Neutrophil-Derived Exosomes Induce M1 Macrophage Polarization and Prime Macrophage Pyroptosis via miR-30d-5p in Sepsis

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Research

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Abstract

Background: Polymorphonuclear neutrophils (PMNs) have been demonstrated to play a role in proinflammatory M1 activation and macrophage (Mϕ) pyroptosis in sepsis. Accumulating evidence suggests PMN-derived exosomes as a new subcellular entity acting as a fundamental link between PMN-driven inflammation and tissue damage. However, the role of PMN-derived exosomes in sepsis remains unclear. This study aimed to determine whether PMN-derived exosomes play a role in proinflammatory M1 activation and Mϕ pyroptosis in sepsis and explore the potential mechanisms involved.

Methods: Exosomes were isolated from the supernatant of PMNs activated with phosphate buffered saline (PBS) or tumor necrosis factor (TNF)-α, cocultured with Raw264.7 macrophages or BMDMs, and then assayed for macrophage polarization and pyroptosis. To examine the role of exosomes *in vivo*, PMN-derived exosomes were administered to mice, and then examined for lung inflammation.

Results: After activated by TNF-α, PMNs released exosomes (TNF-Exo) to promote M1 macrophage activation both *in vivo* and *in vitro.* In addition, TNF-Exo primed macrophages for pyroptosis by upregulating NLRP3 inflammasome expression through NF-κB signaling pathway. Mechanistic studies demonstrated that miR-30d-5p mediated the function of TNF-Exo by targeting SOCS-1 and SIRT1 in macrophages. Furthermore, treatment of miR-30d-5p inhibitors *in vivo* significantly decreased cecal ligation and puncture (CLP) or TNF-Exo-induced M1 macrophage activation and macrophage death in the lung. Lung injury was also alleviated by miR-30d-5p inhibitors.

Conclusions: In this study, we identified a novel mechanism of PMN-Mϕ interaction in sepsis, demonstrating that exosomal miR-30d-5p from PMNs induced M1 macrophage polarization and primed Mϕ for pyroptosis by activating NF-κB signaling. These findings suggest a previously unidentified role of neutrophil-derived exosomes in sepsis and may lead to new therapeutic approaches.

Introduction

Sepsis is defined as a global health priority by the World Health Organization, with a reported death rate of 30–45% in hospitalized patients(1, 2). Mortality in sepsis is primarily due to multiple organ dysfunction syndrome including acute lung injury (ALI) as a common and important component(3). Sepsis elicits dysregulated immune responses manifested by a cytokine/chemokine elevation (also known as ‘cytokine storm’), which correlates well with increased disease severity and poor prognosis. However, the molecular mechanism remains to be fully elucidated(4, 5).

Polymorphonuclear neutrophils (PMNs) are the most abundant leukocytes in mammals, playing a double-edged sword role in sepsis. A number of studies have demonstrated that regulation of neutrophils plays a crucial role in the treatment of sepsis(6). In addition to PMNs, macrophages (Mϕ) are predominant immune cells that mediate the inflammatory process(7). Recent studies have proven that the crosstalk between PMNs and Mϕ plays an important role in regulating inflammation following trauma, hemorrhagic shock, endotoxemia and other pathologies(8–11).

Macrophages are roughly classified as M1 macrophage with proinflammatory effects, or M2 macrophage with anti-inflammatory effects. M1 macrophage encourages inflammation by secreting proinflammatory cytokines, such as interleukin 1β (IL-1β), IL-12, IL-6, tumor necrosis factor-α (TNF-α), all of which contribute to the development of sepsis-related lung injury(12, 13). Besides, pyroptosis is a programmed cell death process associated with inflammation(6, 14). In sepsis, pyroptosis promotes pore formation in the plasma membrane, leading to cell swelling and membrane rupture, finally resulting in the leakage of abundant inflammatory factors out of cells(15, 16). Accumulating evidence indicates that PMNs play a role in proinflammatory M1 activation and pyroptosis of Mϕ(4, 11, 17). However, the exact role of PMNs and the underlying mechanism need to be further addressed.

A recent study(18) reported that circulating plasma exosomes induced macrophage M1 activation during sepsis-related ALI. Exosomes are small extracellular vesicles secreted by various cell types, with size ranging from 30 to 150 nm(18). They can transfer a multitude of proteins and genetic material (including DNA, mRNA, and microRNA [miRNA]) to target cells, playing a key role in cell-to-cell communications(19). Neutrophil-derived exosomes participate in the pathogenesis of chronic lung injury, such as chronic obstructive pulmonary disease, bronchopulmonary dysplasia and asthma(20, 21). However, whether neutrophil-derived exosomes play a role in sepsis-related ALI remains unclear.

MicroRNAs (miRNAs) are a group of small single-stranded noncoding RNAs that regulate gene expression by binding to the 3’ untranslated region of target mRNAs, resulting in the degradation of mRNA as well as inhibition of translation(22). Exosome-contained miRNAs may serve as an important mediator of intercellular communication in sepsis(23). However, whether neutrophil-derived exosomal miRNAs could modulate Mϕ activation or death during sepsis needs to be further addressed.

In this study, we identified a novel mechanism of PMN-Mϕ interaction in sepsis, and demonstrated that exosomal miR-30d-5p from neutrophil induced macrophage M1 polarization and prime Mϕ for pyroptosis by activating NF-κB signaling in sepsis. We further demonstrated that exosomal miR-30d-5p activated NF-κB signaling by targeting SOCS-1 and SIRT1. These findings suggest a previously unidentified pathway of PMN-Mϕ cross-talk, which could enhance Mϕ activation and death, and subsequent exaggerated post-sepsis inflammation.

Materials And Methods

More detailed information on methods is provided in Additional file 1: supplementary methods.

Animals

Wild type (WT) male C57BL/6J mice aged 6–8 weeks (Shanghai Sippr-BK Laboratory Animal Co., Ltd., Shanghai, China) were fed under a specific pathogen-free environment in Xinhua Hospital Animal Laboratory (Shanghai, China). All animal experiments were conducted under the rules approved by Ethics

Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, and the approval number is XHEC-F-2020-019.

PMNs isolation and activation

PMNs were induced in the peritoneal cavity of the mice as previously described(24). After isolation, PMNs were suspended in complete culture medium (RPMI 1640 containing 10% exosome-free FBS, supplemented with 50 mg/ml penicillin/streptomycin) at a concentration of 10^6 cells/ml. PMNs activation was induced upon incubation with phosphate buffered saline (PBS) or 20 ng/mL TNF-α for 12 h at 37°C.

Exosome Isolation And Characterization

Exosomes were isolated from the supernatant of PMNs using Total Exosome Isolation Reagent (#4484450; Thermo Fisher Scientific, Waltham, MA, USA). The detailed isolation procedure and the methods used to determine exosomal morphology, size distribution, and surface marker expression are described in the supplementary material.

Macrophage Culture And Transfection

Raw264.7 macrophages or BMDMs were treated with PMN-derived exosomes (100 μg/ml) at 37°C for 24 h. To induce pyroptosis, macrophages were primed with exosomes for 24 h before stimulation with 5 mM ATP (#HY-B2176; MedChemExpress, Monmouth Junction, NJ, USA) or 20 μM nigericin (#HY-100381; MedChemExpress) for 2 h.

Raw264.7 macrophages were then transfected with microRNA control (50nM) and miR-30d-5p inhibitor (Guangzhou Ribobio Corporation, Guangzhou, China) for 24 h prior to be cocultured with exosomes as per the manufacturer's instructions.

Statistics

The normal distribution of the data was tested using the Shapiro-Wilk test. Normally distributed data are presented as means ± SEM. Comparisons between 2 groups were performed by the 2-tailed Student's t test. Multiple group comparisons were performed by one-way ANOVA followed by calculating the least significant difference to compare means. A value of $P < 0.05$ was considered statistically significant.

Results

Characterization of exosomes isolated from the supernatant of PMNs stimulated ex vivo.

TNF-α plays an important role as a potent inducer of inflammatory response and a key regulator of innate immunity in sepsis-related ALI, and is often used to activate neutrophils(25–27). We thus used TNF-α-activated PMNs to mimic sepsis *in vitro.* Exosomes isolated from the supernatant of PMNs stimulated with PBS (PBS-Exo) or TNF-α (TNF-Exo) were first characterized morphologically by transmission electron microscopy (TEM). The isolated microvesicles displayed a round, cup-shaped morphology with a diameter about 100 nm (Fig. 1A-1B). Western blot showed high expression of exosome-specific markers CD9, CD63 and TSG101 (Fig. 1C). The protein concentration in TNF-Exo was higher than that in PBS-Exo (Fig. 1D). After 3-h incubation of BMDMs *in vitro* with Dil-labeled exosomes, Mϕ internalization of exosomes was observed (Fig. 1E). To determine the signaling pathway changes after PMN-Exo treatment, BMDMs were collected for TMT-based proteomics. The results showed that TNF-Exo upregulated the expressions of inflammatory cytokines (IL-1α and IL-1β), M1 macrophage marker (inducible nitric oxide synthase, iNOS) and NLRP3 complexes (NLRP3, Caspase-1) (Fig. 1F, **Additional file 2: Table 1**). Enrichment pathway analysis showed that NF-κB signaling pathway was within the 20 most enriched pathways (Fig. 1G). In addition, the protein levels of NLRP3, pro-IL-1β and NF-κB p-p65 were increased in TNF-Exo exposed BMDMs as demonstrated by Western blot (Fig. 1H). All these results indicate that PMNs released exosomes to upregulate NF-κB signaling activity, promote M1 activation and increase NLRP3 inflammasome expression in macrophages during sepsis.

PMN-derived exosomes promote M1 macrophage activation both in vivo **and** in vitro.

To verify whether PMN-derived exosomes promoted M1 macrophage activation *in vivo*, C57bl/6 WT mice were injected with PBS-Exo or TNF-Exo (300 μg/mouse, *i.p.*) for 24 h. It was found that TNF-Exo significantly increased the proportion of M1 macrophage (CD11c + CD206-) in peritoneal macrophage (PMϕ) (Fig. 2A). To explore whether PMN-Exo mediated sepsis-related ALI, Dil-labeled exosomes were injected intraperitoneally into naive mice. Fluorescence imaging showed that PMN-Exo accumulated in the lung 12 h after injection (Fig. 2B). 24 h after exosome injection, the lung morphological changes were observed by H&E staining, demonstrating that TNF-Exo promoted lung injury as compared with PBS-Exo (Fig. 2C). Furthermore, qRT-PCR showed the same result that the expressions of pro-inflammatory mediators (iNOS, IL-1β and TNF-α) in the lung were increased by TNF-Exo (Fig. 2D). The immunofluorescence results showed that the numbers of macrophages (F4/80 + cells) and M1 macrophages (iNOS + F4/80 + cells) were markedly increased in the lung after TNF-Exo injection (Fig. 2E). All these data indicate that TNF-Exo promoted M1 macrophage activation and then induced pulmonary inflammation *in vivo*.

To further confirm that TNF-Exo promoted M1 macrophage activation *in vitro*, PMN-Exo and Raw264.7 macrophages/BMDMs were cocultured. The results showed that TNF-Exo promoted macrophage M1 polarization as assessed by flow cytometry, qRT-PCR and ELISA (Fig. 2F-2K).

PMN-derived exosomes prime macrophage for pyroptosis.

As indicated by TMT-based proteomics, TNF-Exo increased the expression of NLRP3 inflammasomes and gasdermin D (GSDMD, the core event in pyroptosis) in macrophages. We thus determined the role of

PMN-Exo in macrophage pyroptosis, and found that TNF-Exo increased PMϕ death after injection intraperitoneally, as shown by double staining of Annexin V and PI (Fig. 3A). To further determine the type of PMϕ death, PMϕ were detected for nuclear fragmentation, caspase-1 activation (the characteristics of pyroptosis) by staining the cells with TMR-Cell Death Reagent and Alexa Flour 488-labeled caspase-1 FLICA. Flow cytometry showed ~ 8% pyroptotic PMϕ at 24 h after TNF-Exo injection (Fig. 3B).

Next, we assessed the effect of PMN-Exo on macrophage pyroptosis *in vitro*. Surprisingly, treatment with TNF-Exo did not promote macrophage death or pyroptosis, while ATP/nigericin significantly upregulated pyroptotic cell death in TNF-Exo-primed macrophages (Fig. 3C-3D). The cleaved GSDMD N-terminus and IL-1β secretion were also increased in TNF-Exo plus ATP or nigericin group (Fig. 3E-3F). In addition, TNF-Exo increased NLRP3 and caspase-1 mRNA expression (Fig. 3G-3H). NLRP3 inflammasome expression levels are known to be regulated by the proinflammatory transcription factor NF-κB(28), and the previous result of the present study showed that TNF-Exo upregulated NF-κB signaling activity (Fig. 1H).

Collectively, these results indicate that TNF-Exo primed macrophages for pyroptosis by upregulating NLRP3 inflammasome expression through NF-κB signaling pathway.

miRNA analysis of PMN-derived exosomes.

We screened PMN-derived exosomes for miRNAs and detected 26 miRNAs that were increased ≥ 2-fold in TNF-Exo compared with PBS-Exo (Fig. 4A, **Additional file 2: Table 2**). Enrichment pathway analysis was also performed to identify the most enriched pathways related to signaling transduction for these 26 miRNAs, and the data showed that NF-κB signaling pathway was within the 20 most enriched pathways (Fig. 4B).

Next, we searched the literature and found that miR-30d-5p was reported to positively regulate NF-κB signaling pathway(29). We thus used qRT-PCR to verify the expression of miR-30d-5p in TNF-Exo was significantly higher than that in PBS-Exo (Fig. 4C). However, in PMNs exposed to TNF-α, miR-30d-5p was decreased, indicating that miR-30d-5p relocated from the cellular compartment to exosomes (Fig. 4D). Interestingly, macrophages treated with TNF-Exo exhibited higher levels of miR-30d-5p than PBS-Exo (Fig. 4E). These results indicate that TNF-α could enhance miR-30d-5p loading into exosomes and transfer to recipient macrophages. Thus, we hypothesized that PMN-derived exosomes may transfer miR-30d-5p into macrophages and then activate NF-κB signaling pathway during sepsis.

PMN-derived exosomes promote M1 macrophage activation and prime macrophage for pyroptosis through miR-30d-5p.

To test the above hypothesis, we transfected Raw264.7 macrophages with miR-30d-5p inhibitors prior to coculture with PMN-derived exosomes. It was found that transfection of miR-30d-5p inhibitors reversed the upregulation of M1 macrophage markers and pro-inflammatory cytokines induced by TNF-Exo (Fig. 5A-5C). In addition, inhibition of miR-30d-5p significantly decreased NF-κB p-p65 protein expression in recipient macrophages treated with TNF-Exo (Fig. 5D).

Moreover, treatment of Raw264.7 macrophages with an miR-30d-5p inhibitor prior to coculture with TNF-Exo decreased mRNA levels of NLRP3 and caspase-1 (Fig. 5E). Intracellular caspase-1 activation was also measured by flow cytometry and Western blot. Transfection of miR-30d-5p inhibitors exhibited a significant suppressive effect on caspase-1 activation in response to TNF-Exo and ATP (Fig. 5F-5G). The cleaved GSDMD N-terminus upregulated by TNF-Exo plus ATP stimulation was also inhibited by miR-30d-5p inhibitors (Fig. 5H).

Altogether, these data show that exosomal miR-30d-5p promoted M1 macrophage activation and primed macrophage for pyroptosis via an miR-30d-5p-NF-κB signaling-dependent pathway.

Exosomal miR-30d-5p activates NF-κB in macrophage via targeting SOCS-1 and SIRT1.

Next, we sought to understand the mechanism through which miR-30d-5p activated NF-κB signaling pathway. Bioinformatics analysis showed that SOCS-1 (suppressor of cytokine signaling) and sirtuin 1 (SIRT1) were putative target genes for miR-30d-5p, and also negative regulators of NF-κB signaling pathway. As predicted by Targetscan in our study, miR-30d-5p may conserve the binding sites in the 3’ UTR of SOCS-1 and SIRT1 (Fig. 6A). To validate this bioinformatic prediction, we conducted a dual luciferase reporter assay and found that luciferase activity was markedly reduced by miR-30d-5p overexpression in SOCS-1/SIRT1 3'-UTR WT group, but not in 3'-UTR Mut group (Fig. 6B). In Raw264.7 macrophages, overexpression of miR-30d-5p suppressed both the mRNA and protein levels of SOCS-1 and SIRT1 (Fig. 6C-6D). All these results demonstrate that SOCS-1 and SIRT1 were the direct target genes of miR-30d-5p.

To determine whether exosomal miR-30d-5p targeted SOCS-1/SIRT1 in recipient macrophages, we investigated SOCS-1 and SIRT1 levels in PMN-Exo-treated PMNs, and found that both mRNA and protein expressions of SOCS-1 and SIRT1 were concomitantly inhibited in recipient macrophages (Fig. 6E-6F), while miR-30d-5p inhibitor significantly upregulated the expression of SOCS-1 and SIRT1 (Fig. 6G). In addition, previous studies demonstrated that SIRT1 reduced NF-κB activity by decreasing the acetylation levels of lysine 310 of the NF-κB p65 subunit(30). We thus examined lysine 310 acetylation of p65 subunit and found that TNF-Exo enhanced p65 lysine 310 acetylation (Fig. 6F), while inhibition of miR-30d-5p decreased lysine 310 acetylation of p65 (Fig. 6G).

All the above results demonstrate that exosomal miR-30d-5p targeted SOCS-1 and SIRT1 in macrophage, and subsequently activated NF-κB partly by increasing acetylation of Lysine 310 of p65.

Exosomal miR-30d-5p promotes lung injury during sepsis in vivo.

We next investigated the functional role of miR-30d-5p in PMN-derived exosomes during sepsis by using the cecal ligation and puncture (CLP) mouse model to mimic sepsis *in vivo*. It was found that miR-30d-5p expression was significantly increased in the lung tissue of CLP group (Fig. 7A). Injection of TNF-Exo also upregulated miR-30d-5p level in the lung tissue (Fig. 7B). Next, miR-30d-5p inhibitor or scrambled negative control was administered via the tail vein of mice before conducting the CLP model or injecting

TNF-Exo, and the expression levels of proinflammatory cytokines and NLRP3 inflammasome in the lung tissues were examined. The results showed that miR-30d-5p inhibitor significantly reversed the upregulation of IL-6, iNOS, NLRP3 mRNA levels induced by CLP, and repressed IL-1β, iNOS, NLRP3, caspase-1 mRNA expression following TNF-Exo injection (Fig. 7C-7F). In addition, inhibition of miR-30d-5p decreased CLP or TNF-Exo-induced M1 macrophage activation and macrophage death in the lung (Fig. 7G-7J). All these data demonstrate that exosomal miR-30d-5p enhanced M1 macrophage activation and macrophage death, and then promoted lung inflammation during sepsis.

Discussion

In the current study, we demonstrated a previously unidentified role of PMN-Mϕ interaction in promoting inflammation in sepsis. In addition, exosomal miR-30d-5p from neutrophils induced macrophage M1 polarization and primed Mϕ for pyroptosis in sepsis by activating NF-kB signaling, which in turn enhanced inflammation. We further demonstrated that exosomal miR-30d-5p activated NF-kB signaling by targeting SOCS-1 and SIRT1.

In the early stage of sepsis, neutrophils are thought to be the primary innate immune cells that cause damage to host tissues(31). In addition to releasing important cytokines, chemokines, ROS and NETs, some studies suggested that PMN-derived exosomes were a new subcellular entity, working as a fundamental link between PMN-driven inflammation and tissue damage(21). TNF-α plays a central role in the pathogenesis of sepsis, and is an early regulator of the immune response(32). Previous studies(33, 34) showed that TNF-α and IL-1β produced by macrophages activated neutrophils during sepsis, and high concentrations of TNF-α and IL-1β have been reported in BALF from ARDS patients. Therefore, in this study, we used TNF-α to activate PMNs, and found an increased number of PMN-derived exosomes after TNF-α stimulation.

Macrophages have been shown to be the recipient cells for exogenous exosomes and in direct contact with peripheral serum exosomes(35). A recent study showed that peripheral serum exosomes promoted M1 macrophage polarization and inflammation during sepsis-related ALI, but the study did not address the cellular origin of circulating exosomes(36). Our results showed that exosomes isolated from the supernatant of PMNs stimulated with TNF-α promoted M1 macrophage activation both *in vivo* and *in vitro*. We also observed that TNF-Exo resulted in a significant lung inflammatory response, suggesting that exosomes released from TNF-α-activated PMNs are a kind of pro-inflammatory exosome and play an important role in sepsis-related ALI.

In addition, we observed that ATP/nigericin significantly upregulated pyroptotic cell death in TNF-Exo-primed macrophages. Induction of pyroptotic cell death *in vitro* usually needs two signals: the priming signal and the secondary signal. The priming signal upregulates NLRP3 and pro-IL-1β expression levels through the transcription factor NF-κB. After the priming phase, the secondary signal, such as ATP/nigericin, initiates the assembly of several protein complexes, including NLRP3, ASC and pro-caspase-1, by regulating the formation of the ASC pyroptosome and splicing of caspase-1 into its active

form(37). Based on our results, TNF-Exo serves as a priming signal to increase NLRP3 inflammasome expression through activating NF-κB signaling pathway, which needs the secondary signal to finally induce pyroptotic cell death. High concentrations of extracellular ATP have been implicated in multiple *in vivo* inflammatory responses, including lung inflammation and fibrosis, systemic inflammation and tissue damage during endotoxemia(38, 39). Almost all mammalian cells, including myeloid cells, platelets, leukocytes, epithelial and endothelial cells, can release ATP(40). That may explain why TNF-Exo could promote macrophage pyroptosis *in vivo*, as TNF-Exo or cytokines may stimulate other cells to release high concentrations of ATP. However, the exact mechanisms need to be further addressed.

Exosomally transferred miRNAs have emerged as novel regulators of cellular function. miRNA sequencing and literature search in our study suggest that miR-30d-5p may be the functional molecule within TNF-Exo. Our study here reported a novel function of miR-30d-5p in exosomes as a regulator of macrophage polarization and pyroptosis in sepsis. The exosome-mediated inflammatory pathway may be a new mechanism responsible for the development of lung inflammation by promoting PMN-Mϕ communication.

Finally, we demonstrated that miR-30d-5p played a functional role in PMN-derived exosomes during sepsis-related ALI. The expression level of miR-30d-5p was significantly increased in the lung tissue after TNF-Exo injection, suggesting that miR-30d-5p could be transferred to the lung tissue via exosomes. miR-30d-5p loss-of-function markedly reduced M1 macrophage activation and death in the lung, and ameliorated lung inflammation, indicating that miR-30d-5p contributed to sepsis-related ALI.

Conclusions

We demonstrated that exosomal miR-30d-5p from neutrophil induced macrophage M1 polarization and primed Mϕ for pyroptosis through activating NF-kB signaling. Exosomal miR-30d-5p mediated the cross-talk between PMNs and macrophages by targeting SOCS-1 and SIRT1. miR-30d-5p played a critical role in the development of lung inflammation and injury during sepsis. These findings suggest that exosomal miR-30d-5p derived from PMNs may represent a new therapeutic target for the progression of sepsis-related ALI.

Abbreviations

PMNs
polymorphonuclear neutrophils; Mϕ:macrophage; BMDMs:bone marrow-derived macrophages; PBS:phosphate buffer saline; TNF-α:tumor necrosis factor-α; Exos:Exosomes; SOCS-1:suppressor of cytokine signaling; SIRT1:sirtuin 1; ALI:acute lung injury; IL-1β:interleukin 1β; miRNAs:microRNAs; WT:wild type; RPMI:roswell park memorial institute; FBS:fetal bovine serum; TEM:transmission electron microscopy; iNOS:inducible nitric oxide synthase; NLRP3:NOD-like receptors; PMϕ:peritoneal macrophage; GSDMD:gasdermin D; CLP:cecal ligation and puncture; RT-PCR:Real-time polymerase chain reaction; H&E:Hematoxylin and eosin.

Declarations

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YJ, TZ and CMZ contributed equally to this work. YJ, ZLM and XYS contributed to the conception and design. YJ, TZ, CMZ and HYJ performed the experiments, analyzed data, and wrote the manuscript. XYT and RX participated in the animal experiments. WW helped to revise the manuscript. All authors read and approved the final manuscript.

Ethics approval

All animal experiments were conducted under the rules approved by Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, and the approval number is XHEC-F-2020-019.

Consent for publication

All listed authors consent to the submission and all data are used with the consent of the person generating the data.

Competing interests

The authors declare that they have no competing interests.

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Figures

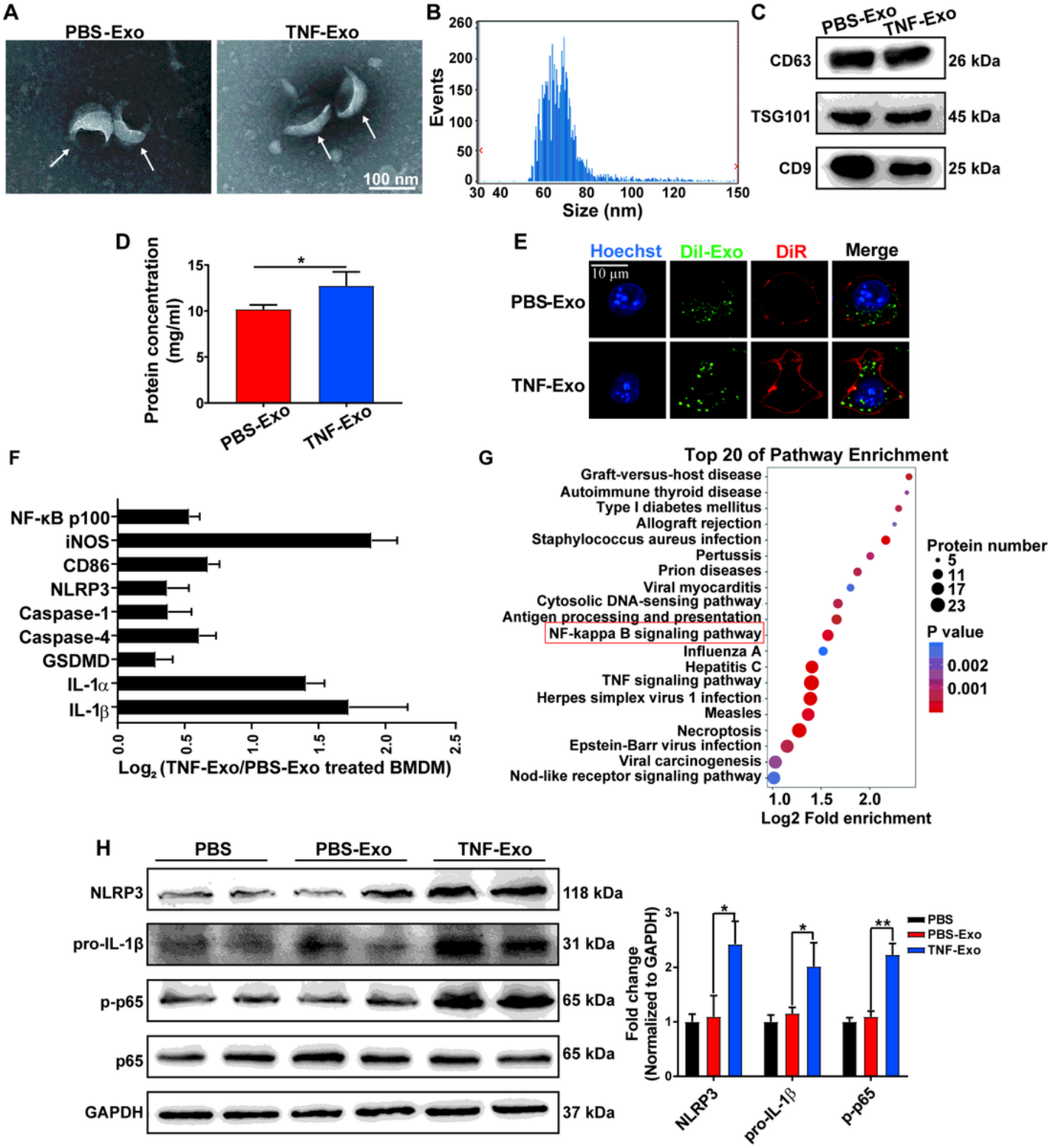

Figure 1

Characterization of exosomes isolated from the supernatant of PMNs stimulated ex vivo. A Electron micrograph of exosomes (indicated by arrows) derived from the supernatant of PMNs stimulated with PBS (PBS-Exo) or 20 ng/mL TNF-α (TNF-Exo) for 12 h. Scale bar, 100 nm. B Measurement of exosome size distribution by NanoSight tracking analysis. C Quantification of CD63, TSG101 and CD9 protein expressions in exosomes by Western blot loaded with equal amounts of exosome protein (60 μg). D

Protein quantification of exosomes by BCA. E Immunofluorescence images showing bone marrow-derived macrophages (BMDMs) incubated with DiI-labeled exosomes (green) for 3 h. Cell membranes and nuclei were counterstained with DiR (red) and Hoechst (blue) respectively. Scale bar, 10 μm. F-H Treatment of BMDMs with PBS-Exo/TNF-Exo for 24 h. F BMDMs for tandem mass tags (TMT)-based proteomic quantification. G Enrichment pathway analysis showed that NF-κB signaling pathway was within the 20 most enriched pathways. H Western blot of NLRP3, pro-IL-1β, NF-κB p-p65 and p65 in BMDMs after coculture with exosomes. Student's t test (D) or one-way analysis of variance with Tukey's multiple comparisons test (H) was used for the analysis. Graphs represent means ± SEM, n≥3; *P < 0.05, **P < 0.01 compared within two groups.

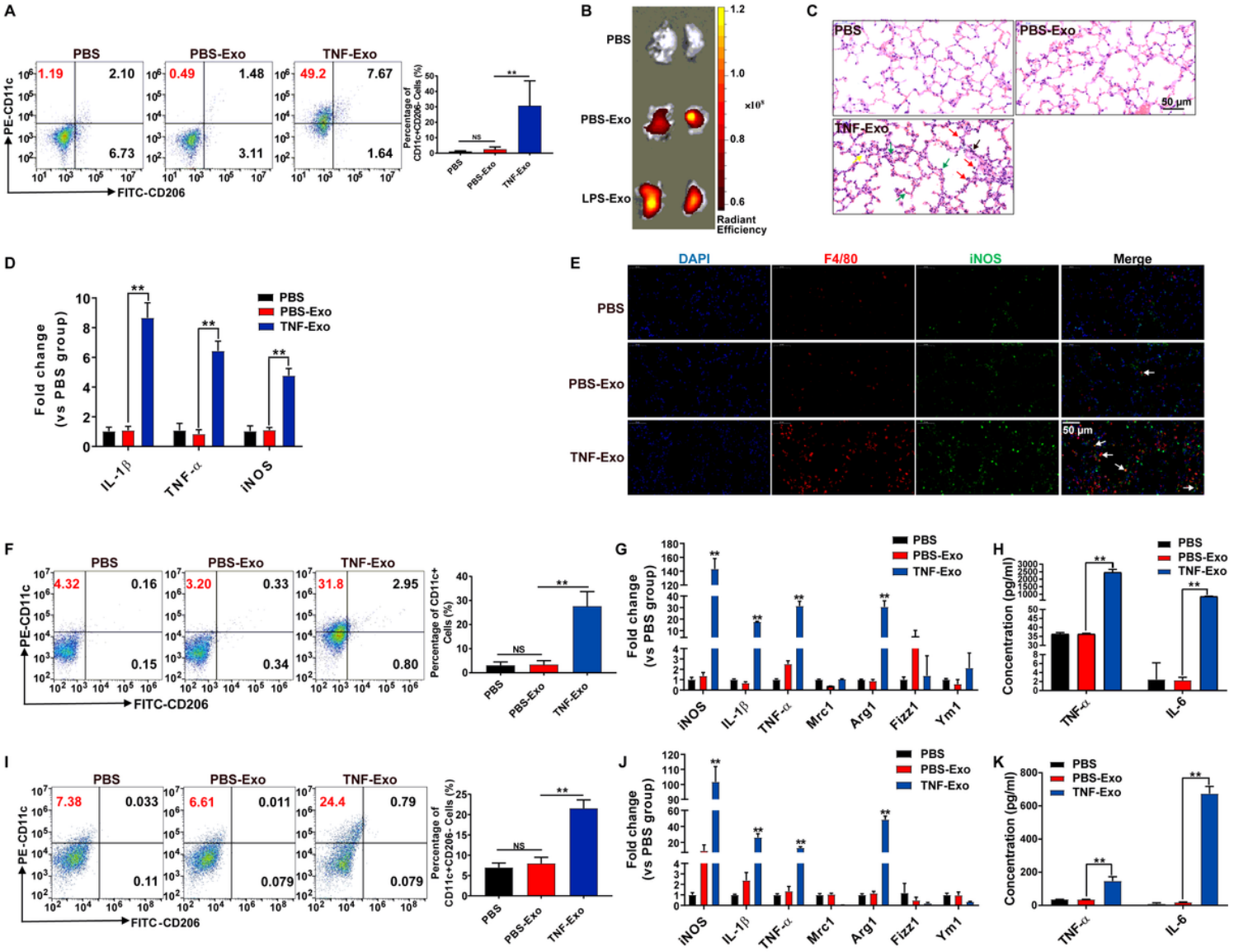

Figure 2

PMN-derived exosomes promote M1 macrophage activation both in vivo and in vitro. A-E Treatment of WT C57BL/6 mice with PBS or exosomes (300 μg/mouse) isolated from the supernatant of PMNs stimulated with PBS/TNF-α through i.p. injection for 24 h. A Flow cytometry detection of CD11c and CD206 expression on peritoneal Mϕ (PMϕ) after being gated with macrophage marker F4/80. B Ex vivo

fluorescence signals in the lungs of mice injected i.p. with DiI-labeled exosomes. C Evaluation of lung histology by H&E staining (magnification ×400). The lung tissue in Sham group is normal with thin alveolar walls and few alveolar macrophages. Green arrows indicate neutrophils in the alveolar and interstitial space, red arrows indicate alveolar macrophages, yellow arrows indicate hyaline membranes, and black arrows indicate thickening of the alveolar walls. Scale bar, 50μm. D Detection of inflammatory cytokine mRNA (IL-1β, TNF-α) and iNOS mRNA expression in the lung tissue by real-time PCR. E Representative images of direct immunofluorescence staining of DNA (blue), F4/80 (red) and iNOS (green) in the lung sections, and white arrows indicate iNOS positive macrophages. Scale bar, 50 μm. F-H Treatment of Raw264.7 macrophages with PBS or exosomes for 24 h. F Flow cytometry detection of CD11c and CD206 expression on Raw264.7 macrophages. G Detection of expression levels of iNOS, IL-1β, TNF-α, Mrc1, Arg1, Fizz1 and Ym1 mRNA by real-time PCR. H Detection of the concentration of inflammatory cytokines (IL-6, TNF-α) in the supernatant of Raw264.7 macrophages by ELISA. I-K Treatment of bone marrow-derived macrophages (BMDMs) with PBS or exosomes for 24 h. I Flow cytometry detection of CD11c and CD206 expression on BMDMs. J Detection of expression levels of iNOS, IL-1β, TNF-α, Mrc1, Arg1, Fizz1 and Ym1 mRNA by real-time PCR. K Detection of the concentration of inflammatory cytokines (IL-6, TNF-α) in the supernatant of BMDMs by ELISA. One-way analysis of variance with Tukey's multiple comparisons test was used for the analysis. Graphs represent means ± SEM, $n \geq 3$; *$P < 0.05$, **$P < 0.01$ compared within two groups.

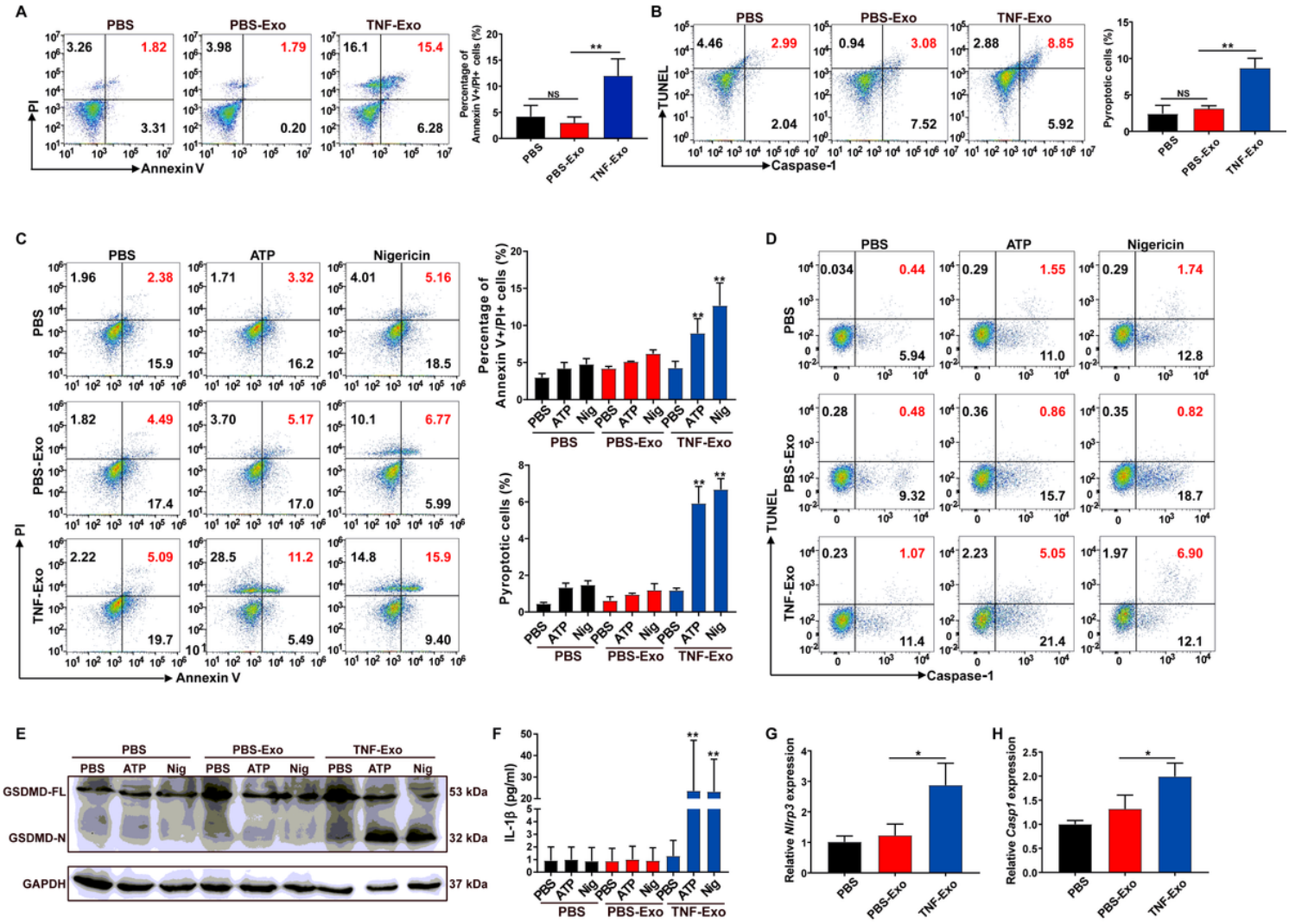

Figure 3

PMN-derived exosomes prime macrophage for pyroptosis. A-B Treatment of WT C57BL/6 mice with PBS or exosomes (300 μg/mouse) isolated from the supernatant of PMNs stimulated with PBS/TNF-α through i.p. injection for 24 h. A Representative flow cytometry plots of Annexin V/PI staining of PMϕ identified as F4/80+, and analysis of Annexin V/PI double-stained cells by dying. B Representative flow cytometry plots and quantitation of PMϕ pyroptosis (caspase1/TUNEL double-positive cells). C-F Stimulation of Raw264.7 macrophages with PBS/PBS-Exo/TNF-Exo for 24 h and treatment with PBS, 5 mM ATP or 20 mM nigericin for 2 h. C Flow cytometry evaluation of macrophage death by Annexin-V and PI double-staining. D Flow cytometry evaluation of macrophage pyroptosis by caspase-1 and TUNEL double-staining. E Cleavage of GSDMD by immunoblotting. GSDMD-FL full-length GSDMD, GSDMD-N N-terminal cleavage products of GSDMD. F Analysis of culture supernatants for IL-1β secretion by ELISA. G-H Stimulation of Raw264.7 macrophages with PBS/PBS-Exo/TNF-Exo for 24 h, and detection of NLRP3 and caspase-1 mRNA expression by real-time PCR. One-way analysis of variance with Tukey's multiple comparisons test was used for the analysis. Graphs represent means ± SEM, n ≥ 3; *P < 0.05, **P < 0.01 compared within two groups.

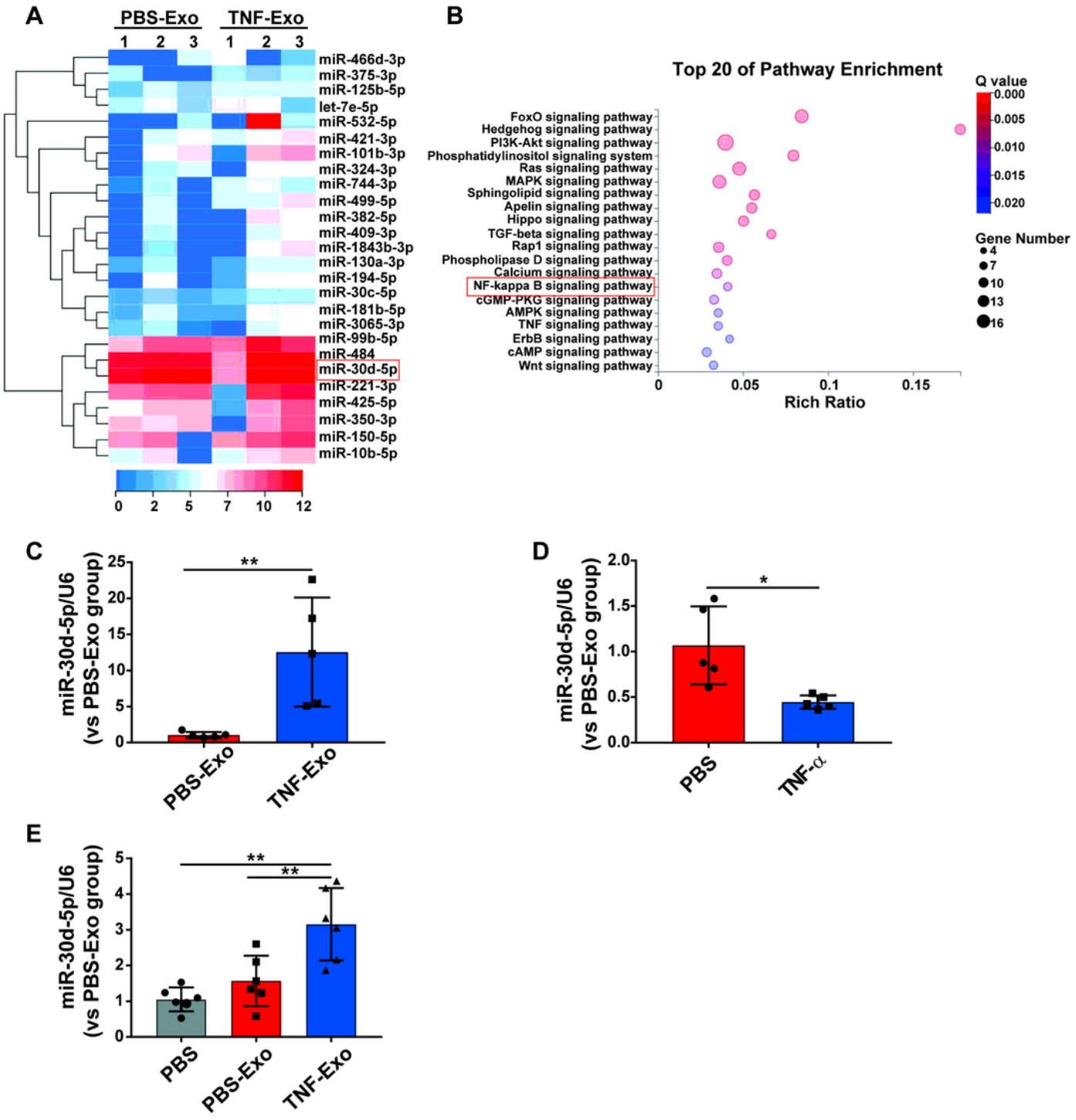

Figure 4

miRNA analysis of PMN-derived exosomes. A Heat map of exosomal miRNA-seq (n=3). The fluorescence intensity of 26 differentially expressed miRNAs (≥ 2-fold) is illustrated from high (red) to low (blue). B Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis on differentially expressed exosomal miRNAs; the 20 most enriched pathways related to signaling transduction are shown. The rich ratio indicates the number of genes in the miRNA target list over the total genes in the respective canonical

pathway. C-E Expression of miR-30d-5p in PMN-derived exosomes, PMNs stimulated with PBS/TNF-α and recipient Raw264.7 macrophages treated with PBS/PBS-Exo/TNF-Exo. Student's t test (C-D) or one-way analysis of variance with Tukey's multiple comparisons test (E) was used for the analysis. Graphs represent means ± SEM, n≥3; *P < 0.05, **P < 0.01 compared within two groups.

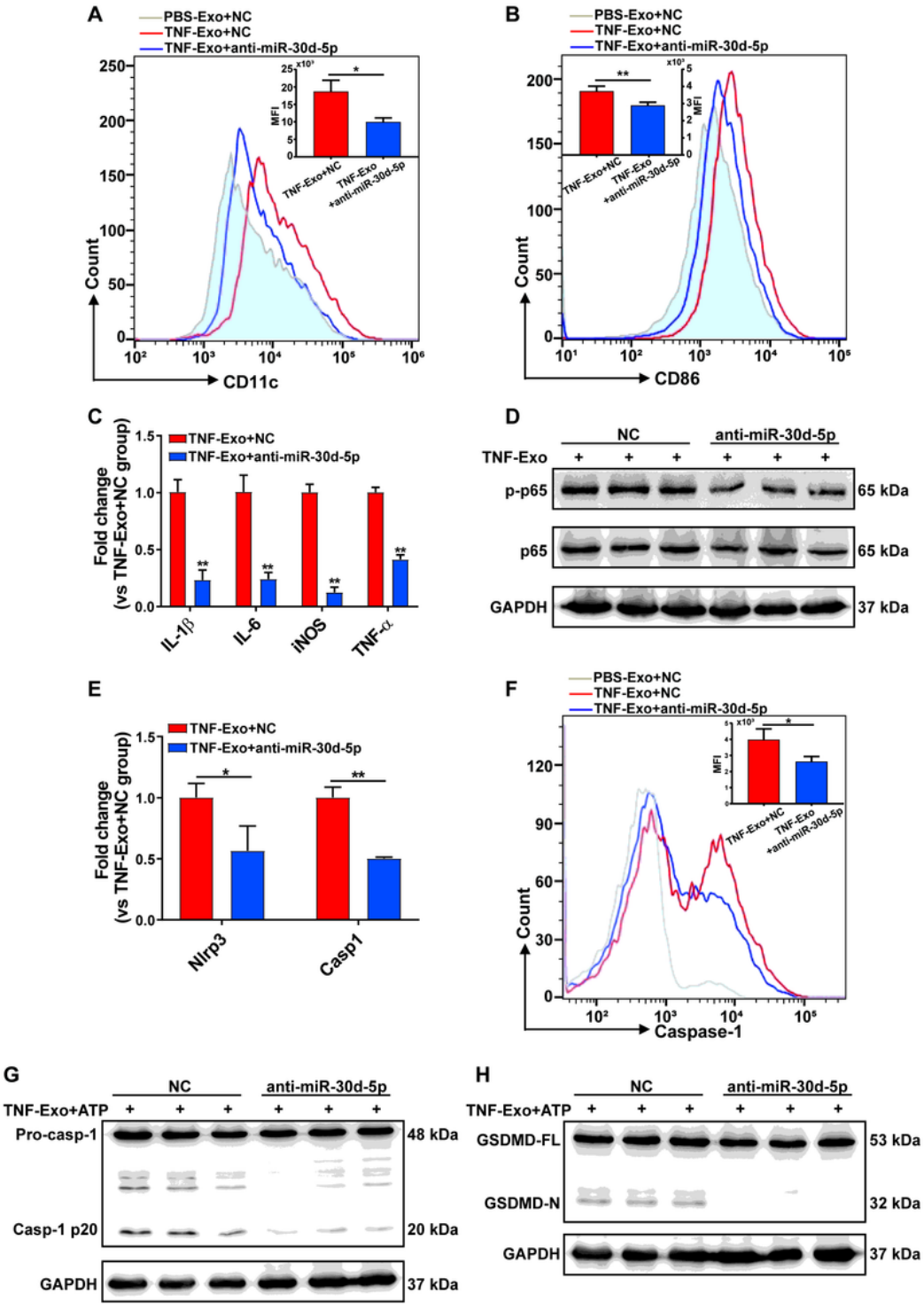

Figure 5

PMN-derived exosomes promote M1 macrophage activation and prime macrophage for pyroptosis through miR-30d-5p. A-E Prior to coculture with TNF-Exo for 24 h, Raw264.7 macrophages were transfected with control or miR-30d-5p inhibitors for 24 h. A-B Flow cytometry detection of CD11c and CD86 expression. C Detection of inflammatory cytokine mRNA (IL-6, IL-1β, TNF-α) and iNOS mRNA expression by real-time PCR. D Western blot of NF-κB p-p65 and p65 in Raw264.7 macrophages. E Detection of NLRP3 and caspase-1 mRNA expression by real-time PCR. F-H Transfection of Raw264.7 macrophages with control or miR-30d-5p inhibitors, followed by stimulation with TNF-Exo plus ATP. F Flow cytometry detection of Alexa Fluor 488-labeled caspase-1 FLICA expression. G Western blots of pro-caspase-1 (Pro-casp-1) and activated/cleaved caspase-1 (Casp-1 p20) in whole-cell lysates of Raw264.7. H Cleavage of GSDMD by immunoblotting. Student's t test was used for analysis. Graphs represent means ± SEM, $n \geq 3$; *$P < 0.05$, **$P < 0.01$ compared within two groups.

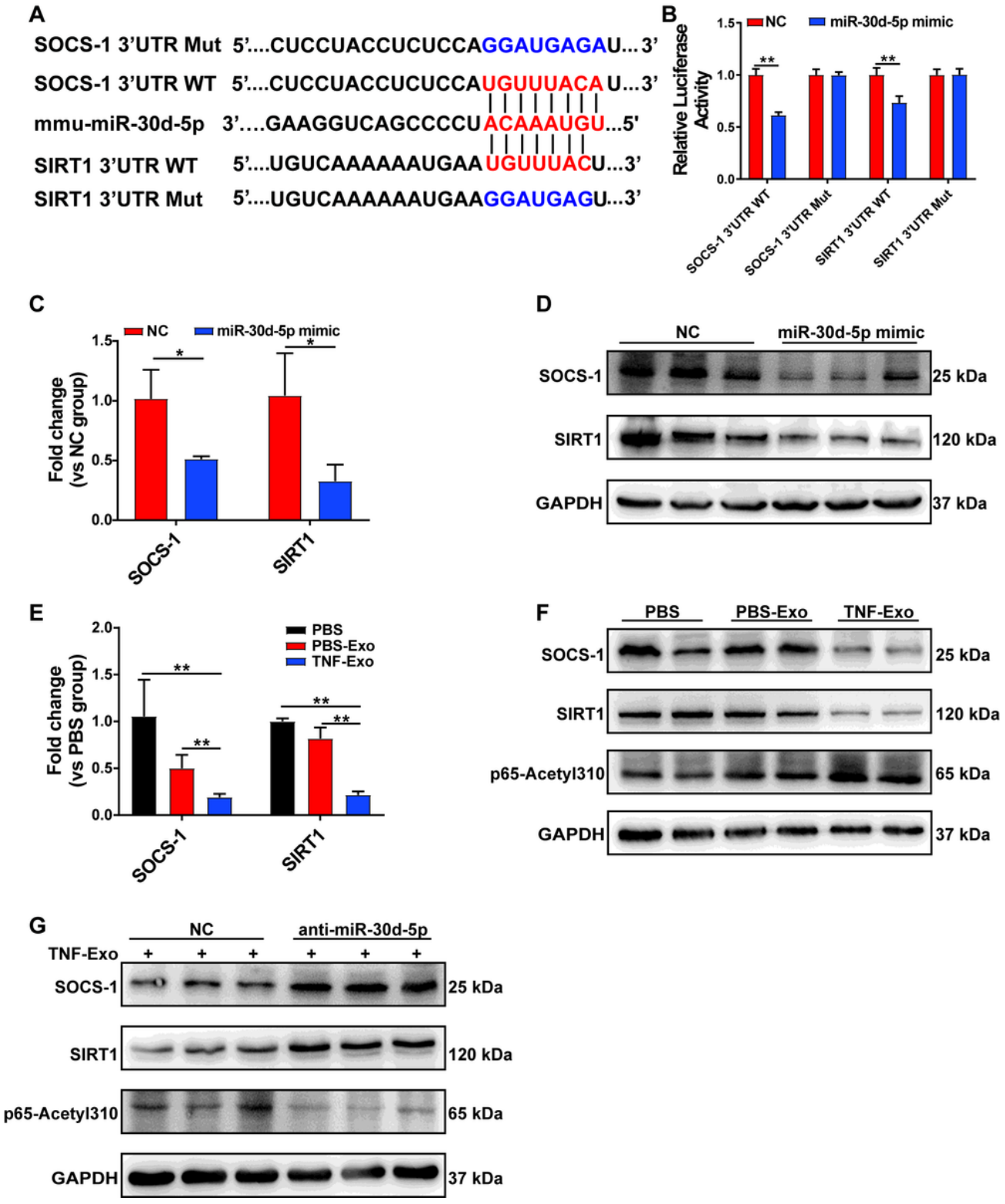

Figure 6

Exosomal miR-30d-5p activates NF-κB in macrophage via targeting SOCS-1 and SIRT1. A Sequence alignment between miR-30d-5p and its putative binding sites (in red letters) in the SOCS-1/SIRT1 3'-UTR. Mutation of the miR-30d-5p target sites (in blue letters) is also shown. B Detection of the relative luciferase activities of WT and Mut SOCS-1/SIRT1 reporters by luciferase reporter assay, using Renilla luciferase vector as the internal control. qRT-PCR analysis of relative SOCS-1/SIRT1 mRNA levels (C) and

Western blot (D) of SOCS-1 and SIRT1 in Raw264.7 macrophages transfected with miR-30d-5p mimics as indicated. E-F Treatment of Raw264.7 macrophages with PBS/PBS-Exo/TNF-Exo for 24 h. E Detection of mRNA levels of SOCS-1 and SIRT1 by qRT-PCR. F Western blot analysis of SOCS-1, SIRT1 and p65-Acetyl 310. G Prior to coculture with TNF-Exo for 24 h, Raw264.7 macrophages were transfected with control or miR-30d-5p inhibitors for 24 h. The expression levels of SOCS-1, SIRT1 and p65-Acetyl 310 in Raw264.7 cells were measured by Western blot. Student’s t test (B-C) or one-way analysis of variance with Tukey's multiple comparisons test (E) was used for the analysis. Graphs represent means ± SEM, $n \geq 3$; *P < 0.05, **P < 0.01 compared within two groups.

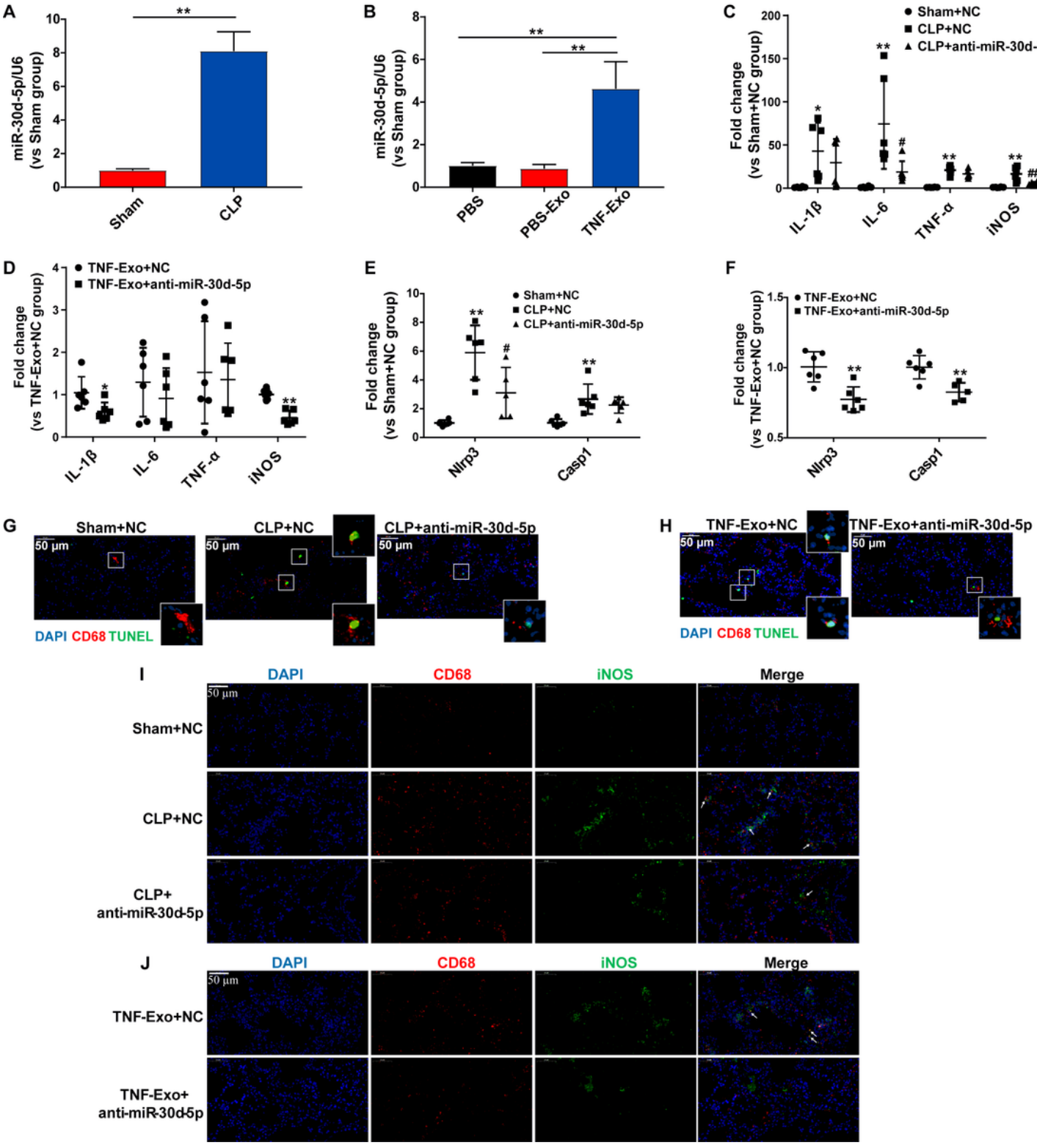

Figure 7

Exosomal miR-30d-5p promotes lung injury during sepsis in vivo. Mice were subjected to sham or CLP for 24 h, or treated with exosomes (300 μg/mouse) isolated from the supernatant of PMNs stimulated ex vivo through i.p. injection. The miR-30d-5p inhibitor or negative control was injected into each mouse 1 day before CLP surgery or TNF-Exo injection. Relative expression levels of miR-30d-5p (A-B), inflammatory cytokine mRNA (IL-6, IL-1β, TNF-α) and iNOS mRNA (C-D), NLRP3 and caspase-1 mRNA

expression (E-F) in the lung tissues were measured by qRT-PCR. G-H Representative images of direct immunofluorescence staining of DNA (blue), CD68 (red) and TUNEL (green) in the lung sections. Scale bar, 50 μm. I-J Representative images of direct immunofluorescence staining of DNA (blue), CD68 (red) and iNOS (green) in the lung sections, and white arrows indicate iNOS positive macrophages. Scale bar, 50 μm. Student’s t test or one-way analysis of variance with Tukey's multiple comparisons test was used for the analysis. Graphs represent means ± SEM, n ≥ 3; *P < 0.05, **P < 0.01 compared within two groups (C and E *,**P compared with Sham+NC group, #,##P compared with CLP+NC group).

Supplementary Files

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