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Lipopolysaccharide mediates time-dependent macrophage M1/M2 polarization through the Tim-3/Galectin-9 signalling pathway

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ABSTRACT

Macrophages are dynamic cells whose phenotypes and functions are regulated by surrounding inflammatory mediators after pathogenic infection. Imbalanced polarization of classically activated (M1) and alternatively activated (M2) macrophages is closely associated with infection-related complications and their severity. The pathway of T-cell immunoglobulin mucin 3 (Tim-3)/galectin-9 (Gal-9) plays an important role in infection by regulating macrophage function. However, the effects of Tim-3/Gal-9 signalling on M1/M2 macrophage polarization are unclear. Bone marrow-derived macrophages (BMDMs) were stimulated with 0.1 μg/mL lipopolysaccharide (LPS). M1/M2 phenotypic macrophage markers were measured 0, 1, 3, 6, 12, and 24 h after stimulation, α-lactose was used to inhibit Gal-9, anti-mouse Tim-3 antibody was used to block Tim-3, recombinant mouse-Gal-9 (rm-Gal-9) was used to activate Tim-3, which were aimed to verify the role of the Tim-3/Gal-9 pathway in the balance of M1/M2 macrophages when stimulated with LPS. Short-term LPS stimulation upregulated Gal-9 expression and secretion, enhanced the association between Gal-9 and Tim-3, and activated the Tim-3/Gal-9 signalling pathway, eventually inhibiting M1 polarization. Long-term stimulation downregulated Gal-9 expression and secretion, reduced the association between Gal-9 and Tim-3, and inhibited the Tim-3/Gal-9 signalling pathway, eventually promoting M1 polarization, however, decreased M2 polarization and Gal-9 autocrine functions. Overall, LPS had a biphasic effect on BMDMs polarization through the Tim-3/Gal-9 pathway, which was time-dependent.

1. Introduction

Macrophages form an important defence against pathogens through a variety of pattern recognitions and scavenger receptors [1]. During infectious diseases, macrophages have the ability to eliminate pathogens or repair inflammatory injuries, and play an important role in innate immunity, antigen presentation, and anti-infectious immune regulation [2]. During inflammatory conditions, macrophages are activated and can differentiate into two subtypes, involving classically activated macrophages (M1) and alternatively activated macrophages (M2) [3]. M1 macrophages express surface markers such as CD86 and secrete proinflammatory cytokines such as inducible nitric oxide synthase (iNOS), promote inflammatory responses, and clear pathogens, while M2 macrophages are associated with immune regulation and tolerance, expressing surface markers such as CD206, secreting anti-inflammatory cytokines such as Arginase-1 (Arg-1), limiting excessive inflammatory responses, and promoting tissue repair [1,4,5]. In the development of inflammation, the two macrophage phenotypes are in a dynamic equilibrium, which is affected by different cytokines and downstream signalling pathways [6,7]. Furthermore, M1/M2 macrophage population imbalance in infectious diseases often causes serious complications such as sepsis, a life-threatening condition resulting from organ dysfunction due to a dysregulated host response to infection [8].

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), involving diffuse pulmonary pathological changes induced by excessive activation and uncontrolled cascade inflammation, are the

Abbreviations: Tim-3, T-cell immunoglobulin mucin 3; Gal-9, galectin-9; BMDMs, Bone marrow-derived macrophages; LPS, lipopolysaccharide; rm-Gal-9, recombinant mouse-Gal-9; rGal-9, recombinant galectin-9 protein; iNOS, inducible nitric oxide synthase; Arg-1, Arginase-1; ALI, Acute lung injury; ARDS, Acute respiratory distress syndrome; Th1, helper T1; DMEM, Dulbecco minimal essential medium; ELISA, Enzyme-linked immune sorbent assay; SD, Standard deviations; ANOVA, One-way analysis of variance; AML, acute myelocytic leukaemia; CLSM, confocal laser scanning microscopy; IP, immunoprecipitation; PI3K, phosphatidylinositol 3-kinase

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most common complications after sepsis or severe pneumonia [9–11]. The regulation of the phenotype and function of alveolar and lung macrophages is closely related to the inflammatory process of ALI/ARDS; it mediates a series of changes in the barrier function of the respiratory system from injury to recovery [1]. The number of M1 macrophages in lungs decreases, and the number of M2 macrophages significantly increases, after anti-inflammatory drugs are used to treat ALI [12]. In the late stages of ALI/ARDS, macrophages may undergo programmed death, which leads to the hypo-function of phagocytosis and limiting inflammatory, eventually resulting in an expanding and uncontrolled lung inflammation, further aggravating lung injury [13]. The clinical severity and prognoses differ in ALI/ARDS patients, so we examined the effects of different stimulation times of lipopolysaccharide (LPS) on macrophages to investigate the differences in clinical severities in ALI/ARDS.

T-cell immunoglobulin mucin 3 (Tim-3) is a recently discovered immunomodulatory molecule, which is highly expressed in helper T1 (Th1) cells and cytotoxic T cells, and produces inhibitory signals that lead to Thl and Tcl cell apoptosis [14]. Tim-3 displays diverse regulatory roles in innate immune cells such as NK cells, NKT cells, and the mononuclear phagocyte system [15]. Galectin-9 (Gal-9) is a ligand of Tim-3 that can be expressed in almost any cell *in vivo*. When it specifically binds to Tim-3 in Thl cells, it provides a negative co-stimulatory signal to T cells that results in programmed death of Th1 cells, ultimately inducing peripheral immune tolerance [16]. The effects of the Tim-3/Gal-9 pathway on innate immunity are different from that of adaptive immunity. In a mouse model of sepsis associated ARDS, recombinant galectin-9 protein (rGal-9) can inhibits macrophage function, thereby downregulating the release of inflammatory factors and producing a protective lung effect [17]. Increased Tim-3 expression may alleviate liver injury by regulating macrophage activation [18]. However, in human macrophages infected with *Mycobacterium tuberculosis*, interactions between Tim-3 and Gal-9 may induce macrophage activation and growth control of tuberculosis by producing the proinflammatory cytokine interleukin-1β [19].

Numerous studies have reported the anti-inflammatory effects of M2 macrophages in ALI/ARDS and other diseases; however, the detailed relationships between macrophage M1/M2 polarization and Tim-3/Gal-9 siganlling pathway have not been fully elucidated. Therefore, we investigated whether bone marrow-derived macrophages (BMDMs) treated with LPS for different times would display different levels of M1/M2 polarization, and whether this process is mediated by Tim-3/Gal-9 pathway.

2. Experimental procedures

2.1. Isolation and culture of BMDMs

Murine BMDMs were isolated from the femurs of C57/BL/6J mice (Shanghai Model Organisms Centre, Shanghai, China) as described previously [20,21]. In brief, mice were sacrificed by rapid cervical dislocation. Bone marrow was flushed from femurs with phosphate-buffered saline solution (PBS; Hyclone, Loughborough, UK). Then collected bone marrow cell suspensions were centrifuged for 10 min at 500 × *g* at room temperature. Red Blood Cell Lysis Buffer (Solarbio Life Sciences & Technology, Beijing, China) was used to treat cell pellets on ice for 10 min, and then centrifuged for 10 min at 500 × *g* at room temperature. Then the cell pellets were resuspended in Dulbecco minimal essential medium (DMEM) (Hyclone) with 10% fetal bovine serum (FBS; ScienCell, San Diego, CA, USA), with 10 ng/mL macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA). The cells were seeded in 6-well plates in 2 mL complete DMEM at a concentration of 1×10^6 cells/mL and incubated at 37 °C and 5% CO_2 for 3 days. On day 4, 1 mL medium in each well was replaced with fresh DMEM. The adherent cells turned into 95% pure macrophages after 7 days of culture. It confirms the existence of cell-surface marker F4/80. These cells were used in subsequent experiments.

2.2. Flow cytometry

Adherent BMDMs were incubated with LPS (LPS from *Escherichia coli*; Sigma-Aldrich, Steinheim am Albuch, Germany) in medium without FBS for 0, 1, 3, 6, 12, or 24 h at a concentration of 0.1 μg/mL. The other BMDMs were pretreated with the anti-mouse Tim-3 antibody (RMT3–23, BioLegend, Inc., San Diego, CA, USA) and α-lactose (Solarbio) at concentrations of 10 μg/mL and 40 μM, diluted with DMEM without FBS for 1h before LPS stimulation and then incubated with LPS (0.1μg/mL) for 3h. The cells were detached with 0.25% trypsin (Genom Biomedical Technology, Hangzhou, China) and centrifuged for 5 min at 350 × *g* and washed twice using cold PBS with 1% BSA. The pellets were resuspended in 100 μL PBS with 1% BSA at a concentration of 1×10^7 cells/mL, Purified Rat Anti-Mouse CD16/CD32 (BD Biosciences, San Jose, CA, USA) was used to block non-antigen-specific binding of immunoglobulins to the FcγIII (CD16) and FcγII (CD32), and possibly FcγI on macrophages. Then, 0.5 μg APC conjugated Anti-Mouse F4/80 (Cat. No. 70-AM048005, MultiSciences Biotech, Co., Ltd., Hangzhou, China) and 0.4 μg PE conjugated Anti-Mouse CD86 (Cat. No. 12-0861-81, Thermo Fisher Scientific, California, US) were added to each tube and incubated for 30 min at room temperature. After washing, fixation, and permeabilization as described in the Fixation/Permeabilization Reagents manual (eBioscience, San Diego, CA, USA), BMDMs were incubated with 0.5 μg FITC conjugated Anti-Mouse CD206 (Cat. No. 141703, BioLegend, San Diego, CA, US) for 45 min, cells were also incubated for same time with respective isotype control antibodies, Mouse IgG2a Isotype Control, APC (MultiSciences Biotech), Rat IgG2b kappa Isotype Control, PE (Thermo Fisher Scientific, California, US), and FITC Rat IgG2a, κ Isotype Ctrl Antibody (BioLegend, San Diego, CA, US), then washed and analysed by flow cytometry (FACSVERSE; Becton Dickinson, San Jose, CA, USA).

2.3. Immunofluorescence

After washing, fixation, permeabilization and blocking as described previously [22]. BMDMs were then incubated with Tim-3 Mouse antibody (1:500, Cat. No. 60355-1-Ig Proteintech Group, Inc., IL, US) and Gal-9 Rabbit antibody (1:400, Cat. No. 17938-1-AP, Proteintech) overnight at 4 °C. Then washing three times with PBS, and the cells were incubated with Alexa Fluor 594 conjugated Goat Anti-Mouse IgG(H + L) (1:400, Cat. No. SA00006-3, Proteintech) and Alexa Fluor 488 conjugated Goat Anti-Rabbit IgG (H&L) (1:500, Cat. No. ab150077; Abcam, Cambridge, MA, USA) for 1 h in the dark at room temperature. The cells were again washed three times with PBS and treated for 10 min with DAPI (1:2000, Cat. No. D9564; Sigma-Aldrich) to stain the cell nuclei. Finally, the cells were washed three times again in PBS and observed under a confocal microscope (Olympus FV-1000).

2.4. Western blotting

Total proteins of BMDMs were extracted after treatment and analysed using western blotting as described previously [22], Blots were incubated with iNOS (1:1000, #13120; Cell Signalling Technology, Inc., Danvers, MA, US), Arg-1 (1:1000, # 93668; Cell Signalling Technology), Gal-9 (1:2000, Cat. No. 17938-1-AP, Proteintech), Tim-3 (1:4000, Cat. No. 11872-1-AP, Proteintech), GAPDH (1:1000, #5174; Cell Signalling Technology) antibodies at different concentration at 4 °C overnight. Finally, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, Cat. No. A0208, Beyotime) and developed by enhanced chemiluminescence kit (Cat. No. 70-P1421; MultiSciences Biotech) and exposed to X-ray film.

2.5. ELISA

Culture medium was removed at each time point and stored at −80 °C. When BMDMs were treated for 1 h with rm-Gal-9 (R&D Systems, Minneapolis, MN, USA), the culture medium was replaced with fresh medium without FBS, and the supernatants were collected after 3 h and stored at −80 °C until analyses. The level of s-Gal-9 in supernatants of the cultures was determined using ELISA kits (Westang Biotech, Shanghai, China) based on the manufacturer's recommendations.

2.6. Immunoprecipitation

To detect Tim-3 associated with Gal-9, total protein was extracted from BMDMs after treatment, then the protein concentration was adjusted to equal the results of protein quantification. Cell lysates for immunoprecipitation were prepared following Protein A SureBeads Starter Kit manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). After incubating with Tim-3-protein A magnetic beads overnight at 4 °C, the beads were washed and then boiled with loading buffer, transferred to immunoblots, incubated with Tim-3 (1:4000, Cat. No. 11872-1-AP, Proteintech) and Gal-9 (1:2000, Cat. No. 17938-1-AP, Proteintech) antibodies, and then the association between Tim-3 and Gal-9 was quantified. The ratio of Gal-9 (IP) to Tim-3 (IP) represented the binding ability of Tim-3/Gal-9.

2.7. Quantitative real-time PCR (qRT-PCR)

The total RNA was extracted and reverse-transcribed to cDNA in a total volume of 10 μl, in accordance with the manufacturer's instructions (Cat. No. RR036A; TaKaRa, Tokyo, Japan). Fold inductions were calculated using the cycle threshold ΔΔCt method. qPCR was performed at 95 °C (30 s) followed by 40 cycles at 95 °C (5 s)/60 °C (30 s). SYBR green intercalating dye (Cat. No. RR820L; TaKaRa) was used for signal detection. For each sample, the number of cycles required to generate a given threshold signal (Ct) was recorded, and the results are shown as 2-ΔΔCt.

Sequences of the primers used in this study were as follows: GAPDH: 5′-TGGATTTGGACGCATTGGTC-3′ (sense) and 5′-TTTGCACTGGTACG TGTTGAT-3′ (antisense); Gal-9: 5′- ATGCCCTTTGAGCTTTGCTTC-3′ (sense) and 5′- AACTGGACTGGCTGAGAGAAC-3′ (antisense).

2.8. Statistical analysis

All data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for multiple-groups comparisons. GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA) was used for analysis. The difference was statistically significant at $P < 0.05$.

3. Experimental results

3.1. BMDMs polarization after treatment with LPS at multiple time points

We investigated the effects of different stimulation times of LPS treatment on BMDMs polarization. Macrophage marker F4/80, M1 phenotype marker CD86, and M2 phenotype marker CD206 were estimated using flow cytometry, and the M1 phenotype marker iNOS, and M2 phenotype marker Arg-1 were quantified by Western blotting. The percentage of $F4/80^+CD86^+$ cells increased in the 6, 12, and 24 h groups compared to the 0 h group (Fig. 1**a, c**) and iNOS protein expression increased with increasing stimulation times (Fig. 1**e, f**). The percentage of $F4/80^+CD206^+$ cells and the expression of Arg-1 were upregulated after stimulation with 0.1 μg/mL LPS for 1 h and peaked at 3 h; then the percentage of $F4/80^+CD206^+$ cells gradually decreased to the 0 h level. Moreover, the expressions of Arg-1 were significantly downregulated when stimulated with 0.1 μg/mL LPS for 12 h and 24 h (Fig. 1**b, d, e, g**).

3.2. Expressions of Tim-3/Gal-9 in BMDMs at different time after stimulated with LPS and levels of secretory galectin-9 (s-Gal-9) in supernatants of the cultures

To investigate changes in Tim-3/Gal-9 expression in BMDMs after LPS treatment for different times, the expression of Tim-3/Gal-9 was quantified by Western blotting, and s-Gal-9 in supernatants of the cultures of BMDMs was quantified using an enzyme-linked immune sorbent assay (ELISA). Western blotting showed that the expression of Tim-3 was increased when stimulated with 0.1 μg/mL LPS for 1 h and 3 h; peaked at 3 h, however, it was downregulated when stimulated with 0.1 μg/mL LPS for 24 h (Fig. 2**a, b**). The expression of Gal-9 was upregulated when stimulated with 0.1 μg/mL LPS for 1, 3, and 6 h, peaked at 3 h and gradually decreased to the 0 h level (Fig. 2**a, c**). The ELISAs showed that s-Gal-9 levels in supernatants of the cultures of BMDMs were increased in the 1, 3, and 6 h groups compared to the 0 h group, peaked at 3 h, and then gradually decreased to the 0 h level (Fig. 2**d**)

3.3. Binding ability of Tim-3/Gal-9 in BMDMs after treatment with LPS for different times

Whether the interaction of Gal-9 and Tim-3 was associated with M1/M2 polarization in BMDMs was studied. Double-labelling immunofluorescence combined with confocal laser scanning microscopy (CLSM) showed that the interaction between Gal-9 and Tim-3 in BMDMs was regulated by LPS in a time-dependent manner. Using co-localization analyses, Pearson's correlation coefficient, which represented the quantitative degree of co-localization, was increased in the 1, 3, and 6 h groups and peaked in the 3 h group, while it decreased in the 12 and 24 h groups (Fig. 3**a, b, d**). Similarly, co-immunoprecipitation (co-IP) showed that Tim-3/Gal-9 binding increased in the 3 and 6 h groups, while it decreased in the 24 h group **(**Fig. 3**c, e)**.

3.4. LPS-induced BMDMs M1/M2 polarization depends on the Tim-3/Gal-9 pathways

We used α-lactose (40 μM), an inhibitor of Gal-9 to evaluate the effects of Gal-9 signalling on BMDMs polarization induced by LPS. We chose to use 0.1 μg/mL LPS treatment for 3 h because this treatment resulted in the lowest percentage of M1 macrophages and the highest Gal-9 expression. In contrast with the basal level of LPS treatment, the percentage of M1 phenotype macrophages upon being stimulated with LPS (0.1 μg/mL) + α-lactose (40 μM) for 3 h was significantly increased (Fig. 4**a, c**), while the percentage of M2 phenotype macrophages was significantly decreased (Fig. 4**b, d**). Next, we used anti-mouse Tim-3 antibody (Tim-3 mAb, 10 μg/mL), a function blocking agent of Tim-3, to evaluate the Tim-3 inhibition on BMDMs polarization induced by LPS. We chose to use 0.1 μg/mL LPS treatment for 3 h because this treatment resulted in the most Tim-3 expression. In contrast to the basal level of LPS treatment, the percentage of M1 phenotype macrophages upon being stimulated with LPS (0.1 μg/mL) + Tim-3 mAb (10 μg/mL) for 3 h was significantly increased (Fig. 4**a, c**); however, the percentage of M2 phenotype macrophages was significantly decreased (Fig. 4**b, d**). The iNOS and Arg-1 protein expressions reflected similar trends as the results of flow cytometry (Fig. 4**e, f**).

Western blotting and ELISA revealed that LPS + α-lactose and LPS + Tim-3 mAb treatments all downregulated Gal-9 and Tim-3 protein expression (Fig. 5**a-c**), and s-Gal-9 secretion (Fig. 5**d**) compared to LPS alone treatment. CLSM co-localization analyses (Fig. 6**a, b, d**) and a co-IP assay (Fig. 6**c, e**) revealed that both LPS + α-lactose treatment and LPS + Tim-3 mAb treatment suppressed the Tim-3/Gal-9 binding increased by LPS. Taken together, these results suggest that the Tim-3/Gal-9 pathways mediated time-dependent BMDMs polarization induced

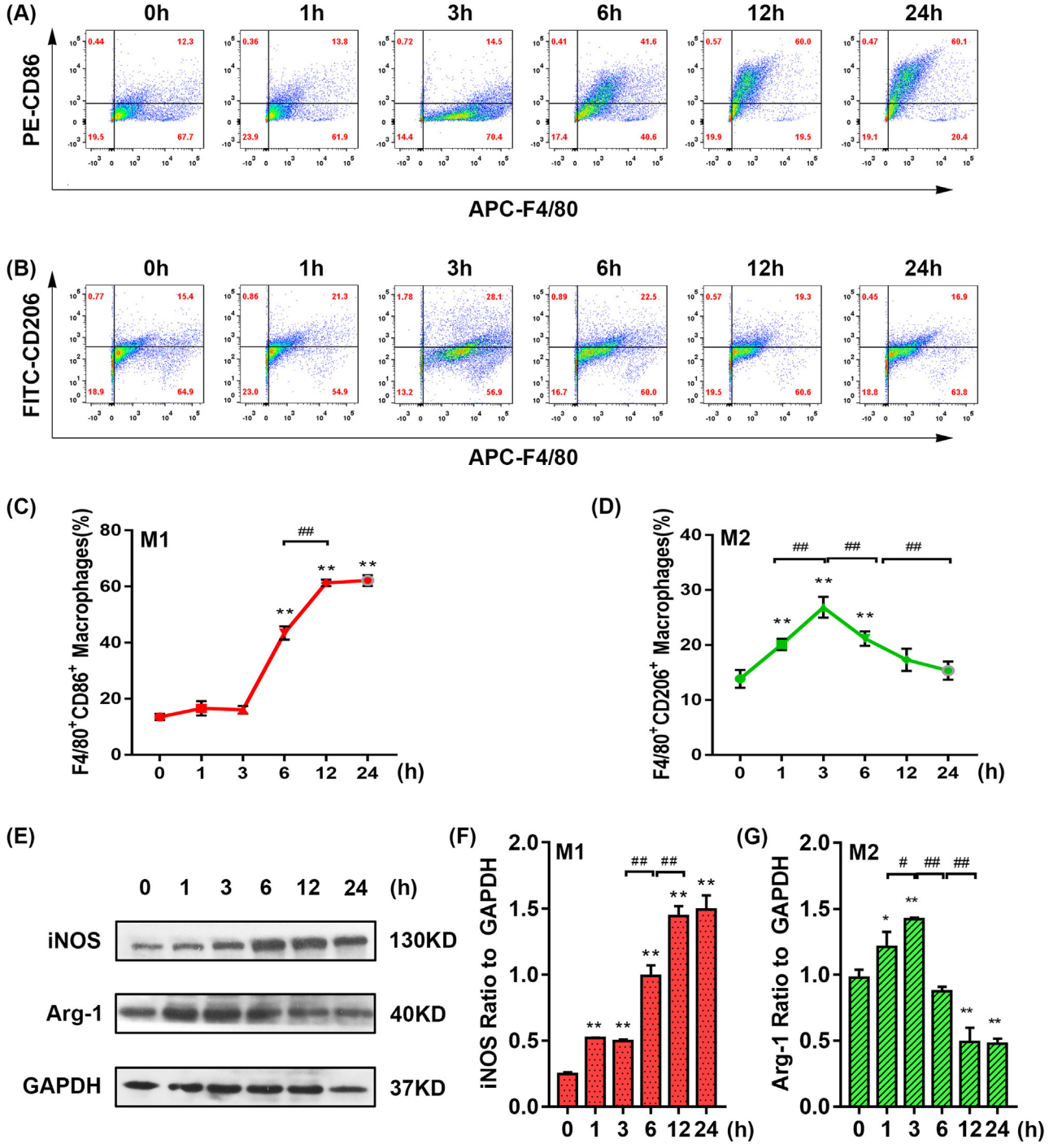

Fig. 1. Bone marrow-derived macrophage polarization after treatment with lipopolysaccharide at multiple time points. (**a, b**) The expressions of macrophage marker F4/80, M1 phenotype marker CD86, and M2 phenotype marker CD206 were measured using flow cytometry. (**c**) The percentage of M1 subtype macrophages ($F4/80^+$ $CD86^+$) was increased in the 6, 12, and 24 h groups compared to the 0 h group (all, $P < 0.01$). (**d**) The percentage of M2 subtype macrophages ($F4/80^+$ $CD206^+$) were increased in the 1, 3, and 6 h groups compared to the 0 h group (all, $P < 0.01$), peaked at 3 h and gradually decreased to the 0 h level. (**e**) The M1 phenotype marker iNOS and M2 phenotype marker Arg-1 were measured using Western blotting. (**f, g**) The expression of iNOS protein increased with increasing stimulation time compared to that in the 0 h group (all, $P < 0.01$). Arg-1 protein expression was upregulated in the 1 h ($P < 0.05$) and 3 h ($P < 0.01$) groups, and downregulated in the 12 and 24 h groups (all, $P < 0.01$). Data are from three independent experiments, error bars represent the standard deviation, $^{*}P < 0.05$ vs. control group, $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

by LPS.

3.5. Tim-3 is involved in LPS-induced Galectin-9 autocrine by macrophages

We speculated that Gal-9 was also regulated by its autocrine function in macrophages, which might be mediated by the Tim-3 signalling. We used 40 μM α-lactose and 10 μg/mL Tim-3 mAb to pre-treat the BMDMs, and then recombinant mouse Gal-9 (rm-Gal-9; 5 μg/mL) was used to stimulate BMDMs for 3 h, replaced medium with fresh medium and cultured for another 3 h. mRNA levels of Gal-9 and s-Gal-9 in supernatants of the cultures were upregulated with rm-Gal-9 stimulation, while α-lactose and Tim-3 mAb abrogated the increased mRNA levels

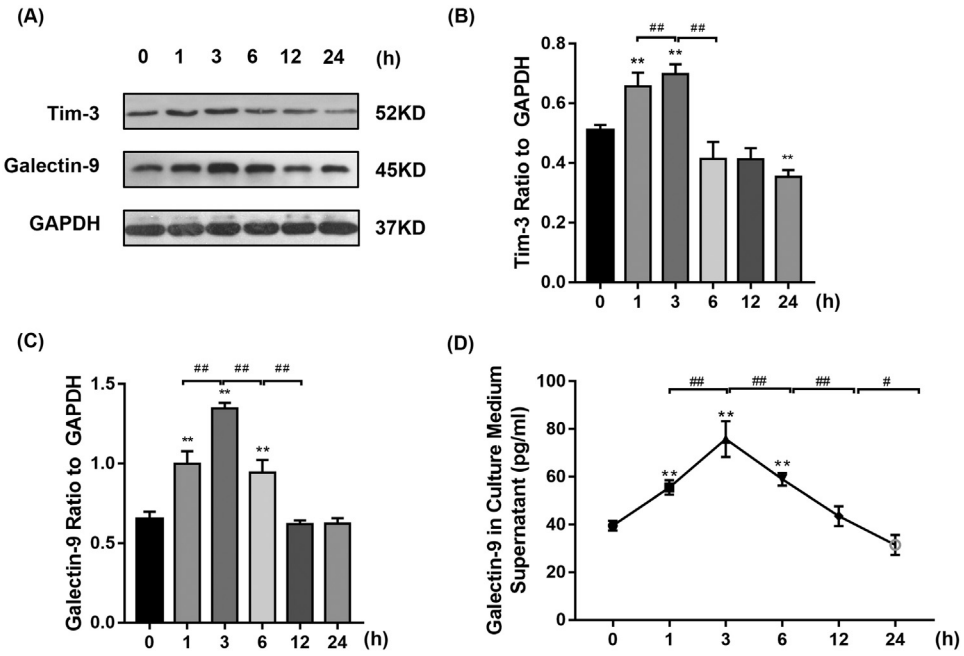

Fig. 2. The expressions of Tim-3, Gal-9 and secretory galectin-9 (s-Gal-9) in supernatants of the cultures of bone marrow-derived macrophages treated with lipopolysaccharide (LPS) for different times. (**a**) Expressions of Tim-3 and Gal-9 were evaluated by Western blotting. The relative protein levels are expressed as the ratio of the target protein to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), results are representative of three independent protein preparations. (**b, c**) The expression of Tim-3 were increased when stimulated with 0.1 μg/mL LPS for 1 h and 3 h, peaked in the 3 h group and decreased in 24 h group (all, $P < 0.01$). The levels of Gal-9 were remarkably upregulated in the 1, 3, and 6 h groups, and peaked in the 3 h group (all, $P < 0.01$). (**d**) The levels of s-Gal-9 in supernatants of the cultures were increased in the 1, 3, and 6 h groups (all, $P < 0.01$), peaked at 3 h and gradually decreased to the 0 h level. While there were no significant differences between 12, 24, and 0 h groups. Data are from three independent experiments.

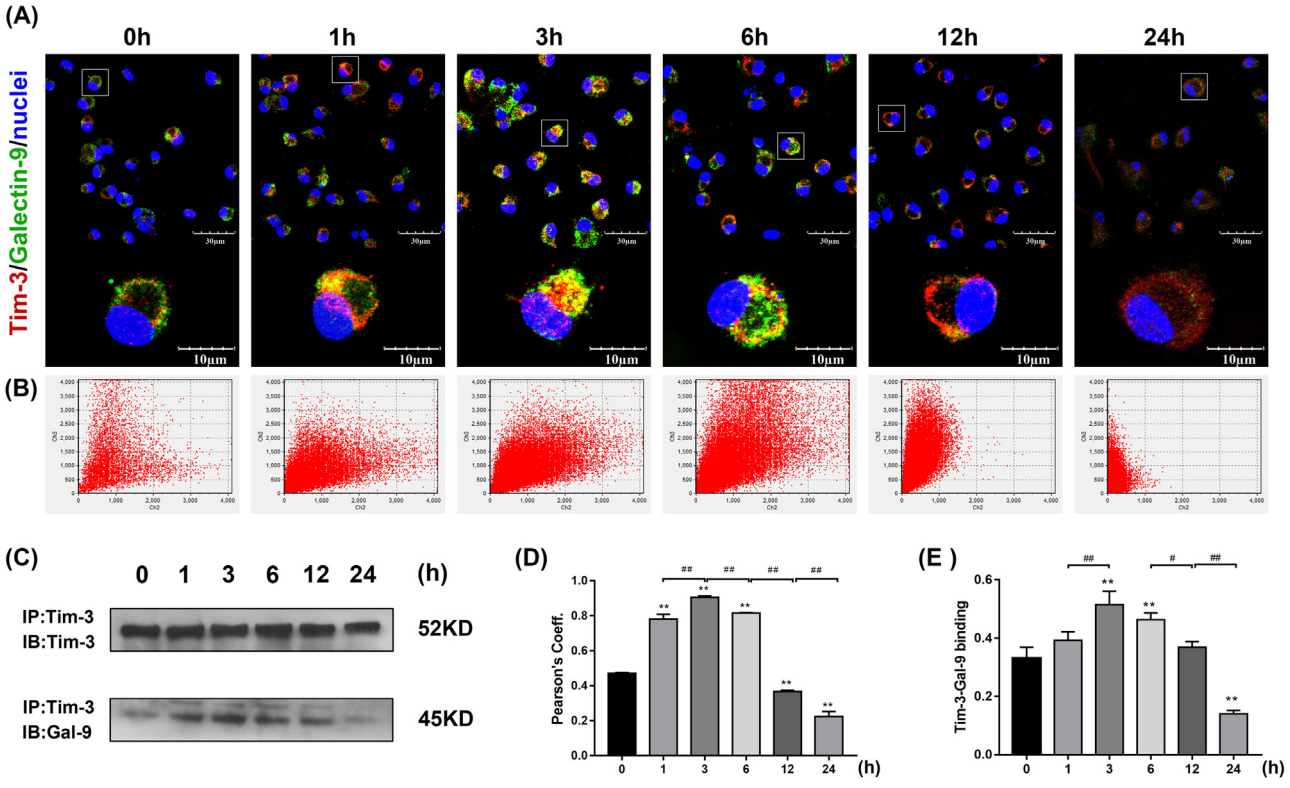

Fig. 3. The effects of different time stimulations of lipopolysaccharide (LPS) on Tim-3/Gal-9 binding ability. (**a**) Detection of Tim-3/Gal-9 co-localization in bone marrow-derived macrophages treated with 0.1 μg/mL LPS for 1, 3, 6, 12, and 24 h using confocal microscopy. In a typical merged image, green represents Gal-9, red represents Tim-3, blue represents the nuclei, and yellow represents Tim-3/Gal-9 co-localization. Amplified images are shown below. (**b**) The two-dimensional scatter diagram representing pixel greyscale information for each channel was obtained using Olympus FV-1000 co-localization analysis software. (**d**) Pearson's coefficient calculated using co-localization analysis software showed that co-localization was increased in the 1, 3, and 6 h groups (all, $P < 0.01$), and was decreased in the 12 and 24 h groups compared to the 0 h group (all, $P < 0.01$). (**c, e**) Detection of the physical association between Gal-9 and Tim-3 by immunoprecipitation. Tim-3/Gal-9 binding was remarkably increased in the 3 and 6 h groups while it was decreased in the 24 h group compared to the 0 h group (all, $P < 0.01$). Scale bar: 30 μm (normal image) and 10 μm (amplified image). Data are from three independent experiments.

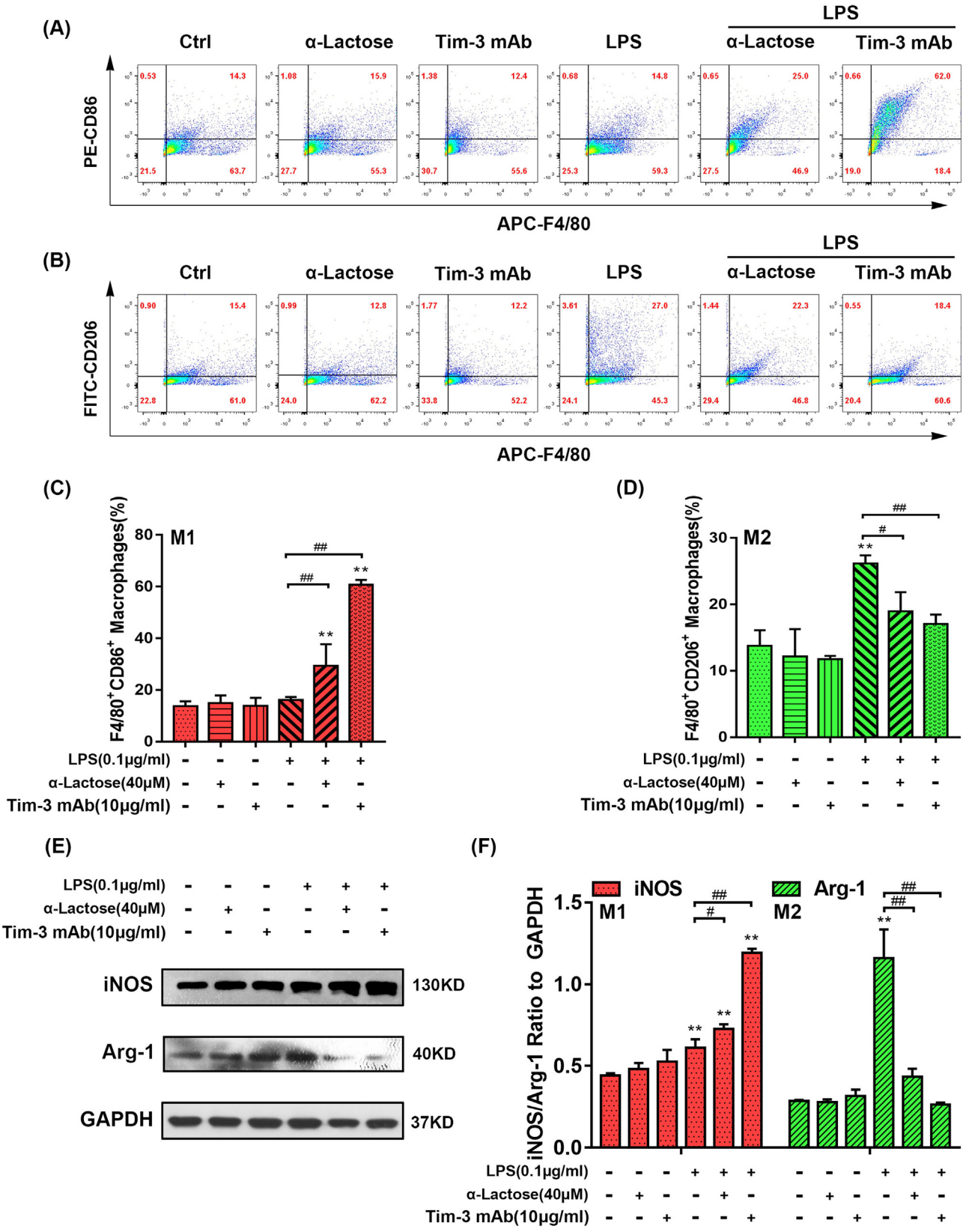

Fig. 4. LPS-induced bone marrow-derived macrophage polarization after Gal-9 and Tim-3 inhibition. (**a, b**) The expressions of macrophage marker F4/80, M1 phenotype marker CD86, and M2 phenotype marker CD206 were measured using flow cytometry. (**c**) The percentage of M1 subtype macrophages (F4/80$^+$ CD86$^+$) were increased in the LPS + α-Lactose (40 μM) group and the LPS + Tim-3 mAb (10 μg/mL) group compared to the LPS group (all, $P < 0.01$). (**d**) The percentage of M2 subtype macrophages (F4/80$^+$CD206$^+$) were remarkably decreased in the LPS + α-Lactose group ($P < 0.05$) and the LPS + Tim-3 mAb group ($P < 0.01$) compared to the LPS group. (**e**) The M1 phenotype marker iNOS and M2 phenotype marker Arg-1 were measured using Western blotting. (**f**) The expression of iNOS protein increased in the LPS + α-Lactose group ($P < 0.05$) and the LPS + Tim-3 mAb group ($P < 0.01$) compared to the LPS group. Arg-1 protein expression was significantly decreased in the LPS + α-Lactose group and the LPS + Tim-3 mAb group compared to the LPS group (all, $P < 0.01$). There is no significant difference between control group, α-Lactose group and Tim-3 mAb group. Data are from three independent experiments.

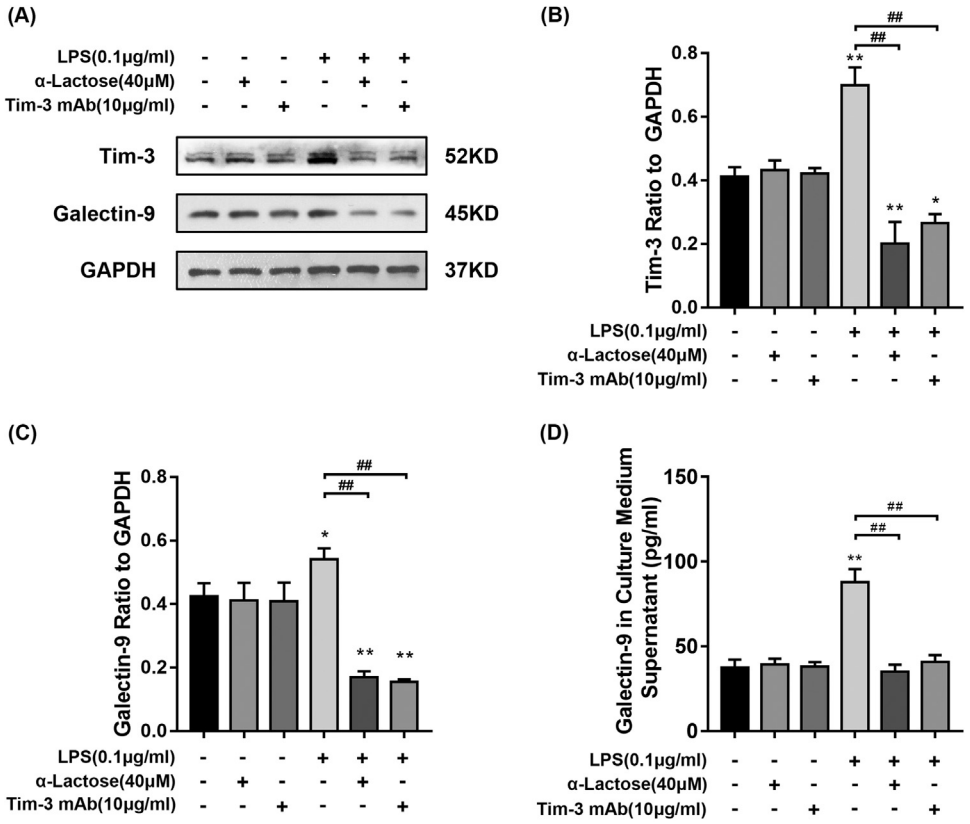

Fig. 5. The expressions of Tim-3, Gal-9 and secretory galectin-9 (s-Gal-9) in supernatants of the cultures of LPS induced bone marrow-derived macrophages after Gal-9 and Tim- 3 inhibition. (**a**) Expressions of Tim-3 and Gal-9 were evaluated by Western blotting. (**b**) The levels of Tim-3 were significantly down-regulated in the LPS + α-Lactose group ($P < 0.01$) and the LPS + Tim-3 mAb group ($P < 0.01$) compared to the LPS group. (**c**) The levels of Gal-9 were significantly down-regulated in the LPS + α-Lactose group ($P < 0.01$) and the LPS + Tim-3 mAb group ($P < 0.01$) compared to the LPS group. (**d**) The levels of s-Gal-9 in supernatants of the cultures were decreased in the LPS + α-Lactose group and the LPS + Tim-3 mAb group compared to the LPS group (all, $P < 0.01$). Results are representative of three independent protein preparations. There is no significant difference between control group, α-Lactose group and Tim-3 mAb group.

and protein secretion (Fig. 6**f, g**).

4. Discussion

When the body is infected with pathogenic microorganisms, circulating monocytes are recruited and differentiate into macrophages and maintain homeostasis with resident macrophages in tissues [1]. During the early stages of bacterial infection, macrophages are generally polarized to the M1 phenotype. When the pathogen-associated molecular pattern is present in bacteria, it is recognized by pathogen recognition receptors, for instance, Toll-like receptors, macrophages are activated and secrete proinflammatory cytokines, including iNOS and TNF-α, which can clear pathogens and promote adaptive immune responses [19]. Continuously activated neutrophils and macrophages/monocytes usually produce large amounts of cytokines, known as a "cytokine storm," which causes more harm than good, especially in the lungs, such as during ARDS [23]. A previous study showed that, with the persistent upregulation of MCP-1, alveolar macrophages in ARDS patients are polarized to the M1 phenotype, which is significantly correlated with the severity of the disease [24]. Another research of *Pseudomonas aeruginosa* infection induced acute lung injury gave a discovery that inflammatory macrophages M2 polarization may be related to the resolution phase of ALI, and the failure of the M1 to M2 phenotype transition may contribute to the pathogenesis of non-resolving ALI, it indicates that the evolution of distinct macrophage phenotype in the lung can regulate the inflammatory response and its resolution [25]. A novel finding of the current study was the demonstration of a biphasic effect of LPS; we found that M1/M2 polarization in BMDMs induced by LPS was time-dependent. Flow cytometry and Western blotting showed that M2 phenotype surface markers were significantly upregulated in the early stages after LPS stimulation; however, they were significantly downregulated in the late stages after LPS stimulation. Furthermore, M1 phenotype surface markers were persistently upregulated over time, but only slightly upregulated in the early stages after LPS stimulation. Overall, short-term LPS stimulation promoted macrophage M2 polarization, which may have played a role in the ability of self-repair and homeostasis regulation.

Tim-3 has great effects on regulating macrophages activity and preventing immune-associated injury. It is generally accepted that this receptor regulates macrophage functions by binding extracellular ligands, either soluble or presented by other cells, via a trans association [26]. And Gal-9, a kind of β-galactoside lectin, is involved in many processes of immune diseases such as autoimmune diseases, asthma, and immunomodulation of macrophages [16]. Gal-9 is a Tim-3 ligand, which can phosphorylate tyrosine residues at the tail of Tim-3. Furthermore, a peptide derived from Tim-3 can interact with an SH2 domain of the p85 adaptor subunit of phosphatidylinositol 3-kinase (PI3K), which works on immune pleiotropism [27,28]. A previous study showed that an extracellular Gal-9 interaction with Tim-3 on monocytes/macrophages affects inflammatory cytokine production [26]. We evaluated the amount of Tim-3/Gal-9 in BMDMs treated with LPS, and found a time-dependent change in binding ability of Tim-3/Gal-9, which increased in the early stages after LPS stimulation, but decreased in the late stages after this stimulation. A recent research found that overexpression and secretion of Gal-9 are closely related to macrophages M2 polarization, whereas its downregulation promotes macrophages M1 polarization [29]. Our results are consistent with those findings, as we found that differentiation of BMDMs into M1 and M2 macrophage phenotypes was followed by marked variation in Tim-3/Gal-9 expression and secretion, which was upregulated when M2 phenotype macrophages increased but downregulated when M2 phenotype macrophages decreased and M1 phenotype macrophages increased. We also found that both α-lactose (inhibitor of Gal-9) and Tim-3 mAb (Tim-3 function blocking agent) decreased the percentages of M2 phenotype but increased the percentages of M1 phenotype of BMDMs with short-term LPS stimulation, which reveals that Tim-3/Gal-9 signalling is associated with M1/M2 polarization in BMDMs.

Tim-3-galectin-9 autocrine loop as a biosynthetic and secretory

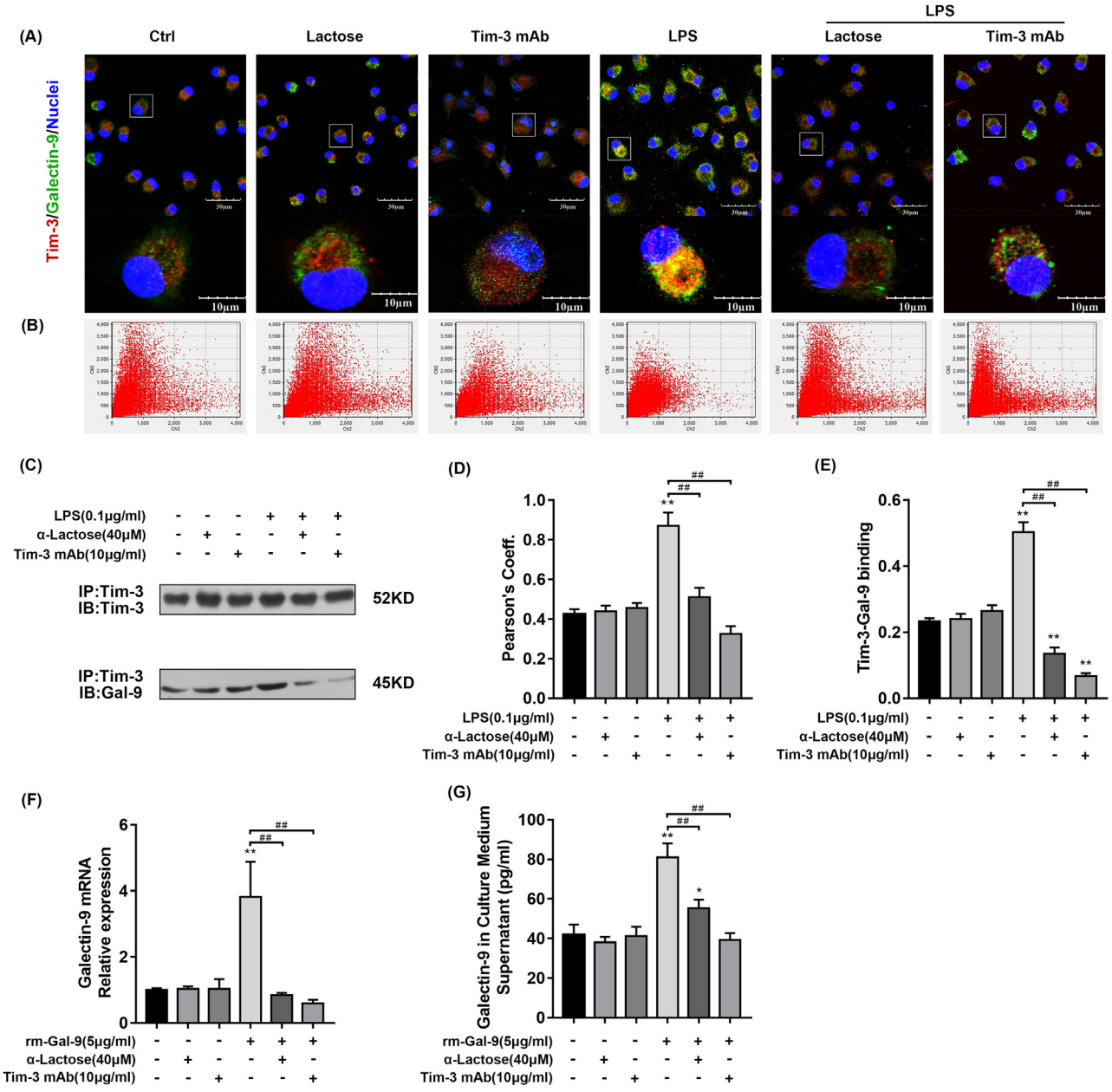

Fig. 6. The effects of Tim-3/Gal-9 inhibition on Tim-3/Gal-9 binding ability induced by LPS and the autocrine function of Gal-9 stimulated by recombination mouse Gal-9(rm-Gal-9). Detection of Tim-3/Gal-9 co-localization in bone marrow-derived macrophages using confocal microscopy. In a typical merged image, green represents Gal-9, red represents Tim-3, blue represents the nuclei, and yellow represents Tim-3/Gal-9 co-localization. Amplified images are shown below. (**b**) The two-dimensional scatter diagram representing pixel greyscale information for each channel was obtained using Olympus FV-1000 co-localization analysis software. (**d**) Pearson's coefficient calculated using co-localization analysis software showed that co-localization was decreased in the LPS + α-Lactose group and the LPS + Tim-3 mAb group compared to the LPS group (all, $P < 0.01$). (**c, e**) Detection of the physical association between Gal-9 and Tim-3 by immunoprecipitation. Tim-3/Gal-9 binding was remarkably decreased in the LPS + α-Lactose group and the LPS + Tim-3 mAb group compared to the LPS group (all, $P < 0.01$). (**f, g**) Levels of Gal-9 mRNA and s-Gal-9 protein in supernatants of the cultures were significantly upregulated after rm-Gal-9 stimulation compared to the control group (all, $P < 0.01$) and downregulated in the rm-Gal-9 + α-lactose and rm-Gal-9 + Tim-3 mAb groups compared to the rm-Gal-9 group (all, $P < 0.01$). There were no significant differences between the control, α-lactose, and Tim-3 mAb groups. Data are from three independent experiments, scale bar: 30 μm (normal image) and 10 μm (amplified image).

pathway was found in human acute myelocytic leukaemia (AML) cells, which was related to a decrease of immune surveillance and promotion of disease progression [30]. Tim-3 and Gal-9 autocrine loop is also critical for stem cell self-renewal and development in human AML [31]. We speculated that the Tim-3 and Gal-9 autocrine loop also exist in macrophages and mediates the regulation of M1/M2 polarization, so we detected intracellular Gal-9 expression and secretion in supernatants of the cultures, we subsequently found that rm-Gal-9 upregulated mRNA levels and protein secretion of Gal-9, and that α-lactose/Tim-3 mAb abrogated the increased Gal-9 mRNA levels in BMDMs and s-Gal-9 levels in supernatants of the cultures, suggesting that Gal-9 autocrine regulation by LPS is involved in macrophage polarization. Therefore, Tim-3-galectin-9 autocrine loop will be a potential therapeutic target for sepsis and its complications in the future.

In addition, it's reported that the PI3K/Akt pathway activated by Tim-3 signalling suppresses Th1 by inhibiting NF-kB activation [32], and the cascade of the MAPK and NF-kB responses to LPS is suppressed by the activation of the PI3K pathway in monocytes, it leads to the

reduction of TNF-α [33], which also demonstrate a negative regulatory part for Tim-3 signalling that reflects new adaptive compensatory and protective mechanisms in sepsis victims [34]. In further studies, we will investigate the downstream effectors of Tim-3/Gal-9 and its interaction, we will also detect the effects of the Tim-3/Gal-9 signalling pathway on the pulmonary macrophage function of the sepsis model *in vivo*.

5. Conclusion

Based on our findings, we conclude that Tim-3/Gal-9 interactions regulate macrophage polarization, and in the early phase of LPS stimulation, upregulate Tim-3/Gal-9 interaction and activate Tim-3 signalling pathway, eventually inhibiting M1 polarization and promoting Gal-9 autocrine functions. However, in the later phase of LPS stimulation, downregulated Tim-3/Gal-9 interaction and inhibited Tim-3 signalling pathway, eventually promoting M1 polarization, with decreases in M2 polarization and Gal-9 autocrine functions.

Author contributions

WZ and YTZ wrote the main manuscript text, YXH, XYW and QF participated in part of the experiments and analysed the data.

Conflict of interest

The authors declare no conflicts of interest with the contents of this article

Ethical statement

All animal studies were approved by the Animal Care and Use Committee of Zhejiang University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Informed consent was obtained from all participants in accordance with the guidelines of the Human Subjects Committee of the Medical Ethical Commission of the First Affiliated Hospital of Zhejiang University (China) and the declaration of Helsinki.

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