANOVA.
Our findings build on the emerging correlation of elevated circulating neutrophil counts with the development of unstable atherosclerotic plaques previously documented in both experimental animals and human patients with atherosclerosis (8–11). In human atherosclerosis, high-circulating neutrophil ratios have been associated with characteristics of rupture-prone atherosclerotic lesions (10). In experimental animals, neutrophilia promoted by hyperlipidemia particularly accelerates early atherosclerosis, which is apparent within 4 weeks of HFD feeding (8). The sizes of atherosclerotic lesions were shown to be positively correlated with circulating neutrophils counts, and the depletion of neutrophils was shown to reduce the initial phase of atherosclerotic lesion burden (8). However, the fundamental nature and underlying mechanisms of proatherosclerotic neutrophils are poorly appreciated or understood.

Our data fill this critical void and reveal that, in addition to the elevated counts of neutrophils, the quality of polarized neutrophils may bear more significant relevance to atherosclerosis. We found that mice subjected to chronic injection of pathologically relevant subclinical low-dose LPS developed atherosclerotic plaques with elevated lipid deposition and reduced collagen content, characteristics of unstable plaques. We observed that neutrophils underwent unique polarization in mice injected with low-dose LPS and adopted a skewed nonresolving inflammatory state represented by increased expression of proinflammatory mediators such as LTB4, MPO, and MMP9, known risk factors for unstable plaques (12–14). This was in addition to the reduced expression of resolving mediators such as LRRC32 and miR-126. LRRC32 is responsible for the generation of mature TGFβ, while miR-126 is critically involved in facilitating vascular integrity (18, 20). Through adoptive transfer studies, we demonstrated that the transfer of neutrophils polarized by superlow-dose LPS was sufficient to significantly aggravate atherosclerosis and reduce plaque stability as compared to the transfer of equal numbers of PBS-treated control neutrophils. Our study complements a recent report that the reduction of LRRC32 levels on human immune cells is associated with reduced human atherosclerotic plaque stability (32).

Our data reveal a novel programming dynamic in neutrophils under chronic low-grade inflammatory conditions and are consistent with a recent independent study regarding the effects of endotoxemia on plaque destabilization (13). Mawhin et al. (13) recently reported that mice repeatedly injected with LPS (1.5 mg/kg) (~30 to 60 μg per mouse) exhibited elevated numbers of circulating neutrophils potentially mediated by elevated LTβ4 and severe atherosclerotic vulnerability with reduced collagen content and enlarged necrotic cores. However, that study did not define the fundamental nature of neutrophils affected by LPS and did not provide a causal connection between neutrophils and plaque instability. Our study not only complements the phenotypic observation of unstable plaques elicited by LPS injection but also provides compelling mechanistic principles for neutrophil polarization directly responsible for the development of unstable plaques. Here, we provide both in vitro and in vivo data that define the unique skewing of neutrophils by very low dose LPS into a nonresolving inflammatory state directly responsible for unstable atherosclerosis. Our study also better resembles the pathological conditions in humans and experimental animals with endotoxemia (33–36). Circulating endotoxin levels in humans and experimental animals are extremely low and within the picogram-nanogram per kilogram ranges (37–41), which are thousands-fold less than the microgram-milligram per kilogram range that most studies used. The unique adaptations of innate leukocytes to varying dosages of bacteria endotoxin are highly complex and bear profound pathological relevance (42–45). However, to our knowledge, this study provides the first characterization of unique neutrophil polarization by...
pathologically relevant concentrations of endotoxin. We observed that neutrophils are skewed to a polarized inflammatory state with reduced ability for homeostatic resolution. The identified features of polarized neutrophils such as elevated surface Dectin-1 and CD11b and reduced surface LRRC32 and FPN, as well as increased secretion of MPO, LTβ4, and miR-24 and reduced secretion of miR-126, may serve as potential markers for characterizing neutrophils involved in various chronic inflammatory diseases such as atherosclerosis.

The qualitative nature of neutrophils, rather than the simple elevation of neutrophil counts, have been increasingly implicated in the pathogenesis of other chronic diseases such as cancer (46). Although neutrophils are known to be pleiotropic in nature, the exact characterization of neutrophil polarization and the underlying mechanisms are poorly defined. To fill this critical gap, our study reveals that the novel disruption of peroxisome homeostasis within neutrophils may be one of the critical culprits. We observed that polarized neutrophils by superlow-dose endotoxin exhibit a disruption of proper fusion among peroxisomes and lysosomes, which contributes to the accumulation of ROS and the development of nonresolving inflammatory neutrophils. The accumulation of ROS is associated with elevated oxCAMKII responsible for the expression of LTβ4, MPO, and Dectin-1. On the other hand, polarized neutrophils have reduced ATF4 and KLF2, key transcriptional factors beneficial for the resolution of inflammation through the expression of homeostatic molecules such as LRRC32 and miR-126. ATF4 and KLF2 are also known to closely cooperate with NRF2 [nuclear factor (erythroid-derived 2)–like 2] in reducing the accumulation of ROS and restoring cellular homeostasis. Collectively, our study defines the disrupted molecular balance in neutrophils leading to the accumulation of ROS and the development of a nonresolving inflammatory state conducive for atherosclerosis.

This study further defines a novel and effective strategy in rebalancing the polarized neutrophils back to the homeostatic state through the application of 4-PBA. Our work builds on previous biochemical characterizations of 4-PBA in other cells, which demonstrated that 4-PBA can restore peroxisome homeostasis (31, 47–49). We observed that 4-PBA can restore peroxisome-lysosome fusion in neutrophils and reduce LPS-mediated elevation of neutrophil ROS. 4-PBA can effectively reduce the induction of oxCAMKII, LTβ4, MPO, Dectin-1, CD11b, and miR-24 in neutrophils by superlow-dose LPS and also restore the expression of ATF4, FPN, LRRC32, and miR-126 in neutrophils suppressed by LPS. Functionally, we demonstrated that rejuvenated homeostatic neutrophils in vitro by 4-PBA treatment can potently reduce the pathogenesis of atherosclerosis, as reflected in drastically reduced lesion sizes and elevated collagen contents.

Together, our study not only provides compelling mechanistic data that address a unique aspect of neutrophil polarization relevant to the pathogenesis of unstable atherosclerotic plaques but also demonstrates the novel feasibility of using reprogrammed neutrophils to directly treat experimental atherosclerosis.

MATERIALS AND METHODS

Experimental animals and LPS injection
Male ApoE−/− mice (6 to 8 weeks old) on the C57BL/6 background were purchased from the Jackson laboratory and fed with RD or HFD. Either PBS or subclinical-dose LPS (5 ng/kg body weight) was intraperitoneally injected every 4 days for 3 weeks. Then, the mice were sacrificed, and tissues were harvested for further analyses. 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 Institutional Animal Care and Use Committee of Virginia Tech.

Analyses of neutrophil phenotype in vivo
Peripheral blood cells, BM cells, and splenocytes were harvested from the mice treated as described above. Cells were stained with anti-Ly6G (1:200 dilution; BioLegend, no. 127618), anti-CD11b (1:200 dilution; BioLegend, no. 101206), anti-CD62L (1:200 dilution; BioLegend, no. 104407), anti–Dectin-1 (1:200 dilution; BioLegend, no. 144305), anti-LRRC32 (1:200 dilution; BioLegend, no. 142904), and anti-FPN (1:200 dilution; Novus, no. NB1211502) antibodies. The surface phenotype of Ly6G+ neutrophils was analyzed using FACS Canto II (BD Biosciences). The data were processed by FlowJo (TreeStar).

Adoptive transfer of in vitro primed neutrophils
BM cells were isolated from ApoE−/− mice, and BM neutrophils (Ly6G+ population) were purified by FACS (fluorescence-activated cell sorter) sorting with >99.5% purity. The cells were cultured in complete RPMI with G-CSF (100 ng/ml) in the presence of subclinical-dose LPS (100 pg/ml) or 4-PBA (1 mM) for 24 hours. Cells were washed three times with PBS and suspended in PBS for injection. HFD-fed ApoE−/− mice were transfused with 2 × 10⁶ neutrophils in 200 μl of PBS once weekly through intravenous injection for 4 weeks. One week after the last cell transfer, mice were sacrificed and tissues were harvested for subsequent analyses.

Analyses of atherosclerotic lesions
Histological analyses were performed on fresh-frozen and optimal cutting temperature (OPT) compound–embedded proximal aortic sections (8 μm). Slides were fixed in 4% neutral buffered formalin for 5 min, followed by H&E or Oil Red O staining. Collagen staining was performed using the Picrosirius Red Stain Kit (Polysciences) according to the manufacturer’s instructions. The samples were observed using a light microscope. The percentages of total lesion area, lipid deposition, and collagen composition were calculated. For immunofluorescence staining, proximal aortic sections were fixed with 4% neutral buffered formalin for 5 min, permeabilized with 0.1% saponins, and blocked with 10% normal goat serum (Jackson ImmunoResearch) for 1 hour. To detect lesional neutrophils, the samples were stained with Alexa Fluor 647–conjugated anti-Ly6G antibody (1:50 dilution; BioLegend, no. 127610). To detect lesional macrophages, the samples were co-stained with eFluor 660–conjugated anti-CD68 (1:100 dilution; Thermo Fisher Scientific, no. 50–0681–82) and rabbit anti–SR-A (1:100 dilution; Thermo Fisher Scientific, no. PA5–22956) antibodies, followed by staining with DyLight 488–conjugated goat anti-rabbit immunoglobulin G (IgG) (1:100 dilution; Thermo Fisher Scientific, no. 35552) in the dark at room temperature for 1 hour. To detect oxCAMKII levels, the samples were stained with rabbit anti–oxCAMKII antibody (1:100 dilution; Millipore, no. 07–1387) at 4°C overnight and then with DyLight 488–conjugated goat anti-rabbit IgG (1:100 dilution; Thermo Fisher Scientific, no. 35552) in the dark at room temperature for 1 hour. 4′,6-Diamidino-2-phenylindole was used to stain nucleus. The samples were observed under a confocal microscope.

In vitro neutrophil priming and FACS analyses
BM neutrophils were isolated from C57BL/6 mice and cultured in complete RPMI medium containing 10% fetal bovine serum, 2 mM l-glutamine, and 1% penicillin/streptomycin in the presence of G-CSF.
ELISA and determination of plasma lipids
For in vivo analyses, plasma was collected from the mice at time of sacrificing. For in vitro analyses, purified BM neutrophils were cultured in complete RPMI medium with G-CSF (100 ng/ml) in the presence of PBS, subclinical-dose LPS (100 pg/ml), or 4-PBA (1 mM), and supernatant was collected after 2 days. ELISA kit of MPO was purchased from Thermo Fisher Scientific, and ELISA kits of MMP9, LTB4, and TGFβ1 were purchased from R&D Systems. Cholesterol quantitation kit was purchased from Sigma-Aldrich, and triglyceride quantification kit was purchased from BioVision.

Immunoblotting
Purified BM neutrophils were cultured in complete RPMI medium with G-CSF (100 ng/ml) in the presence of PBS, subclinical-dose LPS (100 pg/ml), oxLDL (10 μg/ml), or 4-PBA (1 mM), and total cell lysate was extracted on day 2. Protein samples were separated with SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were probed with anti-oxCaMKII (1:500 dilution; Millipore, no. 07-1387), anti–5-LOX (1:500 dilution; Cell Signaling Technology, no. 32895S), and anti–β-actin (1:1000 dilution; Santa Cruz Biotechnology, no. sc-47778) primary antibodies, followed by horseradish peroxidase–conjugated anti-rabbit IgG (1:1000 dilution; Cell Signaling Technology, no. 7074S) or anti-mouse IgG (1:1000 dilution; Cell Signaling Technology, no. 7076S) secondary antibodies. Images were developed with an enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific).

Real-time reverse transcription polymerase chain reaction for detection of miRNAs
For in vivo analyses, plasma was collected from the mice at time of sacrificing. Circulating miRNAs were isolated using miRNeasy Serum/Plasma kit (Qiagen). For in vitro analyses, purified BM neutrophils were cultured in complete RPMI medium with G-CSF (100 ng/ml) in the presence of PBS, subclinical-dose LPS (100 pg/ml), or 4-PBA (1 mM), and miRNAs were isolated using the miRNeasy Mini Kit (Qiagen) after 2 days. TaqMan miRNA assay kits for detection of U6, miR-16, miR-24, and miR-126 were purchased from Thermo Fisher Scientific. Real-time reverse transcription polymerase chain reaction (RT–PCR) was performed following the manual. U6 expression was used as the internal control for miRNA expressions in neutrophil cultures, and miR-16 expression was used as the internal control for plasma miRNA expressions.

Confocal microscopy
Purified BM neutrophils were cultured in complete RPMI medium with G-CSF (100 ng/ml) in the presence of PBS, subclinical-dose LPS (100 pg/ml), or 4-PBA (1 mM) for 2 days. To determine lysosome-peroxisome fusion, the cells were fixed with 4% paraformaldehyde, deposited on slides through cytopsin, and permeabilized with 0.2% Triton X-100. The cells were blocked and stained with primary rabbit anti-mouse PMP70 antibody (1:1000) supplied in the SelectFX Fluor Alexa Fluor 488 Peroxisome Labeling Kit (Thermo Fisher Scientific, #S34201), followed by staining with Alexa Fluor 488 goat anti-rabbit secondary antibody (1:1000) supplied in the kit. After extensive washing with PBS, the cells were then stained with Cy3 anti-LAMP1 antibody (1:1000 dilution; Abcam, no. Ab67283) and observed under a confocal microscope.

Statistical analyses
Statistical analysis was performed using Prism 6 software (GraphPad Software, La Jolla, CA), and data were expressed as means ± SEM. The significance of the differences was assessed by Student’s t test (for two groups) or one-way analysis of variance (ANOVA) (for multiple groups). P < 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/2/eaav2309/DC1
Fig. 1. Subclinical endotoxin up-regulates MPO level in HFD-fed mice.
Fig. 2. Subclinical endotoxin exacerbates atherosclerotic pathogenesis in RD-fed mice.
Fig. 3. Subclinical endotoxin causes neutrophil expansion in atherosclerotic mice.
Fig. 4. Subclinical endotoxin primes neutrophils into a proinflammatory state in atherosclerotic mice.
Fig. 5. Subclinical endotoxin induces oxCAMKII elevation in vivo.
Fig. 6. Neutrophils maintain viability after in vitro polarization.
Fig. 7. Transfusion of superlow-dose LPS–polarized neutrophils elevates plasma lipid levels and modulates lesional macrophages.
Fig. 8. Superlow-dose LPS and oxLDL treatment elevates ROS accumulation in neutrophils.
Fig. 9. 4-PBA reverses superlow-dose LPS–induced differential regulation of miR-24 and miR-126 in neutrophils.
Fig. 10. Transfusion of 4-PBA–polarized neutrophils down-regulates plasma lipid levels and reduces lesional macrophage activation.

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