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Innate Priming of Neutrophils Potentiates Systemic Multiorgan Injury

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Abstract

Excessive inflammatory reactions mediated by first-responder cells such as neutrophils contribute to the severity of multiorgan failure associated with systemic injury and infection. Systemic subclinical endotoxemia due to mucosal leakage may aggravate neutrophil activation and tissue injury. However, mechanisms responsible for neutrophil inflammatory polarization are not well understood. In this study, we demonstrate that subclinical low-dose endotoxemia can potently polarize neutrophils into an inflammatory state in vivo and in vitro, as reflected in elevated expression of adhesion molecules such as ICAM-1 and CD29, and reduced expression of suppressor molecule CD244. When subjected to a controlled administration of gut-damaging chemical dextran sulfate sodium, mice conditioned with subclinical dose LPS exhibit significantly elevated infiltration of neutrophils into organs such as liver, colon, and spleen, associated with severe multiorgan damage as measured by biochemical as well as histological assays. Subclinical dose LPS is sufficient to induce potent activation of SRC kinase as well as downstream activation of STAT1/STAT5 in neutrophils, contributing to the inflammatory neutrophil polarization. We also demonstrate that the administration of 4-phenylbutyric acid, an agent known to relieve cell stress and enhance peroxisome function, can reduce the activation of SRC kinase and enhance the expression of suppressor molecule CD244 in neutrophils. We show that i.v. injection of 4-phenylbutyric acid conditioned neutrophils can effectively reduce the severity of multiorgan damage in mice challenged with dextran sulfate sodium. Collectively, our data, to our knowledge, reveal novel inflammatory polarization of neutrophils by subclinical endotoxemia conducive for aggravated multiorgan damage as well as potential therapeutic intervention.

INTRODUCTION

Systemic inflammation and multiorgan damage triggered by infection or injury can lead to severe morbidity and high mortality and poses significant health concern world-wide (1, 2). Despite extensive past research efforts involving clinical and basic studies, no effective

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DISCLOSURES

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therapy is available because of the complex and altered inflammatory dynamics that underlie the pathogenesis of systemic inflammation. Alterations of systemic inflammation appear to occur in two distinct phases (3). Early exacerbation of hyperinflammation and late immune suppression can both contribute to elevated mortality/morbidity of systemic inflammation and multiorgan injury. During the initial hyperinflammatory phase of acute systemic inflammation, injury-/pathogen-triggered hyperinflammatory reactions often lead to an excessive “inflammatory storm” aggravating multiorgan dysfunction. However, mechanisms underlying the altered inflammatory dynamics are still not well understood. This study will focus on defining mechanisms of early inflammatory exacerbation through innate priming.

Pre-existing health conditions such as ageing, obesity, and other chronic diseases associated with subclinical endotoxemia are known risk factors contributing to elevated morbidity/mortality associated with systemic inflammation and injury, likely through priming and aggravating the early phase of innate hyperinflammatory reaction (4). We and others previously reported that preconditioning of experimental animals with subclinical priming dose of endotoxin aggravates mortality of experimental sepsis, whereas preconditioning of animals with higher tolerant dosage of endotoxin alleviates early phase of hyperinflammation and reduces sepsis mortality (5, 6). Among innate immune cells mediating altered inflammation dynamics, neutrophils are the immediate responders to systemic injury and/or infection (7, 8). Previous studies suggest that neutrophils may provide either detrimental or beneficial roles during the pathogenesis of sepsis, potentially depending upon the timing and activation states of neutrophils (9). However, mechanisms for neutrophil activation dynamics are poorly understood, and such lack of understanding hinders the effective prevention and treatment of systemic inflammation as well as multiorgan injury.

Using a well-controlled model of experimental systemic inflammation and multiorgan injury triggered by controlled administration of gut-damaging chemical dextran sulfate sodium (DSS), we, in this study, examined the effect of preconditioning with subclinical endotoxemia on the priming of neutrophils *in vivo* as well as the development of systemic inflammation and multiorgan damage. Previous studies suggest that pre-existing conditions such as obesity and aging increase the risk for more severe ulcerative colitis as well as subsequent comorbidity risks, including sepsis (10–12). Subclinical endotoxemia manifests in humans with obesity and ageing (13–15), which may precondition innate immune system for aggravated systemic inflammation and organ damage following subsequent ulcerative colitic injury. DSS administration is a well-established model for inducing acute gut damage mimicking ulcerative colitis. Based on these studies, we therefore tested the hypothesis that preconditioning with subclinical endotoxemia may invoke more severe systemic inflammation in experimental animals subsequently challenged with mucosal damaging agents such as DSS. In this current study, we observed that subclinical endotoxemia preconditioning led to aggravated systemic inflammation, multiorgan damage, and elevated infiltration of neutrophils into organs such as liver, colon, and spleen in mice challenged with DSS. Further characterization of neutrophil activation revealed an inflammatory polarization state represented by higher levels of adhesion molecule ICAM1 and lower expression of suppressor molecule CD244. Mechanistically, subclinical endotoxemia led to neutrophil polarization through activating SRC kinase and STAT1, and reducing AKT. We also demonstrated that the application of phenylbutyric acid (4-PBA) can effectively balance

neutrophil homeostasis by reducing the activation of SRC and inducing the homeostatic molecule CD244. Therapeutically, we demonstrated that i.v. administration of neutrophils reprogrammed by 4-PBA can effectively alleviate the severity of multiorgan failure triggered by DSS. Taken together, our study defines the novel, to our knowledge, proinflammatory polarization of neutrophils conducive for systemic inflammation and potential therapeutic intervention of neutrophil polarization.

MATERIALS AND METHODS

Experimental animals

C57BL/6 wild-type (WT) mice were purchased from the Jackson Laboratory. WT mice were bred and maintained at the animal facility at Virginia Tech in accordance with the Institute for Animal Care and Use Committee–approved protocol. Mice used in the experiment were 6–10 wk of age and 20–30 g of weight.

Reagents

DSS (MW 47,000; MP Biomedicals) was purchased from MP Biomedicals. LPS (*Escherichia coli* 0111:B4), Percoll, and 4-PBA were from Sigma-Aldrich. Anti-phospho-AKT (S473) and anti-phospho-SRC (Tyr418) were from eBioscience. Anti-phospho-STAT5, anti-phospho-STAT1, and anti-phospho-spleen tyrosine kinase (SYK) Ab were from Cell Signaling Technology. Anti-phospho-AMP-activated protein kinase (AMPK) and anti-PPAR γ Abs were purchased from Bioss Antibodies. Conjugated Abs with fluorescent dyes against Ly6G, CD11b, ICAM-1, CD29, CD244, and CD88 were from BioLegend. Anti-myeloperoxidase (MPO) Ab was from Abcam. G-CSF was from PeproTech. 3, 3'-diaminobenzidine was from Vector Laboratories.

DSS-induced colitis model

WT mice were given 4.0% (w/v) DSS in drinking water continuously for 5 d, followed with regular water for an additional 3 d. Mice were monitored for weight loss, physical body condition, stool consistency, and rectal bleeding. For preconditioning with subclinical endotoxemia, i.p. injection of subclinical dose LPS (5 ng/kg body weight) were started from 1 d before DSS treatment for 5 d (Fig. 1A).

Immunohistochemistry and TUNEL staining

Tissues were imbedded into Optimal-Cutting-Temperature compound, then sectioned (5 μ m). Sections were fixed in 4% PFA for 10 min, then incubated in blocking buffer (PBS containing 10% goat serum and 1% BSA) for 30 min and stained with anti-MPO primary Ab (1:100) for 2 h at room temperature, followed by a biotinylated anti-IgG secondary Ab and ABC Elite Kit (Vector Laboratories), then subjected to substrate (3, 3'-diaminobenzidine) detection and counterstained with methyl green.

For TUNEL staining, tissues were fixed in 4% PFA and embedded into paraffin. Samples were sectioned (5 μ m). After deparaffination, colorimetric IHC Detection Kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions. Multiple viewing

fields from each slide were captured. Images were quantified with National Institutes of Health ImageJ software.

Neutrophil purification and culture

Neutrophils were isolated from WT mice bone marrow. Briefly, after lysis of RBCs, bone marrow cells resuspended in HBSS were layered over a Percoll gradient (62.5%) and centrifuged at $1000 \times g$ for 30 min. The purities of neutrophils were >90% as assessed by flow cytometry analysis. Isolated neutrophils were cultured with RPMI completed medium (10% FBS, 1% penicillin/streptomycin, 1% glutamine) supplemented with 100 ng/ml G-CSF and stimulated LPS (100 pg/ml) or 4-PBA (1 mM) or PBS at 37°C in 5% CO₂ overnight. The cells were harvested next day for further analysis.

Biochemical analysis

Peripheral blood was collected into EDTA-coated tubes. After centrifugation, plasma was collected and stored in -80°C for later analysis. Activities of lactate dehydrogenase (LDH) were measured enzymatically in the plasma samples using commercial assay kit from Biovision according to the manufacturer's instructions. Troponin-1 levels in plasma were analyzed by ELISA using ELISA Kit from Life Diagnostics. Plasma creatinine and blood urea nitrogen (BUN) were detected by Colorimetric Assay Kits from Cayman Chemical and Thermo Fisher Scientific, respectively.

Flow cytometry

Single-cell suspensions were prepared and stained with fluorescently conjugated Abs in the presence of Fc block on ice for 20 min. After washing, the cells were resuspended in flow buffer ($1 \times$ HBSS/2% FBS), and subjected to flow analysis. Intracellular phospho-proteins were detected with Transcription Factor Phospho Buffer Set (BD Pharmingen) according to the manufacturer's instructions. Briefly, after incubation, neutrophils were fixed and permeabilized with TFP buffer for 50 min and perm buffer III for 20 min sequentially, then washed and stained with fluorescent Abs against Ly6G and phosphoproteins for 45 min on ice. Samples were analyzed with a FACSCanto II (BD Biosciences). FACS plots shown were analyzed with FlowJo (Ashland, OR).

Adoptive transfer of neutrophils

WT mice were divided into two recipient groups transfused with either 4-PBA-primed neutrophils or PBS-primed neutrophils as control. All recipients were given 4.0% (w/v) DSS in drinking water continuously for 5 d followed with regular water for 1 d. Neutrophils for adoptive transfer purpose were isolated from naive WT mice and purified by EasyStep Mouse Neutrophil Enrichment Kit (StemCell Technologies) according to the instruction from the manufacturer. Purified neutrophils were cultured in RPMI complete medium supplemented with 100 ng/ml G-CSF and treated with 4-PBA (1 mM) or PBS as control overnight. Then the neutrophils were harvested and resuspended in PBS to the concentration of 25×10^6 cells per milliliter. According to the physiological data summary of C57BL/6J from Jackson Laboratory, the estimation of circulating neutrophil count is $\sim 0.78 \times 10^6$ in an 8-wk-old male mouse. Then, 5×10^6 cells were adoptively transfused each time to recipient

mouse by tail i.v. injection on day 2 and day 5 during the DSS colitis model. Mice were sacrificed on day 6 (Fig. 6A).

Statistical analysis

Data were represented as mean \pm SD. Graphs and statistical analysis were conducted via GraphPad PRISM software. Student *t* test was used for parametric analyses between two groups. Complex data sets were analyzed by one-way ANOVA and followed by Tukey–Kramer test. The $p < 0.05$ was considered statistically significant.

RESULTS

Subclinical endotoxemia exacerbates systemic inflammation and multiorgan damage

To examine the impact of subclinical endotoxemia on early phase of inflammation, WT mice were preconditioned i.p. with a super low dose of LPS (5 ng/kg body weight) or PBS as control as shown in Fig. 1A. Preconditioned mice were then subjected to DSS-induced gut damage and monitored closely. Compared with the control group, the mice preconditioned with the super low dose of LPS showed much severe disease development, including aggravated weight loss (Fig. 1B), severe clinical scores (Fig. 1C), and much shorter colon lengths (Fig. 1D). Meanwhile, elevated bacteria burden was detected in the peripheral blood from LPS-preconditioned mice (Fig. 1E). Sepsis caused by systemic infection can rapidly lead to multiorgan failure. Therefore, we next examined key parameters representative of multiorgan damage. As results shown in Fig. 1F, the levels of LDH, Troponin-1, and creatinine in the plasma were significantly higher in mice injected with a super low dose of LPS, representing tissue damages from liver, heart, and kidney respectively. An independent assay measuring tissue damage with TUNEL staining was also performed on sectioned tissues from heart, liver, and kidney. We observed significantly elevated TUNEL-positive staining in the heart, liver, and kidney tissues collected from LPS-preconditioned mice as compared with PBS-preconditioned mice (Fig. 1G). Collectively, these data reveal that mice preconditioned with the super low dose of LPS displayed severe development of systemic inflammation and multiorgan failure upon DSS challenge.

Subclinical endotoxemia preconditioning exacerbates neutrophil tissue infiltration and activation in vivo

Neutrophil is the first defender of bacterial infection, and neutrophil dysfunction is implicated in the progression of systemic inflammation and multiorgan damage. Previous studies show that excessive and persistent infiltration of neutrophils into tissues can aggravate multiple organ failure accompanying sepsis (16). Thus, we next examine the neutrophil status in the preconditioned mice. We observed that the percentage of neutrophils (Ly6G⁺CD11b⁺) in the blood from LPS-preconditioned mice was significantly higher than that in PBS-preconditioned control mice (Fig. 2A). Moreover, a significantly higher number of neutrophils was also detected in the colon tissues of LPS-preconditioned mice, as measured by elevated MPO-positive staining in the colon sections from the LPS-conditioned group (Fig. 2B). Higher numbers of infiltrating neutrophils were also observed in the spleen and the liver tissues of LPS-conditioned mice, as measured by flow cytometry (Fig. 2C).

Collectively, our data confirm the elevated infiltration of neutrophils in mice conditioned by subclinical endotoxemia.

Subclinical endotoxemia polarizes inflammatory neutrophils by inducing ICAM-1 and reducing CD244

Tissue infiltration and activation of neutrophils are closely regulated by adhesion molecules (17). Septic neutrophils from experimental animals as well as septic human patients often exhibit elevated levels of ICAM-1 (18). We therefore examined the levels of key adhesion molecules such as CD29 and ICAM-1 on neutrophils in mice preconditioned by subclinical dose endotoxemia. We observed that CD29, also known as integrin β 1, which plays essential roles in neutrophil adhesion, aggregation, and activation, was increased in the neutrophils from LPS-preconditioned mice (Fig. 3A). Moreover, the expression of ICAM-1 on the neutrophils was also significantly higher in LPS-preconditioned mice (Fig. 3B). In contrast, CD244 is a known suppressor of myeloid inflammation (19–21). We observed a significant reduction of CD244 on neutrophils harvested from LPS-preconditioned mice, as compared with that in PBS-preconditioned mice (Fig. 3C). Our data suggest that LPS preconditioning potentially skews neutrophils into a proinflammatory state conducive for excessive tissue infiltration during DSS-triggered colitis and contribute to the aggravation of multiorgan injury.

Subclinical endotoxemia increases neutrophil SRC kinase and STAT1/5 and reduces phospho-AKT and phospho-AMPK

To further explore the mechanisms of neutrophil polarization, bone marrow neutrophils were isolated and challenged with super low dose of LPS (100 pg/ml) in an in vitro culture. Consistent with in vivo observations, LPS significantly increased the expression of CD29 and ICAM-1, but reduced CD244 (Fig. 4A). Tyrosine kinases such as SRC are known to be downstream activators of TLR4, and play important roles in the activation of transcription factor STAT1 and induction of ICAM-1 (22, 23). Upon a super low dose of LPS stimulation, we observed that the phosphorylation levels of tyrosine kinase SRC and SYK were significantly increased (Fig. 4B). Moreover, phosphorylation levels of STAT1 and STAT5 were significantly elevated by super low dose of LPS treatment (Fig. 4C), consistent with the increased expression of ICAM-1 and CD29. In contrast, previous studies reveal that the activation of AMPK and AKT can exert anti-inflammatory effects (24, 25). We thus tested the levels of AMPK and AKT in neutrophils polarized by superlow dose LPS. As results shown in Fig. 4D, super low dose of LPS significantly reduced the phosphorylation of AMPK and AKT. Together, our mechanistic studies reveal that super low dose of LPS selectively induces SRC-mediated STAT1/5 activation and suppresses anti-inflammatory AMPK and AKT, leading to inflammatory polarization of neutrophils conducive for tissue infiltration and damage.

4-PBA can potentially reduce SRC kinase and increases CD244 in neutrophils

4-PBA is a potential anti-inflammation compound, known as an inducer for peroxisome homeostasis (26). Therefore, we next tested whether 4-PBA may have anti-inflammatory effects on neutrophil polarization. Purified neutrophils were treated with 4-PBA (1 mM) or PBS. As data shown in Fig. 5A, 4-PBA effectively induced the expression of

immunosuppressive marker CD244. Meanwhile, 4-PBA also significantly reduced the expression of the complement C5a receptor, CD88. Blockage of C5a binding has been reported to reduce the mortality of experimental sepsis (27). Mechanistically, we observed that 4-PBA attenuated the phosphorylation of SRC and SYK (Fig. 5B). In contrast, the expression of PPAR γ was induced by 4-PBA (Fig. 5C), which may be responsible for the elevated induction of CD244.

4-PBA–trained neutrophils can potentially reduce septic mortality

Based on the finding that 4-PBA could potentially switch neutrophils to an anti-inflammatory state, we next test the function of 4-PBA–primed neutrophils *in vivo*. DSS-challenged mice were *i.v.* administered with either 4-PBA–conditioned neutrophils or PBS-conditioned neutrophils on day 2 and day 5 of the DSS regimen as indicated in Fig. 6A. We have previously reported that neutrophils cultured *in vitro* can survive at least 48 h, and that adoptively transferred neutrophils can effectively traffic into mucosal tissues in recipient mice (28, 29). Administration of 4-PBA–conditioned neutrophils prevented weight loss (Fig. 6B) and reduced the shortening of colon length (Fig. 6C). Moreover, the plasma levels of LDH, BUN, and Troponin-1 were significantly reduced in mice receiving 4-PBA–conditioned neutrophils as compared with mice receiving PBS-conditioned control neutrophils (Fig. 6D), suggesting that the administration of 4-PBA–conditioned neutrophils can effectively reduce tissue damage associated with mucosal colitis injury. Moreover, not only the percentage of circulating neutrophils, but also the percentages of tissue-infiltrating neutrophils in the liver and the spleen were significantly reduced in mice receiving 4-PBA–conditioned neutrophils (Fig. 6E). The reduction of adhesion molecules such as CD29 and ICAM-1 was also observed on neutrophils from mice receiving 4-PBA–conditioned neutrophils (Fig. 6F)

DISCUSSION

Our data demonstrate that the inflammatory polarization of neutrophils preconditioned by subclinical endotoxemia leads to elevated neutrophil infiltration into vital organs during experimental ulcerative colitis and aggravates systemic multiorgan injury. Our study further reveals the activation of SRC and downstream STAT1/STAT5 as a potential mechanism for neutrophil polarization by subclinical endotoxemia. We further document that 4-PBA can effectively suppress SRC kinase activation and induce PPAR γ -mediated anti-inflammatory polarization of neutrophils conducive for reducing acute systemic inflammation and multiorgan damage.

Our study expands our understanding of neutrophil priming by subclinical endotoxemia, which bears fundamental and translational significance in the emerging field of programming dynamics of innate immunity and inflammation. Subclinical endotoxemia has been increasingly noticed in humans and experimental animals with chronic diseases (30, 31). Emerging data suggest that subclinical endotoxemia can potentiate the inflammatory polarization of monocytes involved in the pathogenesis of chronic inflammatory diseases such as atherosclerosis and neurologic complications (32, 33). Our current study complements these previous observations and further demonstrates that subclinical dose LPS

is potent enough to enhance the activation of neutrophils as reflected in elevated expression of adhesion molecules such as ICAM-1 and CD29 involved in tissue infiltration. At the translational level, our data showing elevated multiorgan damage in mice conditioned with subclinical endotoxin reconcile the effects of subclinical endotoxemia in humans with chronic conditions prone to the development of severe sepsis (30, 34).

In addition to inflammatory mediators, neutrophils are known to express suppressor molecules such as CD244. CD244 is a known marker of myeloid suppressor cells involved in suppressing the activation of neighboring myeloid cells (20, 21). Upregulation of CD244 on granulocyte-derived suppressor cells has been shown to reduce the severity of sepsis (21). Our current data reveal that neutrophils conditioned with subclinical low dose of LPS not only express inflammatory adhesion molecules such as ICAM-1 and CD29 but also exhibit reduced expression of CD244. The reduced CD244 levels on neutrophils is also observed in vivo in mice conditioned with subclinical dose LPS. The unique polarized induction of ICAM-1 and suppression of CD244 in neutrophils conditioned by subclinical endotoxemia may contribute to the hyperinflammation during the early phase of sepsis and elevate sepsis severity.

At the mechanistic level, our data suggest that SRC kinase and STAT1/5 in neutrophils can be potentially activated by subclinical dose LPS. In contrast, we show that subclinical dose LPS fails to induce anti-inflammatory signaling such as AKT and AMPK in neutrophils. This is in stark contrast to the effects of higher dose LPS, which can broadly induce the activation of diverse signaling pathways including the proinflammatory SRC as well as the anti-inflammatory AKT pathway (35, 36). Our mechanistic data are consistent with the phenotypic observation that neutrophils challenged with subclinical dose LPS exhibit increased expression of ICAM-1 and reduced levels of suppressor molecule CD244.

Our data suggest the potential of reprogrammed neutrophils by 4-PBA in preventing systemic inflammation and multiorgan injury. 4-PBA is a derivative of butyrate acid naturally occurring in healthy commensal microbiota, as well as in humans under strenuous exercise or starvation (37, 38). Previous studies reveal that 4-PBA can enhance peroxisome functions, reduce cellular stress, and reduce proinflammatory polarization of innate leukocytes (29, 39). Our current study extends these previous findings and reveal that neutrophils conditioned with 4-PBA can express the myeloid suppressor molecule CD244. A previous report using another cell stress-reliever TUDCA also reveals the induction of CD244 on granulocytes. TUDCA-treated granulocytes were shown to relieve sepsis severity when injected into mice with experimental sepsis, through CD244-mediated reduction of excessive inflammatory reactions from neighboring innate leukocytes (21). Consistent with these previous studies, we confirm that neutrophils conditioned with 4-PBA have higher expression of CD244, and that 4-PBA-conditioned neutrophils can potentially reduce the severity of experimental systemic inflammation and multiorgan injury when administered in mice. Despite these intriguing observations, this current study serves as an initial and limited attempt in addressing the emerging concept of innate neutrophil memory and its potential relevance in systemic inflammation. The lack of genetic approach precludes the in-depth characterization of detailed molecular mechanisms responsible for neutrophil priming observed in this current study.

Taken together, our study provides evidence supporting the unique inflammatory polarization of neutrophils by subclinical low-dose endotoxin, and reveals potential molecular mechanisms underlying neutrophil polarization. However, we realize that the pathogenesis of multiorgan failure and sepsis triggered by mucosal injuries is a highly complex syndrome, which involves temporal and spatial alterations of innate immune reprogramming. The effects of neutrophils during early and late stages of mucosal injury as well as subsequent multiorgan damage may drastically differ and require further extensive studies. Our current study primarily addressed the potential preventative effects of 4-PBA programmed neutrophils during the progression of mucosal damage and systemic inflammation. At the later stage of disease pathogenesis, neutrophils programmed into an immunosuppressive phenotype may in turn compromise host immune defense. Distinct neutrophil reprogramming strategies other than the one we described in this report may be needed to mitigate immunosuppression and related complications. Future systems studies that use single-cell analysis with detailed time courses are warranted to reveal a comprehensive profile of neutrophil programming dynamics during early and late phases of systemic inflammation collectively leading to multiorgan injuries.

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Abbreviations used in this article:

AMPK	AMP-activated protein kinase
BUN	blood urea nitrogen
DSS	dextran sulfate sodium
LDH	lactate dehydrogenase
MPO	myeloperoxidase
4-PBA	4-phenylbutyric acid
SYK	spleen tyrosine kinase
WT	wild-type

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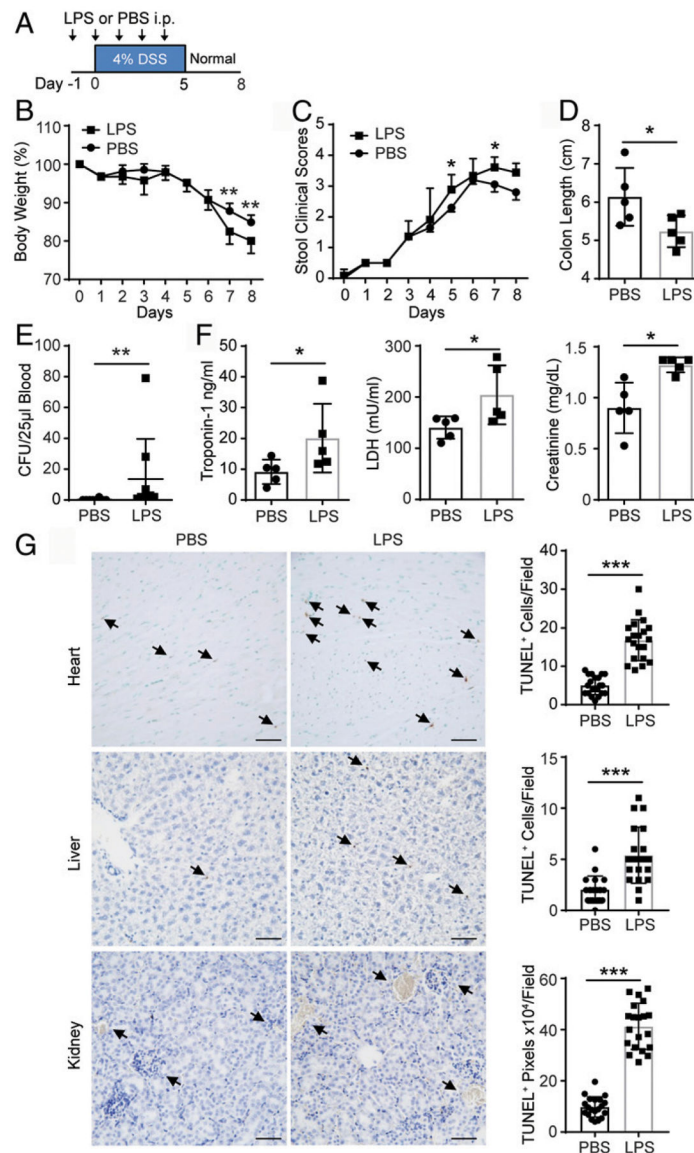


FIGURE 1. Subclinical endotoxemia exacerbates DSS-induced colitis.

(A) Schematic picture of the mouse acute colitis model, preconditioned with subclinical endotoxemia. WT mice ($n = 5$ each group) were preconditioned with subclinical dose of LPS (5 ng/kg body weight) or PBS as control, then subjected to 4% DSS challenge as described in the section of Materials and Methods. (B) Weight loss of the DSS-treated mice preconditioned with LPS or PBS. $**p < 0.001$. (C) Analysis of stool clinical stores based on daily monitors of stool consistency and rectal bleeding. $*p < 0.05$. (D) The length of colons from the DSS-treated mice preconditioned with LPS or PBS. $*p < 0.05$. (E) Bacterial counts in the blood from the DSS-treated mice preconditioned with LPS or PBS. $**p < 0.001$. (F) The measurements of cardiac troponin-1, LDH, and creatinine in the plasma. $*p < 0.05$. (G) Representative images of TUNEL staining of heart, liver, and kidney sections, and quantification of TUNEL-positive (brown color) area ($n = 20$ random fields). Scale bars, 50 μm . $***p < 0.0001$.

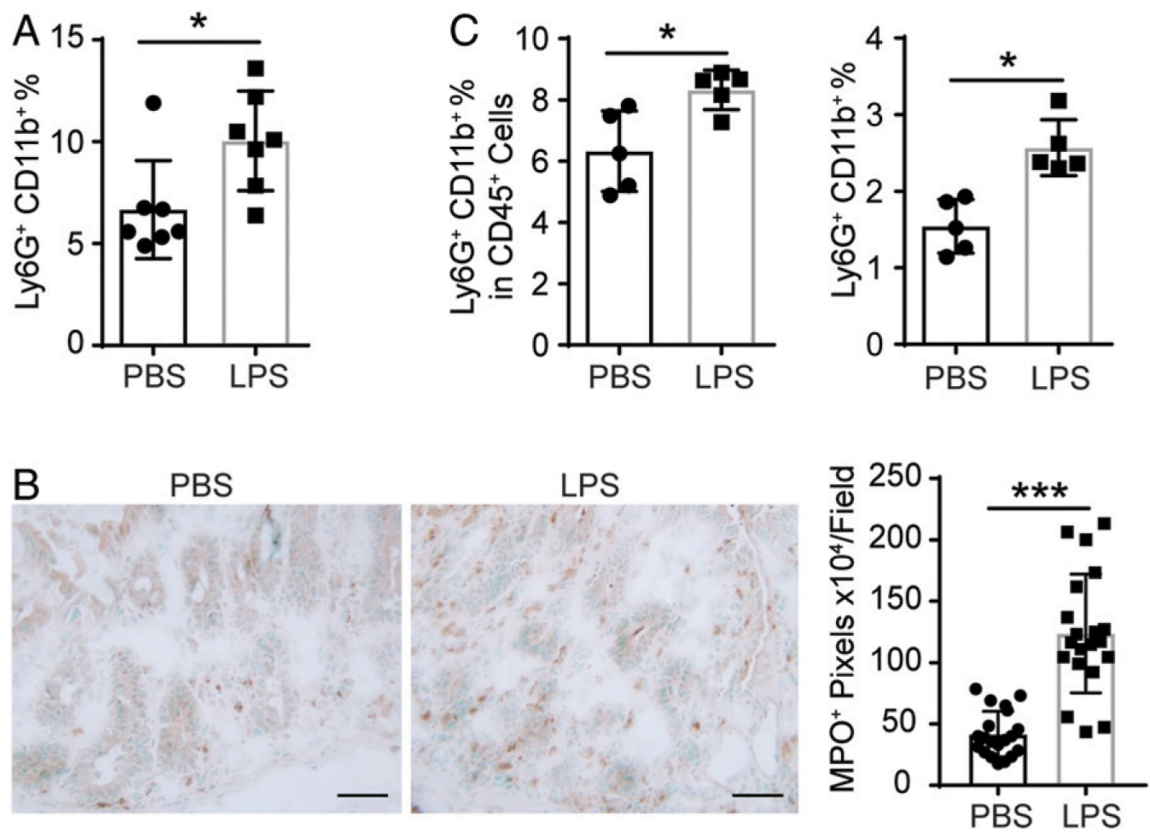


FIGURE 2. Subclinical endotoxemia increased tissue neutrophil infiltration.

(A) Percentages of neutrophils (Ly6G⁺CD11b⁺) in the peripheral blood from the DSS-treated mice preconditioned with LPS or PBS. (B) Representative images of colon MPO staining, and quantification of MPO positive (brown color) area. Scale bars, 50 μ m. (C) The percentage of neutrophils in liver and spleen from the DSS-treated mice preconditioned with LPS or PBS. * $p < 0.05$, *** $p < 0.0001$.

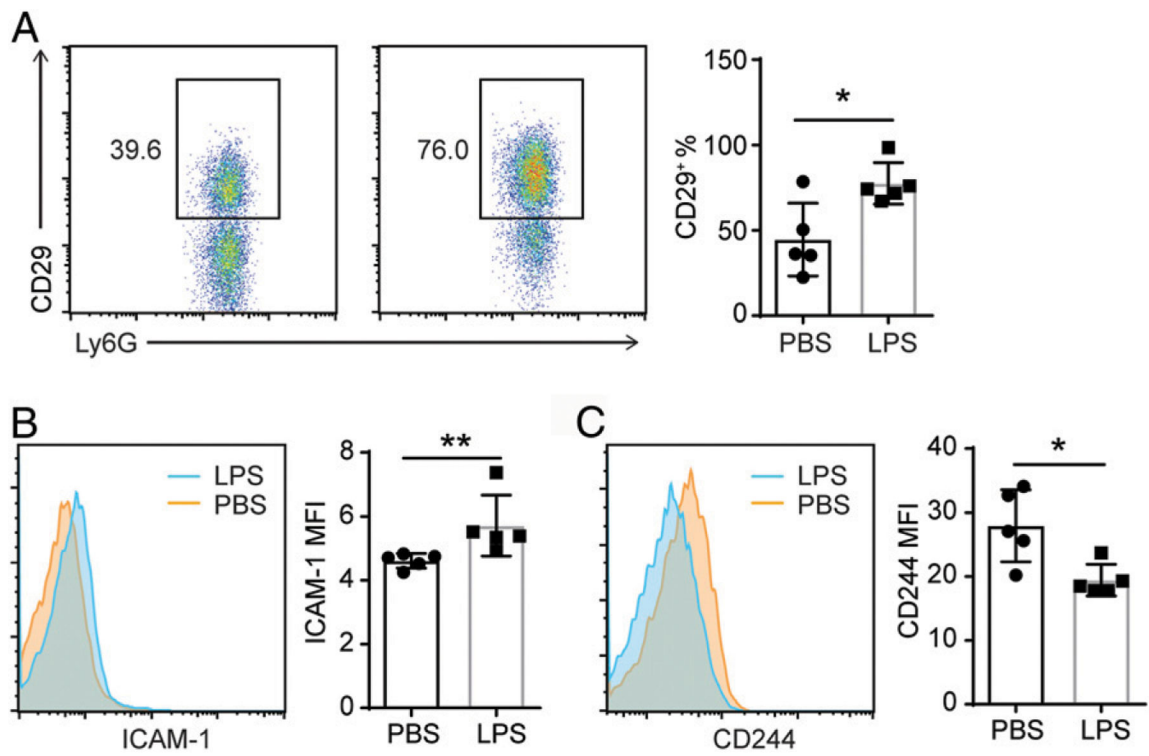


FIGURE 3. Subclinical endotoxemia polarizes inflammatory neutrophils by inducing ICAM-1 and reducing CD244.

In the DSS-treated mice preconditioned with LPS or PBS, the phenotype of neutrophils in the blood were examined by flow cytometry analysis. (A) The percentage of CD29 positive cells in neutrophils (Ly6G⁺CD11b⁺). (B) ICAM-1 expression on neutrophils. (C) CD244 expression on neutrophils. * $p < 0.05$, ** $p < 0.001$.

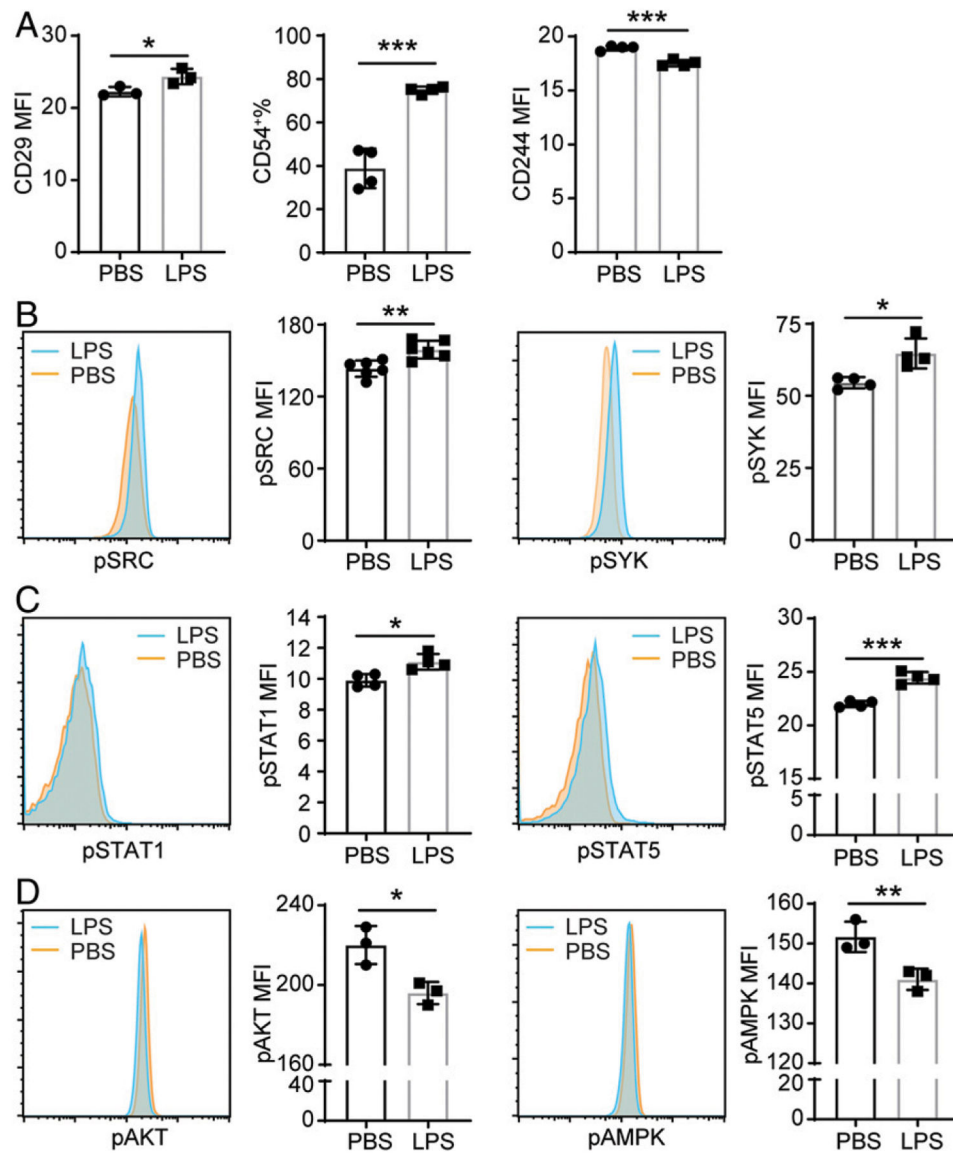


FIGURE 4. Subclinical LPS primes neutrophils into a proinflammatory state in vitro. Bone marrow neutrophils were isolated and treated with super low dose of LPS (100pg/ml), then subjected to flow cytometry analysis. **(A)** Expression of surface marker CD29, ICAM-1, and CD244 on the neutrophils. **(B)** Representative histogram and quantification of phosphorylation levels of tyrosine kinase SRC and SYK in the neutrophils as determined by flow cytometry. **(C)** Representative histogram and quantification of phosphorylation levels of transcription factor STAT1 and STAT5 in neutrophils as determined by flow cytometry. **(D)** Representative histogram and quantification of phosphorylation levels of AKT and AMPK in the neutrophils as determined by flow cytometry. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

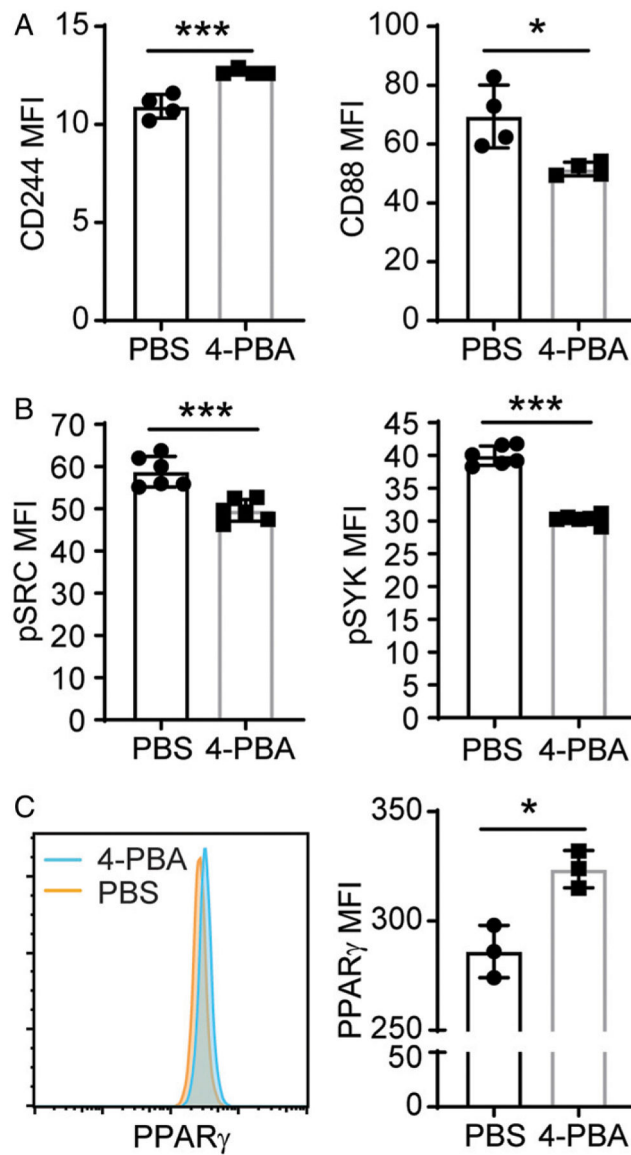


FIGURE 5. 4-PBA can potently reduce SRC kinase and increases CD244 in neutrophils. Bone marrow neutrophils were treated with 4-PBA (1 mM) or PBS as a control, then subjected to flow cytometry analysis. **(A)** Expression of surface molecule CD244 and CD88 on the neutrophils. **(B)** Phosphorylation levels of tyrosine kinase SRC and SYK in the neutrophils. **(C)** Representative histogram and quantification of PPAR γ expression in the neutrophils. * $p < 0.05$, *** $p < 0.0001$.

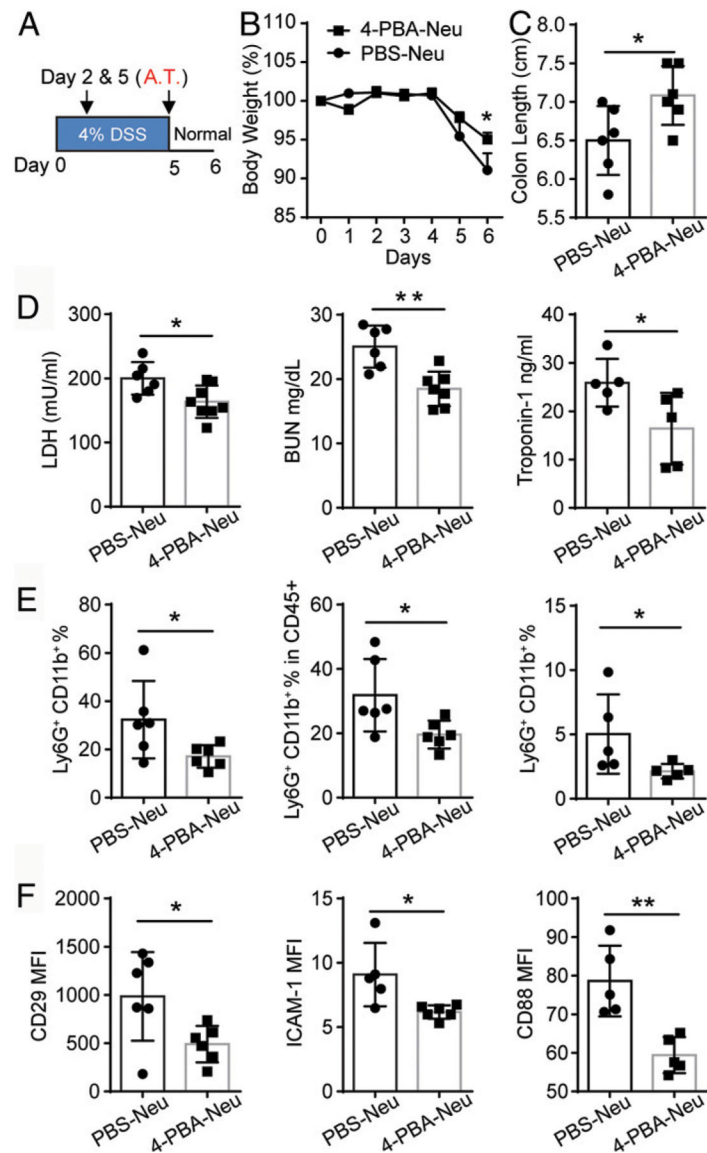


FIGURE 6. 4-PBA-primed neutrophils alleviate DSS-induced colitis.

(A) Schematic experimental design for neutrophil adoptive transfer in the DSS-induced colitis model. DSS-fed WT mice were transfused with 4-PBA- or PBS-primed neutrophils via i.v. injection ($n = 5$ each group). (B) Body weight loss was monitored daily. (C) The measurement of colon length at the end of the experimental regimen. (D) The measurements of LDH, cardiac troponin-1, and BUN in the plasma. (E) Percentages of neutrophils (Ly6G⁺CD11b⁺) in peripheral blood, liver, and spleen. (F) Expression of surface molecules (CD29, ICAM-1, and CD88) on the neutrophils from peripheral blood. * $p < 0.05$, ** $p < 0.001$.