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The deficiency of macrophage erythropoietin signaling contributes to delayed acute inflammation resolution in diet-induced obese mice

Bangwei Luo[a,b,1], Zhishang Wang[b,1], Zhiyuan Zhang[c,1], Zigang Shen[b], Zhiren Zhang[b,*]

[a] Discipline Integrated Chinese and Western Medicine, School of Basic Medicine, Guangzhou University of Chinese Medicine, Guangzhou 510006, Guangdong, China
[b] Institute of Immunology, Army Medical University, 30 Gaotanyan Main Street, Chongqing 400038, China
[c] Department of Pathology, Nanjing Medical University, Hanzhong Road 140, Nanjing 210029, China

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ABSTRACT

Obesity has been linked with altered acute inflammation resolution which contributes to obesity-related clinical complications; however, the mechanisms that contribute to obesity-related unresolved inflammation are not fully known. Here we demonstrated that the deficiency of macrophage erythropoietin (EPO) signaling contributed to delayed acute inflammation resolution in diet-induced obese mice. In zymosan-induced acute peritonitis, in line with the delayed resolution of inflammation, the induction of macrophage EPO signaling was significantly reduced in obese mice relative to normal mice. Exogenous EPO induced macrophage EPO signaling and promoted acute inflammation resolution in obese mice. Efferocytosis of apoptotic cells by macrophages which is central in inflammation resolution was impaired in obese mice and restored by exogenous EPO. Mechanistically, macrophage peroxisome proliferator-activated receptor-γ (PPARγ) was greatly reduced in obese mice and EPO increased macrophage PPARγ to promote efferocytosis in obese mice. Together, our results identify an important mechanism underlying aberrant acute inflammation resolution in obesity, with important implications for regulating unresolved acute inflammation and normalizing macrophage defects in obese and diabetic individuals.

1. Introduction

Obesity is an increasing worldwide health challenge, affecting both developed and developing countries. It is associated with co-morbidities to various diseases, such as type 2 diabetes, cardiovascular disease, cancer and neurological diseases, causing a huge health burden [1,2]. Accumulated data have shown that obesity coincides with a chronic low-grade inflammation, which is important to the development of obesity-associated clinical complications, such as type 2 diabetes, atherosclerosis, cancer, and nonalcoholic fatty liver disease and impairs host defense and wound healing [3,4]. Nevertheless, mechanisms that contribute to obesity-related chronic systemic inflammation remain to be explored.

While factors that may initiate and promote inflammation have been well established to contribute to this process (1–4), recently, factors that may delay the acute inflammation resolution were shown to participate in this process as well (12, 13, 14). Controlled inflammation is a physiologic response to exogenous or endogenous dangerous signals that is normally limited and beneficial to the host [5,6]. Extensive research has shown that the resolution of inflammation is an active programmed response that is activated post the initiation of inflammation to regulate its duration and magnitude [7–9]. The failure of inflammation resolution induces chronic inflammation which has been identified as a common basis of various widely occurring diseases, such as atherosclerosis, pulmonary fibrosis, obesity, rheumatoid arthritis and cancer. Macrophages play essential roles in inflammation resolution, functioning to clear apoptotic cells and tissue debris, produce anti-inflammatory and reparative molecules to regulate resolution. Particularly, the apoptotic cell clearance by phagocytes is important for promoting inflammation resolution, which not only prevents the secondary necrosis of apoptotic cells but also stimulates the production of anti-inflammatory and reparative molecules by phagocytes. During the past decade, lipid-derived mediators, named specialized proresolving mediators (SPMs), including lipoxins, resolvins, protectins, and maresins, were found to play important roles in resolving inflammation [7,10,11]. Moreover, the observations that these SPMs are deficient in obesity and the exogenous SPMs stimulate acute inflammation resolution in obesity have been reported, indicating an important

* Corresponding author at: Institute of Immunology, College of Basic Medicine, Army Medical University, 30 Gaotanyan Main Street, Chongqing 400038, China.
E-mail address: zhangzhiren@yahoo.com (Z. Zhang).
[1] These authors contributed equally to this work.

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contribution of acute inflammation resolution failure to the chronic systemic inflammation in obesity [12] [13,14]. Therefore, understanding the mechanisms underlying altered acute inflammation resolution in obesity is needed to direct new therapeutic approaches for obesity and its secondary complications.

Erythropoietin (EPO) is well known to induce haematopoiesis through EPO receptor (EPOR) expressed on erythroid progenitor cells. However, the expression of EPOR has been found on nonhematopoietic cells and EPO has been observed to exert nonhematopoietic effects over the past two decades [15–21]. Particularly, accumulated data have shown that EPO improve obesity and insulin resistance, suggesting that EPO is a potent regulator of obesity. It has been reported that EPO reduces food intake and preadipocyte differentiation and increases fat oxidation, leading to improved fat mass and insulin resistance in obese mice [22]. However, the involvement of EPO in inflammation regulation in obesity remains poorly known. Interestingly, macrophages also express EPOR [16,23,24] and EPO has been reported to suppress inflammatory macrophage activation [23,25]. Furthermore, we recently found that EPO increased macrophage phagocytosis of apoptotic cells and contributed to acute inflammation resolution [26,27]. In our previous investigations, we found that levels of EPO and EPOR increased during zymosan A-induced peritonitis, EPOR only expressed in macrophages but not in neutrophils and macrophage EPOR signaling was important for promoting inflammation resolution. However, the involvement of EPO in obesity related acute inflammation delay remains unknown. Here we showed that the resolution of acute peritonitis in obesity was delayed in part due to the deficiency of macrophage EPO signaling and exogenous EPO induced macrophage EPO signaling and promoted acute inflammation resolution in obese mice. Efferocytosis of apoptotic neutrophil by macrophages was impaired in obese mice and restored by EPO during the acute peritonitis. Together, our findings suggest that targeting macrophage EPOR or downstream signaling molecules may be a new way to promote acute inflammation resolution and restore phagocyte defects in obese and diabetic individuals.

2. Materials and methods

2.1. Animals

C57BL/6 wild type mice were purchased from the Vital River Laboratories. The C57BL/6 background myeloid-specific *EPOR* [26] or *PPARγ* [28] knockout mice were generated as described previously. The $EPOR^{f/f}LysMCre^{+/+}$ or $PPAR\gamma^{f/f}LysMCre^{+/+}$ littermates were referred to as *EPOR-MKO* and *PPARγ-MKO* mice respectively in this study. Mice between the ages of 10 and 12 weeks were age- and sex-matched for all experiments. Mice were anesthetized with 4% isoflurane. All animal experiments were carried out in accordance with guidelines set by the Ethics Committee for Animal Experiments of Army Medical University.

2.2. Obesity induction and detection

The obese mice were induced by high fat diet (HFD) containing 5240 kcal/kg and 34.9% crude fat as described in our previous report [29]. Following experiment, the mice were sacrificed by cervical dislocation. Their subcutaneous fat deposits and abdominal fat deposits were dissected and weighted. The abdominal and subcutaneous fat mass constituted the fat mass, and the body mass minus the fat mass constituted the lean body mass. In addition, the glucose and insulin tolerance tests were carried out as described in our previous report [29]. Briefly, mice (n = 6/group) were fasted for 16 h and challenged with 1) an intraperitoneal load of glucose (1 g/kg) for glucose tolerance testing, or 2) an intraperitoneal load of human insulin (0.375 IU/kg, Actrapid, Novo Nordic, Denmark) for insulin tolerance testing. Blood samples (10 μl) were taken retro-orbitally from conscious mice at 0, 20, 40, 60, 80, and 100 min after glucose or insulin load. Blood glucose levels were determined with the help of a Onetouch Ultra (Johnson & Johnson, USA).

2.3. Peritoneal inflammation induction and intervention

Peritoneal inflammation in mice was induced by intraperitoneal (i.p.) administration of zymosan A (zymA; 40 mg/kg body weight; Sigma-Aldrich, St Louis, MO, USA) as described previously [30]. For intervention, recombinant human EPO (rhEPO; Sunshine Pharmaceutical, Shenyang, China) or rosiglitazone (RSG; Sigma-Aldrich, St Louis, MO, USA) was intraperitoneal or orally given to peritonitis mice for indicated dosage and time.

The inflammation resolution analysis was performed as described in our previous report. Briefly, the peritoneal exudates were collected by lavage twice with 5 mL of ice-cold PBS at indicated time. Following filtered through a 70 μm cell strainer, the collected cells were incubated with RBC lysis buffer (eBioscience, San Diego, CA, USA) for 5 min at room temperature. Consequently, the leukocytes were collected for following analyses. To determinate exudate cell composition, the total infiltrated leukocyte number was counted by trypan blue. Neutrophils ($Ly6G^+$) and monocytes/macrophage ($F4/80^+$) were enumerated by staining of the exudate cells with anti-mouse Ly6G (1:100, Sungene Biotech, Tianjin, China) and anti-mouse F4/80 antibodies (1:100, Sungene Biotech, Tianjin, China), and then analyzed by flow cytometry [31]. The resolution indices were defined as described by Serhan et al.: Ψ_{max}, the maximal neutrophil numbers; T_{max}, the time point of maximal neutrophil infiltration; Ψ_{50}, 50% of maximal neutrophil; T_{50}, the time point when neutrophil numbers reduce to 50% of maximum; R_i (resolution interval, T_{50}-T_{max}), the time period when 50% neutrophil are lost from exudates [32].

2.4. Flow cytometry

Single-cell suspensions were incubated with anti-CD16/32 antibodies (Sungene Biotech, Tianjin, China) to block unspecific binding and consequently subjected to surface antibody staining for 20 min at 4 °C. Following incubation, cells were washed and analyzed immediately or subjected for intracellular staining which were then fixed and permeabilized with Intracellular Fixation & Permeabilization Buffer and then incubated with indicated antibodies. Following staining, cells were suspended in PBS and then analyzed on CANTO II (Becton Dickinson, Franklin Lakes, NJ, USA). Isotype controls were applied for all staining and following antibodies were used: F4/80 (1:100, M100F1-11A Sungene Biotech, Tianjin, China, BM8), CD11b (1:100, M10117-11C, Sungene Biotech, Tianjin, China, M1/70), EPOR (1:100, sc-697, Santa Cruz, CA, USA), annexin-V (1:20, AO2001-02, Sungene Biotech, Tianjin, China), Ly6G (1:100, M100L8-09B, Sungene Biotech, Tianjin, China, 1A8), PPAR-γ (1:100, Abcam, Cambridge, UK) and p-Jak2 (1:400, Cell Signaling Technology, Danvers, US).

For FACS analysis of inflammatory cytokines, peritoneal fluid was collected, and flow cytometry was applied with a Mouse Inflammation CBA Kit (BD PharMingen, Franklin Lakes, NJ, USA) to determine concentrations of inflammatory cytokines, including IL-6, IL-10, IL-12, MCP-1, IFN-γ and TNF-α according to manufacturer's instruction.

2.5. Apoptotic cell phagocytosis assay

The in vitro phagocytosis assays were carried out as previously described with some modifications [33]. Briefly, murine peritoneal neutrophils were collected from C57/Bl6 mice after 4 h zymA-induced peritonitis, and cultured in complete RPMI 1640 for 24 h to induce cell apoptosis. Then, the apoptotic neutrophils were labeled with pHrodo™ Green (pHrodo, Molecular Probes, Eugene, Oregon, USA) that is a pH-sensitive phagocytosis-dependent indicator according to the manufacturer's instructions. Consequently, the labeled apoptotic neutrophils were incubated with peritoneal macrophages at a 1:5 ratio at 4 °C (negative control) or 37 °C for 60 min in RPMI 1640 supplemented with

10% FBS. Thereafter, macrophages were collected and labeled with anti-mouse CD11b antibody and anti-mouse F4/80 antibodies, and the percentage of $CD11b^+F4/80^+$pHrodo$^+$ macrophages to $CD11b^+F4/80^+$ macrophages were determined by flow cytometry.

The in vivo apoptotic neutrophil phagocytosis assay was carried out as described previously [34], In brief, collected peritoneal leukocytes were incubated with anti-mouse CD16/32 blocking antibody for 5 min and then incubated with anti-mouse F4/80 antibody for 20 min. Thereafter, cells were permeabilized with 0.1% Triton X-100 and then labeled with anti-mouse Ly6G antibody. The percentage of $F4/80^+Ly6G^+$ cell to $F4/80^+$ cells was evaluated by flow cytometry.

2.6. ELISA

Peritoneal fluid from zymA-induced peritonitis mice was collected and the protein concentrations of total TGF-β (R&D Systems, Minneapolis, MN, USA) and EPO (R&D Systems, Minneapolis, MN, USA) were measured by ELISA kit according to the manufacturer's instructions.

2.7. Western blotting

The standard Western blot analysis was performed according to our previous report. Briefly, total protein lysates were collected from exudate leukocytes or peritoneum and 10 μg of total protein was separated on 10% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membrane. Immunostaining was carried out using an enhanced chemiluminescence system BeyoECL Plus (Beyotime Institute of Biotechnology, Haimen, China). Following antibodies were used: EPOR (1:500, Santa Cruz, CA, USA), PPARγ (1:500, Abcam, Cambridge, UK), p-Jak2 (1:500, Santa Cruz, CA, USA), Jak2 (1:500, Santa Cruz, CA, USA), GAPDH (1:1000, Goodhere Biotechnology, Hangzhou, China) and the goat anti-rabbit IgG-HRP secondary antibody (1:1000, Novus Biologicals, Littleton, CO, USA). All conditions were expressed as a ratio of test protein to GAPDH.

2.8. Statistical analysis

Data were analyzed using the statistical software GraphPad Prism 5.0 for windows. The two-tailed unpaired Student's *t*-test was applied to evaluate the statistically significant differences in all analyses. For all statistical analyses, statistical significance was set as $p < 0.05$.

3. Results

3.1. Macrophage EPO signaling activation is deficient in obese mice during acute inflammation

Obesity was induced in male C57BL/6 mice by HFD. Compared with normal diet-feeding mice, high-fat diet greatly increased mouse body weight, particularly fat mass but not lean body mass (Fig. S1A–E). Moreover, glucose disposal rate and insulin sensitivity were altered in obese mice compared to normal mice (Figs. S1F, 1G).

The zymosan A (zymA) induced acute sterile peritonitis was applied to evaluate inflammation resolution and the time period in which the neutrophil number is reduced from the maximum number to 50% (R_i, resolution interval) was used to evaluate resolution speed [7,8,34–36]. Intraperitoneal administration of zymA to normal diet-feeding mice (lean-zymA) induced self-resolving peritonitis. In this model, numbers of neutrophils increased rapidly, peaked at 6 h and followed by a

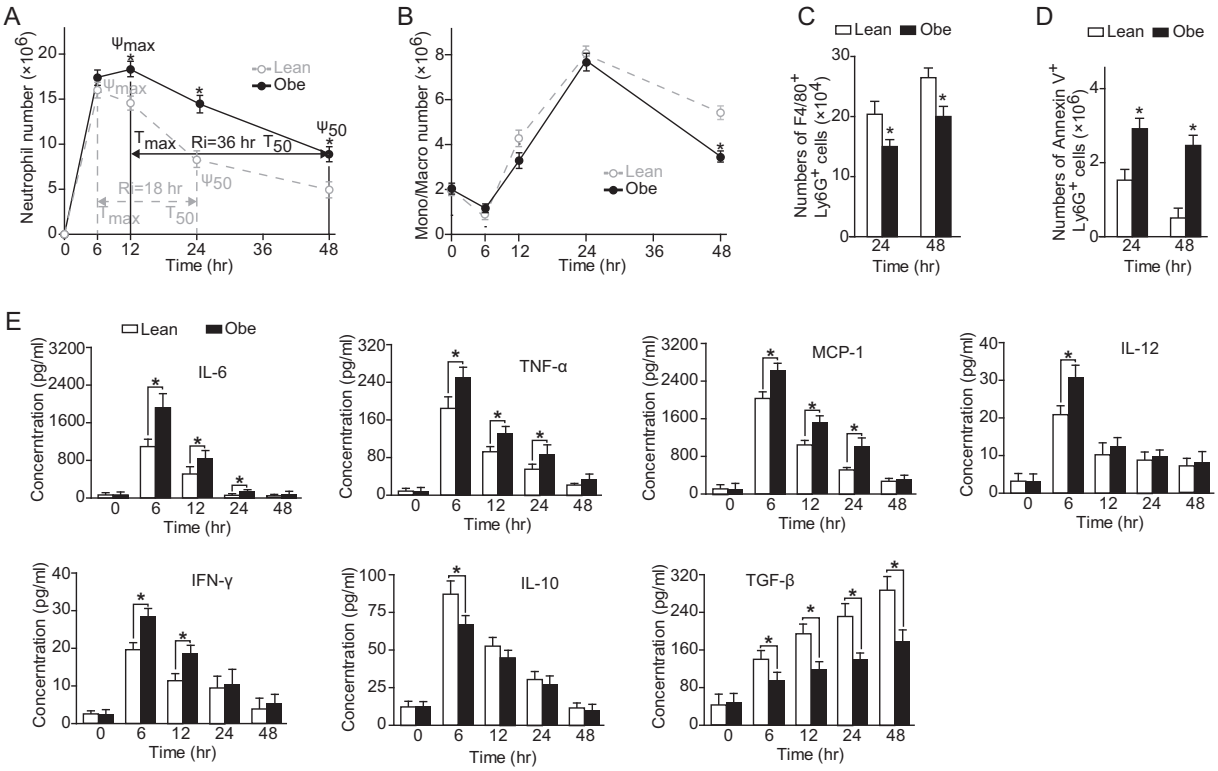

Fig. 1. Acute inflammation is delayed in obese mice.
ZymA (i.p., 40 mg per kg body weight) was applied to induce peritonitis in male lean mice or obesity mice. Lavages were collected at indicated intervals. (A) Neutrophils ($Ly6G^+$) and (B) monocytes/macrophages ($F4/80^+$) were enumerated ($n = 5$). See Table.S1 for the calculation of resolution indices. (C) Efferocytosis ($F4/80^+Ly6G^+$) in mice peritonitis exudates induced by ZymA was measured by flow cytometry. (D) Apoptotic neutrophils ($Ly6G^+$ annexin-V^+) were analyzed by flow cytometry ($n = 5$). (E) Peritoneal fluids were collected and protein levels of certain cytokines were measured by flow cytometry or ELISA ($n = 3$). Representative data from at least two independent experiments are shown. Error bars represent the s.e.m. $^*p < 0.05$.

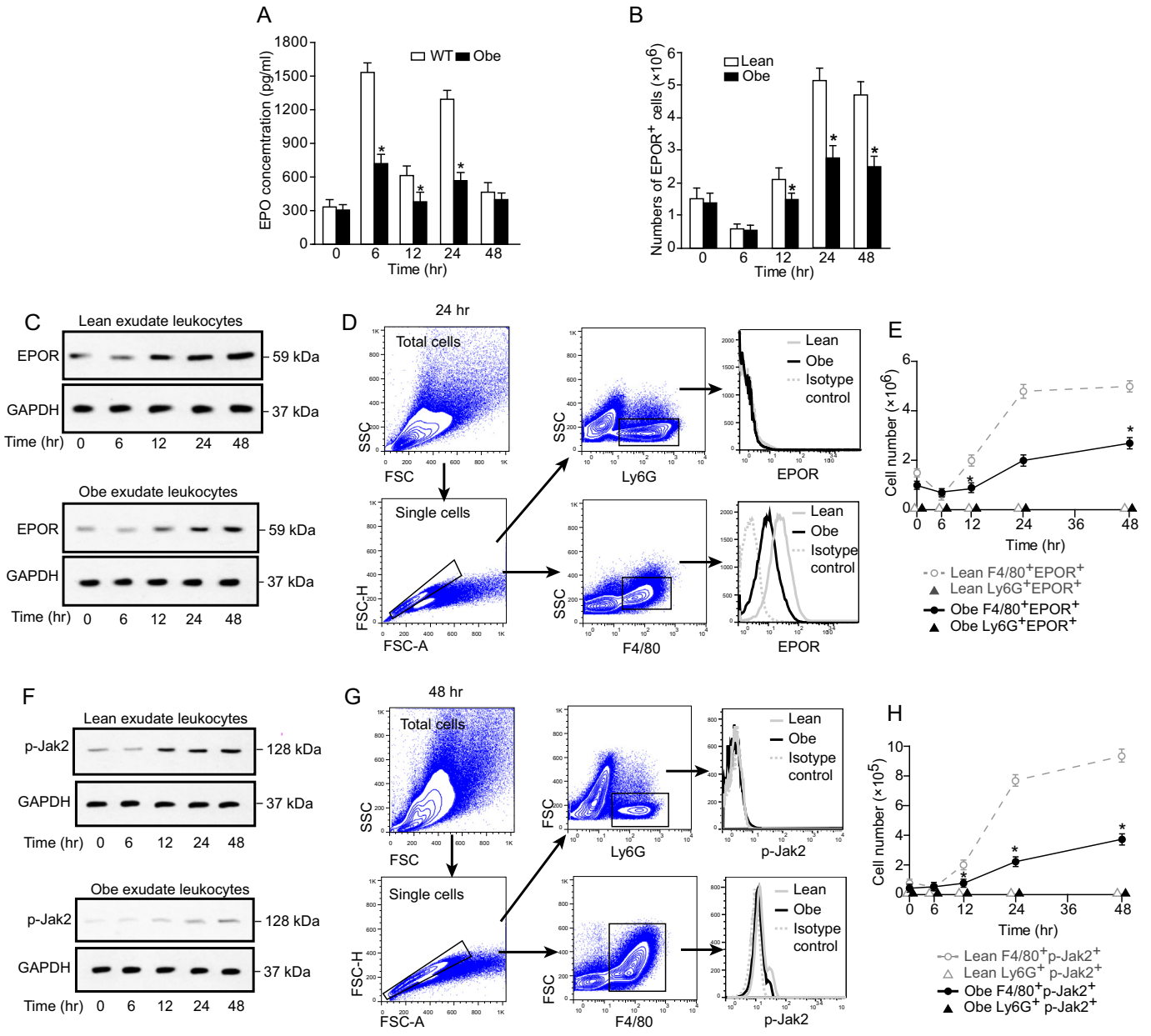

Fig. 2. Macrophage EPO signaling activation is deficient in obese mice during acute inflammation.
ZymA (i.p., 40 mg per kg body weight) was applied to induce peritonitis in male lean mice or obesity mice. Lavages were collected at indicated intervals. (A) EPO concentrations in peritoneal fluids were measured by ELISA ($n = 3$). (B, C) Exudates were collected and levels of EPOR or numbers of EPOR$^+$ cells were detected by flow cytometry (B, $n = 3$) or WB (C, $n = 2$), respectively. (D) Flow cytometric gating strategy for EPOR expression on macrophage and neutrophils surface in peritonitis exudates induced by ZymA in lean and obesity mice ($n = 3$). (E) Expression of EPOR on cell surface in exudates was detected by flow cytometry ($n = 3$). (F) Exudates were collected and levels of p-Jak2 were measured by WB ($n = 2$). (G) Flow cytometric gating strategy for p-Jak2 levels in macrophage and neutrophils of peritonitis exudates induced by ZymA in lean and obesity mice ($n = 3$). (H) Expression of p-Jak2 in exudates was detected by flow cytometry ($n = 3$). Representative data from at least two independent experiments are shown. Error bars represent the s.e.m. $^*p < 0.05$.

gradual decrease, with an R_i of approximately 18 h (Fig. 1A), similar to what has been reported in WT C57BL/6 mice [37]. The macrophage/monocyte counts rapidly decreased first and then increased steadily until they became the predominant exudate leukocytes at 48 h (Fig. 1B). However, in obese mice, zymA delayed inflammation resolution [35]. In the obese mice, more numbers of neutrophils were observed after zymA administration than in the lean mice. Moreover, in the obese mice, the accumulation of neutrophil peaked at 12 h and decreased more slowly with a R_i of approximately 36 h (Fig. 1A). In contrast, in the obese mice, the infiltration of macrophage/monocyte following zymA administration was decreased compared with the lean mice, accounting for approximately 30% of the total exudate leukocytes at 48 h (Fig. 1B). Furthermore, in the obese mice, numbers of apoptotic neutrophils that have been phagocyted by macrophages were lower at 24 and 48 h post-zymA administration than in the lean mice (Fig. 1C). In parallel, numbers of apoptotic neutrophils were much higher at 24 and 48 h following zymA injection in the obese mice than in the lean mice (Fig. 1D). In the obese mice, the concentrations of the inflammatory cytokines IL-6, TNF-α, MCP-1, IL-12 and IFN-γ were increased as well but that of anti-inflammatory cytokines IL-10 and TGF-β were decreased in the peritoneal fluid compared with those in the lean mice (Fig. 1E). So, the overmuch neutrophils, the longer R_i, the decreased macrophages/monocyte accumulation and macrophage efferocytosis and the changed cytokine profile indicated the delayed

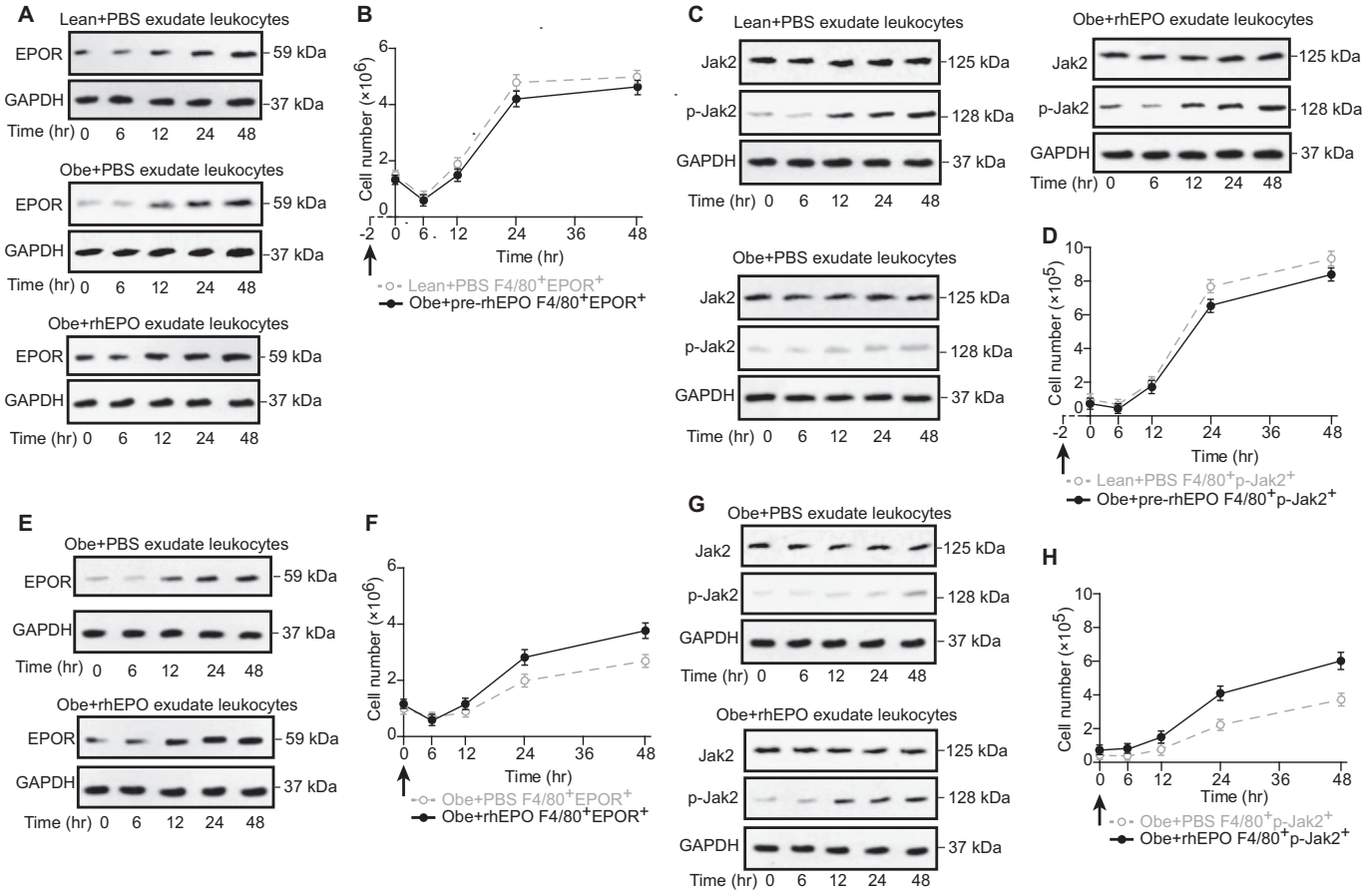

Fig. 3. Treatment with rhEPO restores macrophage EPO signaling in obese mice.
rhEPO (i.p., 5000 IU/kg) or PBS was given to lean or obese mice from 2 days before the instillation of zymA and every 24 h thereafter, exudate leukocytes and peritoneal fluids were collected at indicated time intervals. (A, B) Levels of EPOR in exudate leukocytes and the expression of EPOR on exudate macrophage surface were measured by WB (A, $n = 2$) and flow cytometry (B, $n = 3$), respectively. (C, D) Levels of p-Jak2 in exudates and in macrophages were measured by WB (C, $n = 2$) or flow cytometry (D, $n = 3$), respectively. rhEPO (i.p., 5000 IU/kg) or PBS together with zymA and every 24 h thereafter was given to lean or obese mice, exudate leukocytes and peritoneal fluids were collected at indicated time intervals. (E, F) Levels of EPOR and the expression of EPOR on macrophage surface in exudates were measured by WB (E, $n = 2$) or flow cytometry (F, $n = 3$), respectively. (G, H) Levels of p-Jak2 in exudates and in macrophages were measured by WB (G, $n = 2$) or flow cytometry (H, $n = 3$), respectively. Representative data from at least two independent experiments are shown. Error bars represent the s.e.m. $^{*}p < 0.05$.

resolution in the obese mice.

Thereafter, we analyzed the expression of EPO-related molecules in zymA-induced peritonitis in obese and lean mice. The induction of EPO-associated molecules in normal lean mice peritonitis was in line with our previous observation in health mice. Briefly, a two-peak increase (6 and 24 h) in the EPO concentration was observed in the peritoneal fluid of the normal lean mice as assayed by ELISA (Fig. 2A). The EPOR level in exudate leukocytes decreased at 6 h and then continuously increased by flow cytometry (Figs. 2B, S2) and western blotting (WB) (Fig. 2C). Moreover, the EPOR only expressed on macrophages but not neutrophils during the acute peritonitis (Fig. 2D, E). In addition, similar to the EPOR level, levels of phosphorylated Jak2 (p-Jak2), which indicated the activation of EPO signaling, in exudate leukocytes decreased at 6 h and then increased continuously by WB and flow cytometry. Furthermore, the increase in p-Jak2 was observed in macrophages but not in neutrophils during acute peritonitis (Fig. 2F–H). However, in the obese mice, following zymA administration, the peritoneal fluid EPO concentrations and the macrophage EPOR and p-Jak2 levels were all significantly lower than those in the lean mice at all of the observed time points (Fig. 2A–H). Collectively, these data here indicate that macrophage EPO signaling is deficient in obese mice during acute inflammation.

3.2. Treatment with rhEPO restores macrophage EPO signaling and enhances acute inflammation resolution in obese mice

Thereafter, we sought to explore whether exogenous EPO treatment can restore macrophage EPO signaling and promote inflammation resolution in obese mice. rhEPO (i.p., 5000 IU/kg) was given to obese mice from 2 days before the administration of zymA and every 24 h thereafter. rhEPO nearly normalized macrophage EPOR and p-Jak2 in obese mice to levels seen in lean-zymA mice (Fig. 3A–D). Moreover, rhEPO (i.p., 5000 IU/kg) given together with zymA and every 24 h thereafter also greatly increased EPOR and p-Jak2 levels in exudate leukocytes (Fig. 3E, G) and exudate macrophages (Fig. 3F, H) in obese mice. These data indicate that exogenous EPO can restore macrophage EPO signaling in obese mice and is in line with previous observations that EPO enhances EPOR expression [26,27].

Consequently, we investigated the effects of exogenous rhEPO on delayed inflammation resolution in obese mice. rhEPO (i.p., 5000 IU/kg) was given to obese mice from 2 days before the administration of zymA and every 24 h thereafter. Treatment of obese mice with rhEPO normalized the exudate neutrophil accumulation, the Ri and the apoptotic neutrophil count to that observed in vehicle-treated lean mice during peritonitis (Fig. 4A and B). Furthermore, rhEPO restored the cytokine IL-6, TNF-α, MCP-1, IL-12, IFN-γ, IL-10 and TGF-β

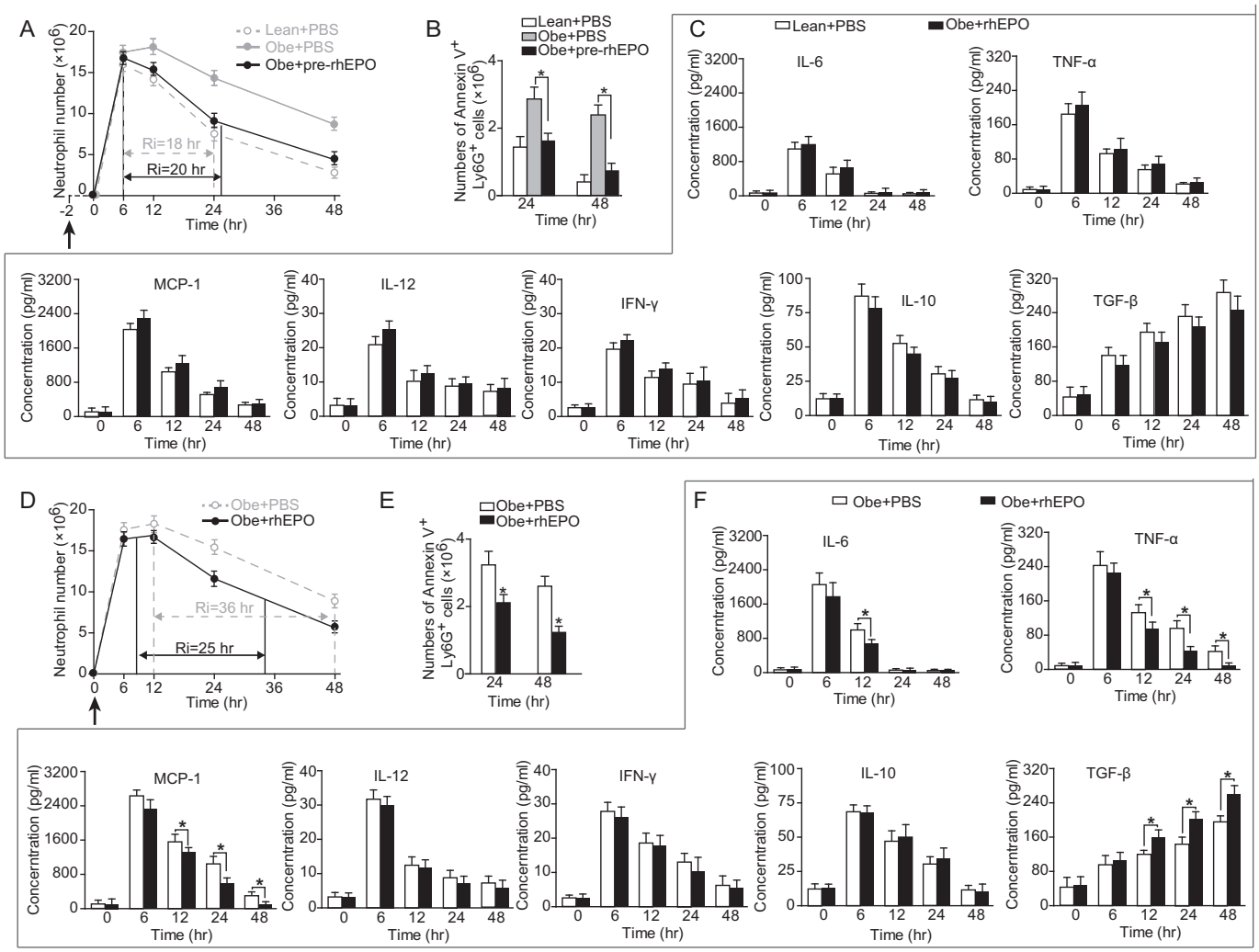

Fig. 4. Treatment with rhEPO enhances acute inflammation resolution in obese mice.
A–C: rhEPO (i.p., 5000 IU/kg) or PBS was given to lean or obese mice from 2 days before the instillation of zymA and every 24 h thereafter, exudate leukocytes and peritoneal fluids were collected at indicated time intervals. (A) Neutrophils were enumerated ($n = 5$) and (B) apoptotic neutrophils were analyzed by flow cytometry ($n = 5$). (C) Peritoneal fluids were collected and protein levels of certain cytokines were measured by flow cytometry or ELISA ($n = 3$). D–F: rhEPO (i.p., 5000 IU/kg) or PBS together with zymA and every 24 h thereafter was given to lean or obese mice, exudate leukocytes and peritoneal fluids were collected at indicated time intervals. (D) Neutrophils were enumerated ($n = 5$) and (E) apoptotic neutrophils were analyzed by flow cytometry ($n = 5$). (F) Peritoneal fluids were collected and protein levels of certain cytokines were measured by flow cytometry or ELISA ($n = 3$). Representative data from at least two independent experiments are shown. Error bars represent the s.e.m. $^*p < 0.05$.

concentration in obese mouse peritoneal fluid to that seen in vehicle-treated lean mice (Fig. 4C). Moreover, rhEPO (i.p., 5000 IU/kg) administrated together with zymA and every 24 h thereafter to obese mice also significantly reduced the exudate neutrophil infiltration, the Ri and the apoptotic neutrophil count compared with vehicle-treated obese mice (Fig. 4D and E, Table S2). Furthermore, rhEPO resulted in reductions in the TNF-α, IL-6 and MCP-1 levels and an increase in the TGF-β level in obese mouse peritoneal fluid (Fig. 4F). In addition, even administrated at the peak of inflammation (12 h after zymA injection) and every 24 h thereafter (i.p., 5000 IU/kg), rhEPO also resulted in a decrease in the R_i, the neutrophil count at 24 h (Fig. 5A), reduced apoptotic neutrophil count (Fig. 5B) and improved exudate cytokine profile (Fig. 5C). These data implicate that exogenous EPO ameliorates delayed resolution in obese mice.

Therefore, these results suggest that exogenous EPO restores macrophage EPO signaling and ameliorates inflammation resolution in obese mice.

3.3. The deficiency of macrophage EPO signaling contributes to obesity-related acute inflammation resolution delay

Consequently, the contributions of macrophage EPO signaling to obesity-associated acute inflammation delay were investigated. The inflammation resolution in obese EPOR-macrophage KO mice, lean EPOR-macrophage KO mice, obese WT mice and lean WT mice was compared with the zymosan-induced peritonitis model. As shown in Fig. 6, the inflammation resolution in obese WT mice was exacerbated compared to that in lean EPOR-macrophage KO mice, indicating the existence of EPO-irrelevant mechanisms that contribute to the obesity-related acute inflammation resolution delay. In line with this observation, the inflammation resolution in obese EPOR-macrophage KO mice was exacerbated as well compared to that in lean EPOR-macrophage KO mice. However, in obese mice, exogenous EPO restored macrophage EPO signaling and ameliorated inflammation resolution to the lean mice level (Fig. 4A), indicating that the macrophage EPO signaling still plays an important role in obesity-related acute inflammation resolution delay.

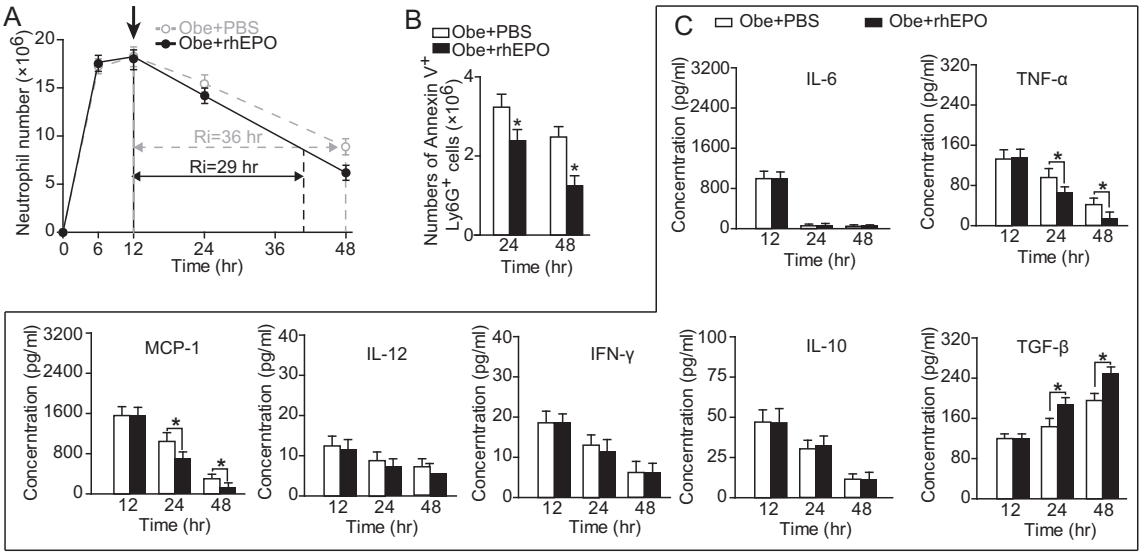

Fig. 5. Therapeutical rhEPO treatment enhances acute inflammation resolution in obese mice.
rhEPO (i.p., 5000 IU/kg) or PBS was given to male obesity mice at peak time point of peritonitis induced by ZymA (i.p., 40 mg per kg body weight) and every 24 h thereafter. (A) Neutrophils were enumerated ($n = 5$) and (B) apoptotic neutrophils were analyzed by flow cytometry ($n = 5$). (C) Peritoneal fluids were collected and protein levels of certain cytokines were measured by flow cytometry or ELISA ($n = 3$). Representative data from at least two independent experiments are shown. Error bars represent the s.e.m. $^{*}p < 0.05$.

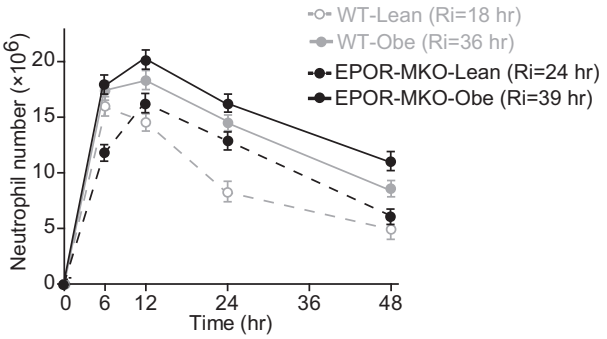

Fig. 6. The deficiency of macrophage EPO signaling contributes to obesity-related acute inflammation resolution delay.
Peritonitis was induced with ZymA (i.p., 40 mg per kg body weight) in WT lean, WT obesity, EPOR-MKO lean or EPOR-MKO obese mice. Lavages were collected at indicated intervals and neutrophils were enumerated ($n = 5$). Representative data from at least two independent experiments are shown. Error bars represent the s.e.m. $^{*}p < 0.05$.

3.4. rhEPO enhances macrophage efferocytosis in obese mice

Considering the essential contribution of efferocytosis to inflammation resolution and the restricted localization of EPOR in macrophages, the effects of EPO on macrophage phagocytosis of apoptotic neutrophils in obese mice during peritonitis were investigated. During zymA-induced peritonitis, the macrophage phagocytosis of apoptotic neutrophils was decreased by about 25% and 20%at 24 and 48 h respectively in obese mice compared with lean mice (Fig. 1C). Furthermore, rhEPO (i.p., 5000 IU/kg) given from 2 days before the administration of zymA and every 24 h thereafter restored macrophage phagocytosis of apoptotic neutrophils to levels observed in vehicle-treated lean mice (Fig. 7A). Moreover, rhEPO dose-dependently increased macrophage phagocytosis of apoptotic neutrophil in the obese mice peritonitis (Fig. 7B). In addition, rhEPO (i.p., 5000 IU/kg) administrated together with zymA and every 24 h thereafter to obese mice, or even at the peak of inflammation (12 h after zymA administration) and every 24 h thereafter, also greatly enhanced efferocytosis of apoptotic neutrophils compared to their respective vehicle control (Fig. 7C, D). So, EPO enhanced macrophage phagocytosis of apoptotic cells in obese mice peritonitis in vivo.

Moreover, EPO effects on peritoneal macrophage phagocytosis of apoptotic cells were measured in vitro. Apoptotic neutrophils were labeled with a pH-sensitive dye (pHrodo), the fluorescence of which increases within acidic phagolysosomes, to distinguish between phagocyted and bound cells (Fig. 7E). Peritoneal macrophages from the obese mice showed greatly decreased engulfment of apoptotic neutrophils compared to that from lean mice in vitro (Fig. 7F); however, rhEPO dose- (Fig. 7G) and time-dependently (Fig. 7H) enhanced obese mice-derived peritoneal macrophage efferocytosis in vitro. Moreover, peritoneal macrophages from rhEPO-pretreated obese mice also exhibited increased engulfment of apoptotic neutrophils (Fig. 7I).

Together, exogenous EPO enhances macrophage efferocytosis in obese mice in vivo and in vitro.

3.5. rhEPO induces macrophage PPARγ to promote efferocytosis in obese mice

We have previously shown that EPO increases efferocytosis through inducing the expression of PPARγ [26], which can regulate the expression of various phagocyte engulfment receptors and bridging molecules. Therefore, we then investigate whether EPO also promotes efferocytosis through PPARγ in obese mice. In line with our previous observations [35], the expression of PPARγ in macrophages was increased during zymA-induced peritonitis (Fig. 8A, B). However, in obese mice, PPARγ up-regulation in macrophages was greatly reduced during zymA-induced peritonitis (Fig. 8A, B) and rhEPO enhanced the expression of PPARγ in macrophage during zymA-induced peritonitis (Fig. 8B), indicating potential regulation by EPO signaling in obese mice. Moreover, pre-treatment with PPARγ agonist restored macrophage phagocytosis of apoptotic neutrophils in obese mice during zymA-induced peritonitis (Fig. 8C). In order to explore whether EPO promotes macrophage phagocytosis of apoptotic cells via PPARγ in obese mice, we applied the self-generated macrophage PPARγ$^{-/-}$ (PPARγ-MKO, *PPARγ$^{f/f}$LysMCre$^{+/+}$*) mice [28] and macrophage EPOR$^{-/-}$ (EPOR-MKO, *EPOR$^{f/f}$LysMCre$^{+/+}$*) mice [26]. PPARγ agonist restored macrophage efferocytosis of apoptotic neutrophils in EPOR-MKO obese mice, but rhEPO did not increase macrophage efferocytosis

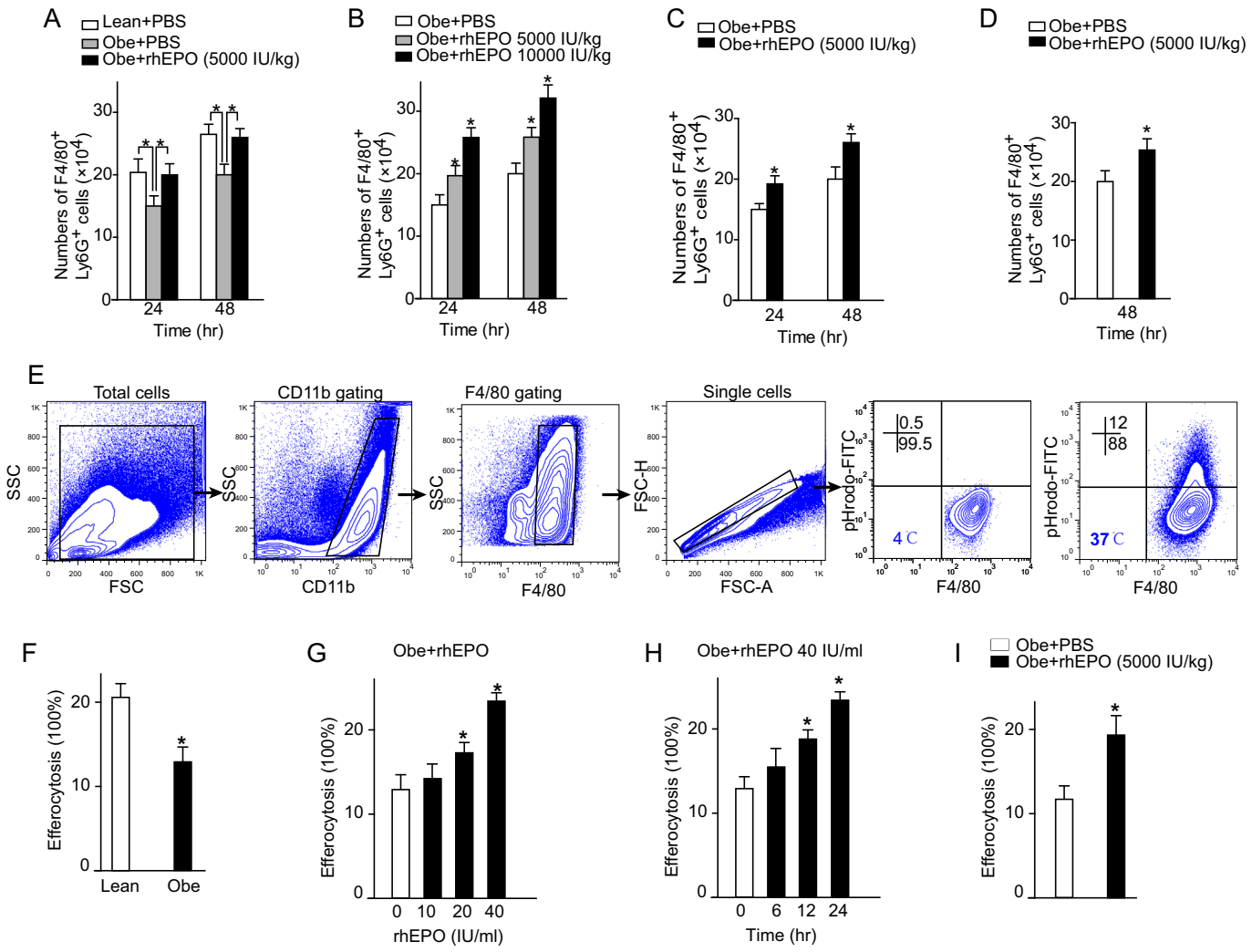

Fig. 7. rhEPO enhances macrophage efferocytosis in obese mice.
rhEPO (i.p.) or PBS was given to lean or obese mice from 2 days before the instillation of zymA and every 24 h, efferocytosis ($F4/80^+Ly6G^+$) at 24 and 48 h and were increased by 5000 IU/kg (A) or 10,000 IU/kg (B) rhEPO ($n = 3$). (C) rhEPO (i.p., 5000 IU/kg) administrated together with zymA and every 24 h thereafter to obese mice significantly increased macrophage efferocytosis ($F4/80^+Ly6G^+$); (D) rhEPO (i.p., 5000 IU/kg) administrated to obese mice at the peak of inflammation (12 h after zymA injection) and every 24 h thereafter significantly increased macrophage efferocytosis ($F4/80^+Ly6G^+$); (E) Flow cytometric gating strategy for in vitro efferocytosis. Mice peritoneal macrophages were incubated with pHrodo-labeled apoptotic neutrophils for 1 h and efferocytosis was assessed as $CD11b^+F4/80^+$pHrodo-$FITC^+$ by flow cytometry ($n = 3$). (F) Peritoneal macrophages from the obese mice exhibited impaired efferocytosis in vitro compared to lean mice peritoneal macrophages. Pre-treatment with rhEPO dose- (G) and time- (H) dependently increased macrophage phagocytosis of apoptotic neutrophils in vitro. (I) Obesity mice received rhEPO (i.p., 5000 IU/kg) treatment for 1 day and their peritoneal macrophages were collected for analysis of efferocytosis of pHrodo-labeled apoptotic neutrophils in vitro ($n = 3$). Representative data from at least two independent experiments are shown. Error bars represent the s.e.m. $^*p < 0.05$.

during zymA-induced peritonitis in PPARγ-MKO obese mice (Fig. 8D), confirming the important role of PPARγ in regulating EPO-enhanced macrophage engulfment of apoptotic cells during acute inflammation in obese mice. Furthermore, in vitro, the PPARγ agonist significantly enhanced EPOR-MKO obese mice-derived peritoneal macrophage engulfment of apoptotic neutrophil (Fig. 8E); but rhEPO did not promote PPARγ-MKO obese mice-derived peritoneal macrophage efferocytosis in vitro (Fig. 8F), affirming that EPO enhances the phagocytosis of dying cells via PPARγ in obese mice.

Furthermore, the inflammation resolution was also impaired in PPARγ-MKO obese mice, and rhEPO did not restore this delayed resolution (Fig. 8G). However, PPARγ agonist restored the delayed inflammation resolution in EPOR-MKO obese mice (Fig. 8H). Moreover, these results further affirm that in obese mice, EPO function through PPARγ to promote inflammation resolution.

Together, these data suggest that EPO function through increasing efferocytosis via PPARγ to promote inflammation resolution in obese mice.

4. Discussion

The failure of inflammation resolution has been linked with the systemic chronic inflammation in obesity, which is a unifying basis for the development of obesity-related clinical complications; however, the mechanisms that result in delayed inflammation resolution in obesity remain unknown. Here we identified that the deficient of macrophage EPO signaling contributed to delayed resolution in diet-induced obese mice (Fig. S3). We determined that the induction of macrophage EPO signaling was significantly reduced in obese mice relative to normal mice during zymosan-induced peritonitis. Furthermore, exogenous EPO induced macrophage EPO signaling and promoted inflammation resolution in obese mice. Finally, EPO increased macrophage PPARγ to promote efferocytosis in obese mice. Together, the failure of macrophage EPO signaling underlies aberrant inflammation resolution in

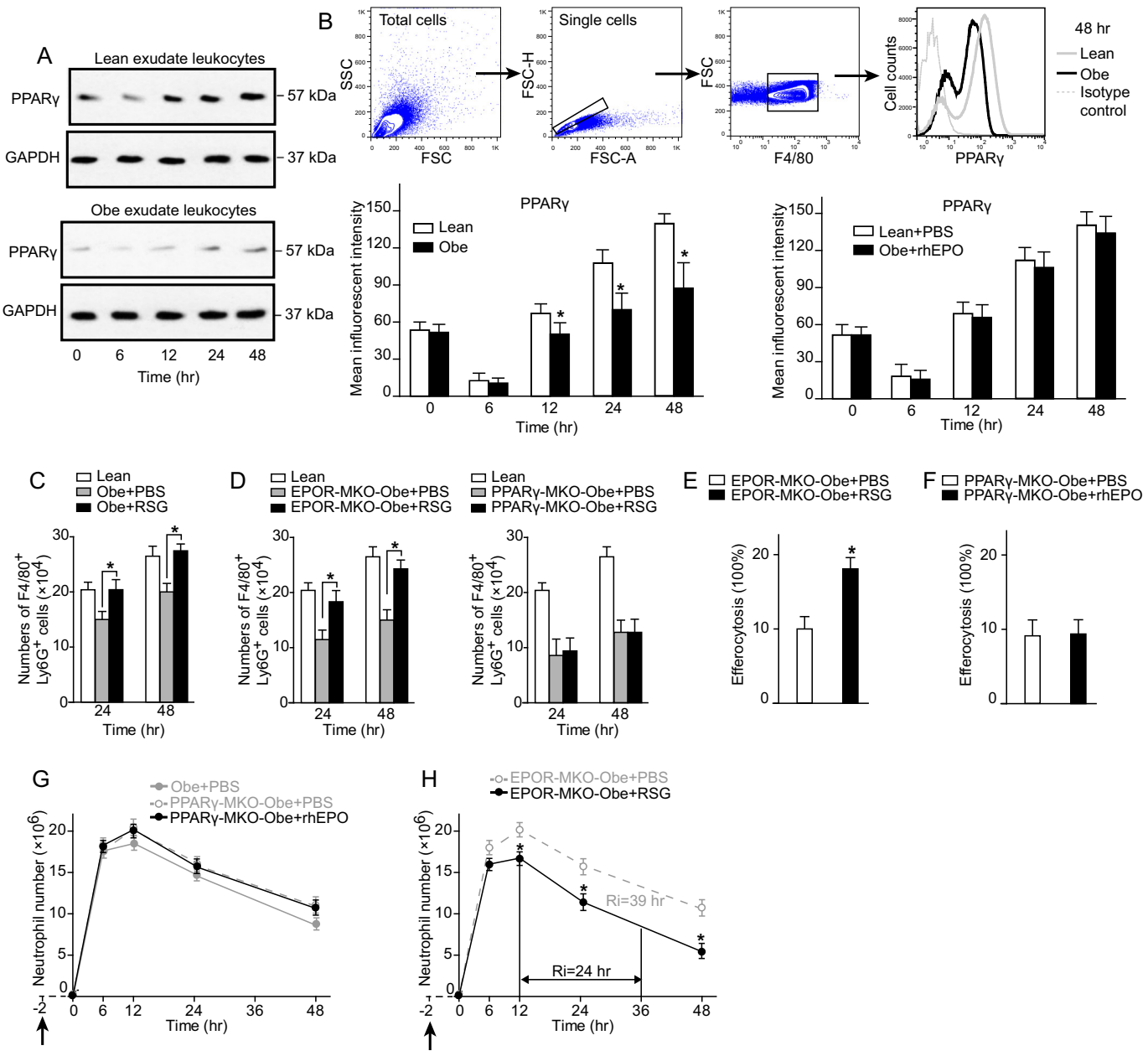

Fig. 8. rhEPO induces macrophage PPARγ to promote efferocytosis in obese mice.
(A) Peritonitis was induced with ZymA (i.p., 40 mg per kg body weight) in lean mice or obesity mice, levels of PPARγ in exudates were measured by WB ($n = 2$). (B) Flow cytometric gating strategy for PPARγ expression in peritonitis exudates induced with ZymA (upper panel). Peritonitis was induced with ZymA (i.p., 40 mg per kg body weight) in lean mice or obesity mice, levels of PPARγ in exudate macrophages were measured by flow cytometry (lower right panel, $n = 3$). rhEPO (i.p., 5000 IU/kg) or PBS was given to lean or obese mice from 2 days before the instillation of zymA and every 24 h thereafter, and expression of PPARγ in exudate macrophages was detected by flow cytometry (lower left panel, $n = 3$). (C) RSG (10 mg/kg via oral gavage) or PBS was given to lean or obese mice from 2 days before the instillation of zymA and every 24 h thereafter, and efferocytosis ($F4/80^+Ly6G^+$) was detected by flow cytometry ($n = 3$). (D) RSG (10 mg/kg via oral gavage) or PBS was given to lean, EPOR-MKO or PPARγ-MKO obese mice from 2 days before the instillation of zymA and every 24 h thereafter, and efferocytosis ($F4/80^+Ly6G^+$) was detected by flow cytometry ($n = 3$). (E) Peritoneal macrophages from EPOR-MKO obese mice were pre-stimulated with RSG (1 μM) for 24 h and in vitro efferocytosis of pHrodo-labeled apoptotic neutrophils were measured by flow cytometry ($n = 3$). (F) Peritoneal macrophages from PPARγ-MKO obese mice were pre-stimulated with rhEPO (40 IU/ml) in vitro for 24 h and then efferocytosis of pHrodo-labeled apoptotic neutrophils were measured by flow cytometry ($n = 3$). (G) rhEPO (i.p., 5000 IU/kg) or PBS was given to PPARγ-MKO obesity mice for 2 days before the instillation of zymA and every 24 h, and neutrophils were enumerated ($n = 5$). (H) RSG (10 mg/kg via oral gavage) or PBS was given to EPOR-MKO obesity mice for 2 days before the instillation of zymA and every 24 h, and neutrophils were enumerated ($n = 5$). Representative data from at least two independent experiments are shown. Error bars represent the s.e.m. $^*p < 0.05$.

obesity, with important implications for promoting inflammation resolution and normalizing phagocyte defects in obese individuals.

Accumulated studies demonstrated chronically activated inflammatory signaling pathways in obesity [3,4,38] [12], which suggests possible defects in inflammation resolution in obese animals, since the failure of inflammation resolution results in chronic inflammation. Several previous studies indeed reported delayed inflammation resolution in obesity and diabetes and indicated that disturbed macrophage phagocytosis of apoptotic cells was involved [13,39–41]. That is in consistent with our present observation of delayed resolution in diet-induced obese mice, with the animal model of zymA-induced peritonitis [7,36]. In this model, an excessive accumulation of neutrophils, a

longer Ri, decreased macrophages/monocyte infiltration and a changed cytokine expression were observed, as well as reduced macrophage efferocytosis, all of which affirmed the impaired resolution in the obese mice.

Macrophage efferocytosis are crucial in the active resolution of inflammation, as impaired clearance of apoptotic cells can cause secondary necrosis that leading to further tissue damage [6,42]. The defects in this process are also an important feature of delayed inflammation resolution and wound healing in obesity and diabetes [13,41,43,44]. Therefore, elucidation of the mechanisms regulating this critical function of macrophages is essential for understanding and treatment of chronic systemic inflammation in obese and diabetic humans.

While EPO is well-known for its activity for promoting erythropoiesis, our previous data have revealed EPO as a novel endogenous pro-resolving molecule [26,27]. We observed that the macrophage EPO signaling was induced during inflammation resolution in zymA-induced peritonitis [26]. Furthermore, the genetic deletion of macrophage EPOR impaired the apoptotic cell phagocytosis in vivo and significantly delayed inflammation resolution; but EPO treatments increased macrophage efferocytosis in vitro and in vivo during inflammation [26]. All these suggest an important role for macrophage EPO signaling in promoting apoptotic cell phagocytosis and thereby contributing to inflammation resolution. Our present data here, for the first time, revealed deficient macrophage EPO signaling activation in obese mice during acute inflammation, as EPO concentrations and levels of macrophage EPOR and its sole direct downstream signaling molecule p-Jak2 were much lower than those in the lean mice during peritonitis acute inflammation. Moreover, exogenous EPO treatments enhanced EPOR expression, restored macrophage EPO signaling and ameliorated delayed resolution in obese mice, which approved the involvement of EPO signaling in delayed resolution of obese animals.

In addition, our previous studies reported that macrophage EPO signaling promoted inflammation resolution through PPARγ induction [26], which is in accordance with previous publications reporting impaired inflammation resolution in myeloid cell-specific PPARγ deficient mice [35,45]. As a transcription factor, PPARγ can regulate expressions of different phagocyte engulfment receptors and bridging molecules [46]. PPARγ can reduce macrophage inflammatory activation while enhancing efferocytosis [47] and plays a crucial role in phenotypic switch of macrophages from pro-inflammatory into anti-inflammatory and pro-healing during wound healing [48]. In our present study, macrophage PPARγ was greatly reduced in obese mice, which could be restored by exogenous EPO. Importantly, it was confirmed that PPARγ contributed to resolution of acute inflammation by working downstream of EPO signaling. PPARγ agonist, but not rhEPO, restored macrophage efferocytosis of apoptotic neutrophils in EPOR-MKO obese mice, confirming the important role of PPARγ in mediating efferocytosis was originally promoted by EPO. PPARγ agonist was shown to promote inflammation resolution in EPOR-MKO obese mice, but EPO couldn't restore the impaired resolution in PPARγ-MKO obese mice, affirming that PPARγ works downstream of EPO signaling. Considering the crucial role of PPARγ in promoting phagocytosis of apoptotic cells and inflammation resolution demonstrated above, our data indicate that mediated by PPARγ induction, macrophage EPO signaling promotes efferocytosis and thereby the inflammation resolution in obese mice. However, PPARγ is a critical transcriptional regulator for fat cell differentiation [49,50], which must be taken into consideration when it comes to translational application. Interestingly, it has been shown that pre-adipocytes expressed EPOR and EPO reduced PPARγ expression and suppressed pre-adipocyte differentiation in vitro and in vivo [51]. So it seems that EPO is more suitable than PPARγ agonists in obesity-related inflammation regulation.

Collectively, our results identify an important mechanism of deficient activation in macrophage EPO signaling underlying aberrant inflammation resolution in obesity, with important implications for normalizing macrophage defects and controlling unresolved inflammation in obese and diabetic individuals, which may be eventually due to the reduced expression of the EPO downstream PPARγ. Mechanistically, EPO signaling promotes macrophage efferocytosis of apoptotic cells through PPARγ induction and thereby contributes to inflammation resolution in obese mice. Considering the close connection between aberrant inflammation resolution and numerous chronic conditions of obese or/and diabetic individuals, its safety as an endogenous pro-resolving molecule, EPO shows potential therapeutic utility for obesity relevant conditions.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflict of interest statement

The authors declare no competing financial interests.

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