MDSCs participate in the pathogenesis of diffuse pulmonary hemorrhage in murine lupus through mTOR-FoxO1 signaling

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ABSTRACT

Diffuse pulmonary hemorrhage (DPH) is a common respiratory complication in patients with systemic lupus erythematosus (SLE). Our previous study found that myeloid-derived suppressor cells (MDSCs) have an important role in SLE pathogenesis. In this study, we further examined the role of MDSCs in the DPH mice model. We first observed an increased proportion of MDSCs and impaired immunosuppressive function in bronchoalveolar lavage fluid (BALF) and peritoneal cavity in the DPH mice model induced by pristane. By injecting anti-Gr-1 antibody, we found that MDSCs clearance can significantly alleviate DPH symptoms. The detection of downstream molecules proved that the mTOR signaling pathway was obviously activated in purified DPH MDSCs. After treatment of DPH model mice with AMPK agonist metformin, mammalian target of rapamycin (mTOR) inhibitor INK128, and rapamycin, respectively, we observed that inhibition of the mTOR signal alleviated DPH symptoms, inhibited the expansion of mononuclear MDSCs (M-MDSCs) and the differentiation of proinflammatory M1 macrophages (M1), which, in turn, promoted the expansion of granulocytes MDSCs (G-MDSCs) and differentiation of anti-inflammatory M2 macrophages (M2). We then demonstrated that inhibition of the mTOR signal increased the expansion of G-MDSCs, promoted M-MDSCs differentiation into M2 and inhibited their differentiation into M1 by administering TLR7 agonist R848 in vitro to simulate lupus environment. In addition, we also observed increased forkhead box-O1 (FoxO1) expression in M-MDSCs and macrophages after mTOR signal inhibition, both in vivo and in vitro. After down-regulation of FoxO1 by siRNA transfection, the regulatory effects of mTOR signal inhibition on M-MDSCs, M1 and M2 were reversed. Taken together, inhibition of the AMPK/mTOR signal could alleviate lupus-like diffuse lung injury by inducing M-MDSCs to differentiate into M2 by up-regulating FoxO1.

1. Introduction

Diffuse pulmonary hemorrhage (DPH) is a rare condition that refers to bleeding into the alveolar space, which results from alveolar microcirculation injury. DPH complicating systemic lupus erythematosus (SLE) remains an overwhelming pulmonary complication of this systemic disease [1], which typically presents with pulmonary capillary vasculitis, extravascular neutrophil interstitial infiltration, inflammation, and necrosis of the alveolar and capillary walls, leading to lung basement membranes damage and leakage of erythrocytes into the alveolar space [2]. Methylprednisolone, cyclophosphamide, and plasmapheresis are frequently used to treat DPH, in addition to rituximab, intrapulmonary rFVIIa, and other methods [2]. Rituximab, a B cell-targeted therapy, can improve the DPH in lupus patients [3]. In the murine lupus model, liver X receptor agonist has been shown to be effective for preventing DPH [4]. Nevertheless, the current treatment options for DPH are limited, and the prognosis is still poor [5,6].

Abbreviations

DPH	Diffuse pulmonary hemorrhage
SLE	Systemic lupus erythematosus
MDSCs	Myeloid-derived suppressor cells
G-MDSCs	granulocytes MDSCs
M-MDSCs	Mononuclear MDSCs
Arg-1	Arginase-1
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
AMPK	AMP-activated protein kinase
FoxO1	Forkhead box-O1
PBMCs	Peripheral blood mononuclear cell
M1	M1 macrophages
M2	M2 macrophages
CFSE	Carboxy fluorescein succinimidyl ester
DMSO	Dimethyl sulfoxide
BALF	Bronchoalveolar lavage fluid
H&E	Hematoxylin and eosin
ROS	Reactive oxygen species

Myeloid-derived suppressor cells (MDSCs) is a heterogeneous cell population consisting of two major groups of cells: granulocytes MDSCs (G-MDSCs) and mononuclear MDSCs (M-MDSCs) [7]. Compared with neutrophils and monocytes, during cancer pathologic conditions, MDSCs are characterized by an immunosuppressive function, which increases the production of reactive oxygen species (ROS) and nitric oxide and the expression of arginase-1 (Arg-1) and prostaglandin E2 [7]. MDSCs have an important role in the development and progression of SLE. MDSCs promote the differentiation of Th17 by secreting Arg-1, promote TH17 differentiation and disease progression in lupus [8]. Moreover, previous studies have shown that the proportion of M-MDSCs is significantly increased in SLE patients, and is positively correlated with disease severity [9]. G-MDSCs could significantly contribute to IFN-I signaling activation of B cells and promote SLE pathogenesis in MRL/lpr mice [10]. In contrast to G-MDSCs, which are non-proliferating cells with a short half-life, M-MDSCs can differentiate into dendritic cells and macrophages in response to environmental stimuli [11,12]. The effect of MDSCs on DPH pathology still remains unclear.

The mammalian target of rapamycin (mTOR) is a serine/threonine-protein kinase in the PI3K-related kinase family, which interacts with several proteins to form two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2) [13,14]. AMP-activated protein kinase (AMPK) is a highly conserved energy homeostasis regulator located upstream of mTOR. Its activation downregulates the mTOR signaling pathway [15]. An increasing number of studies have indicated the involvement of mTOR in SLE. Studies have shown that mTOR activation triggers the expansion and necrosis of $CD4^-CD8^-$ double-negative T cells in SLE [16–18], leads to the loss of TCR in lupus T cells through HRES-1/Rab4-dependent lysosomal degradation [19]. In lupus mice, the activation of the mTOR pathway can inhibit the autophagy and function of Treg cells [20,21]. Simultaneously, the research shows that rapamycin can reduce the renal interstitial infiltration by T cells in mice with lupus nephritis [22–24]. mTOR also affects the pathogenesis of SLE through B cells. mTORC1 could induce SLE by mediating B cell activation and increasing the production of antibodies [25]. The regulatory properties of metformin on AMPK–mTOR–STAT3 signaling inhibited B cell differentiation into plasma cells and spontaneous germinal center formation [26]. B cells may promote the differentiation of Th22 cells through mTOR and TNF-α, thus having a therapeutic role in lupus mice [27]. The aforementioned studies have all focused on adaptive immune cells. Although it has been recognized that mTOR can regulate the

differentiation of MDSCs and its immunosuppressive function [28–32], our laboratory first associated this regulatory mechanism with lupus and found that IRF-8/mir-451a regulates M-MDSCs differentiation through AMPK/mTOR signaling pathway during the development of lupus [33]. It is unknown whether mTOR regulates MDSCs in DPH pathology.

In this study, we examined the role of MDSCs in lupus-like DPH and investigated whether MDSCs are involved in DPH through the mTOR inhibition. Firstly, the proportion and functional changes of MDSCs were detected in the murine lupus DPH model induced by pristane, and then the pathological effects of MDSCs were analyzed by depleting MDSCs with anti-Gr-1 antibody injection. Based on downstream molecular detection, we discussed the importance of the AMPK/mTOR signaling pathway in DPH. Conversely, mTOR pathway inhibitors (INK128, rapamycin and metformin) significantly improved DPH by decreasing M-MDSCs and M1 macrophages (M1), and increasing G-MDSCs and M2. Through the in vitro experiments, we corroborated that forkhead box-O1 (FoxO1) could be the key molecule. Taken together, our results indicated that inhibition of AMPK/mTOR signaling could induce M-MDSCs to differentiate into M2 by upregulating FoxO1, thereby attenuating lupus-like diffuse lung injury.

2. Materials and methods

2.1. Antibodies and reagents

The following antibodies were used for all analyses: CD45-PerCP-Cyanine 5.5 (Cat. No. 103132, clone 30-F11), CD11b-PE/Dazzle 594 (Cat. No. 101256, clone M1/70) or FITC (Cat. No. 101206, clone M1/70), Gr-1-Alexa Fluor 647 (Cat. No. 108418, clone RB6-8C5), Ly-6C-APC (Cat. No. 128015, clone HK1.4) or PE (Cat. No. 128008, clone HK1.4), F4/80-Alexa Fluor 488 (Cat. No. 123120, clone BM8.1), CD86-PE/Cy7 (Cat. No. 105116, clone PO3), CD206-PE (Cat. No. B243217, clone C068C2), Siglec-F-PE/Dazzle 594 (Cat. No. 155529, clone S17007L), CD11c-APC (Cat. No. 117310, clone N418) and CD4-APC (Cat. No. 100412, clone GK1.5) (all from Biolegend), FoxO1-Alexa Fluor 532 (Cat. No. NBP2-31376AF532, clone 83N7F8) or Alexa Fluor 488 (Cat. No. NBP2-31376AF488, clone 83N7F8) (from Novus Biologicals). Zombie NIR (Cat. No. 423106) was from Biolegend. INK128 (Cat. No. S2811), Metformin (Cat. No. S5958) and Rapamycin (Cat. No. S1039) were from Selleckchem (Houston, TX). GM-CSF cytokine (Cat. No. 130-095-739), IL-6 cytokine (Cat. No. 130-096-685), FcR blocking reagent (Cat. No. 130-092-575) and MDSC Isolation Kit (Cat. No. 130-094-538) were from Miltenyi Biotec. Antibodies for p-S6 (Cat. No. 4858S), S6 (Cat. No. 2217S), p-4EBP-1 (Cat. No. 2855S), and 4EBP-1 (Cat. No. 9644T), and horseradish peroxides (HRP)-conjugated anti-rabbit IgG (Cat. No. 7074P2) for Western blot were from Cell Signaling Technology (Danvers, MA, USA). Fixation/Permeabilization Diluent (Cat. No. 00-5521-00), carboxy fluorescein succinimidyl ester (CFSE, Cat. No. 65-0850-84), mouse CD3 functional antibody (Cat. No. 16-0031-86) and mouse CD28 functional antibody (Cat. No. 16-0281-86) were from eBioscience. FoxO1 siRNA (Cat. No. siB160412024858-1-5) was from Guangzhou Ruibo. RFect^{SP} small nucleic acid transfection reagent (Cat. No. 11025) was from Changzhou EMI. Roswell Park Memorial Institute (RPMI) 1640 medium (Cat. No. C3010-0500) were obtained from VivaCell Biosciences. Fetal bovine serum (FBS, Cat. No. 10099141) was purchased from Gibco (Grand Island, NY, USA).

2.2. Animal models

Female C57BL/6 mice (6–8 weeks old) were obtained from the Animal Research Center of Nanjing University (Nanjing, China). All the animals were housed in an environment with a temperature of 22 ± 1 °C, relative humidity of 50 ± 1%, and a light/dark cycle of 12/12 h. The experiments on mice were approved by Institutional Animal Care and Use Committee (IACUC–2003149), Nanjing University, and all experiments were performed in accordance with relevant guidelines and

regulations. This study was carried out in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

A total of 33 female C57BL/6 mice aged 6–8 weeks that weighted 20 ± 2 g were randomly divided into 3 groups (11 mice per groups): Wild-type group (WT group), Control group (treated with DPH) and GR-1 mAb group (treated with DPH and GR-1 mAb). Mice received a single intraperitoneal injection of 0.5 ml pristane to induce DPH. Then, the DPH model received treatment with Gr-1 neutralizing antibody every three days.

A total of 55 female C57BL/6 mice aged 6–8 weeks that weighted 20 ± 2 g were randomly divided into 5 groups (11 mice per groups), namely, (1) Dimethyl sulfoxide (DMSO) group, (2) DPH group, (3) INK128 group, (3) rapamycin group and (4) metformin group. Mice received a single intraperitoneal injection of 0.5 ml pristane to induce DPH. Then, the DPH model received daily i.p. treatment with INK128, metformin, or rapamycin (1 mg/kg prepared in DMSO) or DMSO for 2 weeks. Subsequently, lungs, spleens, peritoneal cells and bronchoalveolar lavage fluid (BALF) cells were harvested.

2.3. Pathological analysis

The lung tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and tissue sections were stained with hematoxylin and eosin (H&E) for histological analysis.

The mice were divided into three groups according to the severity of DPH: non-DPH (no inflammatory cell infiltration, basement membranes destruction, and leakage of erythrocytes); partial DPH (partial inflammatory cell infiltration, basement membranes destruction, and leakage of erythrocytes), and complete DPH (a large number of inflammatory cell infiltration, basement membranes destruction and leakage of erythrocytes).

2.4. Flow cytometry

Mice were asphyxiated with CO_2 under anesthesia. The peritoneal cavity was lavaged with 3 ml cold, sterile PBS, and the intraperitoneal fluid was harvested. Peritoneal cells were collected by centrifugation at 300g for 10 min. Mouse lungs were lavaged by repeated instillations with PBS through the tracheal cannula in order to BALF collection. The cell suspensions were filtered through 70 μm cell strainers, and the cells were collected by centrifugation at 300g for 5 min at 4 °C. Red blood cells were then lysed with lysis buffer for 2 min, and the remaining cells were washed twice with PBS. After washing, the cells were immediately prepared for flow cytometry (CYTEK^{TM} NL-2000).

Cells were treated with FcR blocking reagent for 10 min and then stained with viability dye Zombie NIR for 15 min in the dark. MDSCs were defined as $CD45^+CD11b^+Gr-1^+$. M-MDSCs were defined as $CD45^+CD11b^+Ly6C^{hi}Gr-1^{lo}$. G-MDSCs were defined as $CD45^+CD11b^+Gr-1^{hi}Ly6C^{lo}$. Macrophages from BALF were defined as $CD45^+CD11b^{lo}CD11c^+Siglec-F^+$. M1 from BALF were defined as $CD45^+CD11b^{lo}CD11c^+Siglec-F^+CD86^+$. M2 from BALF were defined as $CD45^+CD11b^{lo}CD11c^+Siglec-F^+CD206^+$. Macrophages from peritoneal cavity were defined as $CD45^+CD11b^+F4/80^+$. M1 from peritoneal cavity were defined as $CD45^+CD11b^+F4/80^+CD86^+$. M2 from peritoneal cavity were defined as $CD45^+CD11b^+F4/80^+CD206^+$. CD45-PerCP-Cyanine 5.5, CD11b-PE/Dazzle 594 or FITC, Gr-1-Alexa Fluor 647, Ly6C-APC or PE, F4/80-Alexa Fluor 488, CD86-PE/Cy7, CD11c-APC, Siglec-F-PE/Dazzle 594 were incubated at 4 °C for 30 min in the dark. After treatment with Fixation/Permeabilization solution for 55 min, CD206-PE and FoxO1-Alexa Fluor 532 or Alexa Fluor 488 were incubated at 4 °C for 30 min in the dark. After washing with buffer, cells were analyzed by flow cytometry (CYTEK^{TM} NL-2000). All data were analyzed with FlowJo software (FlowJo, Ashland, OR).

2.5. MDSC isolation

MDSCs were isolated from spleens of DPH or control mice using a Myeloid-Derived Suppressor Cell Isolation kit (Miltenyi Biotech, Germany) according to the manufacturer's instructions. The mouse spleen was ground and filtered to obtain a single cell suspension. After erythrocyte lysate, 1×10^8 mouse spleen cells were incubated with an Anti-Ly-6G-Biotin antibody and Anti-Biotin MicroBeads. Then, the cells were subsequently applied to LS Column to obtain Gr-1^{high}Ly-6G$^+$ cells (G-MDSCs). The remaining cells were incubated with an Anti-Gr-1-Biotin antibody and Streptavidin MicroBeads and applied to MS Column to obtain M-MDSCs. G-MDSCs and M-MDSCs were finally mixed to get the total MDSCs. The purity of the cells after sorting was >90%.

2.6. MDSC suppressive assay

The normal mouse spleen was ground and filtered to obtain a single cell suspension. After erythrocyte lysate, 1×10^8 normal mouse spleen cells were labeled with 5 μM CFSE. The labeled cells (10^5 cells well^{-1}) were co-cultured with purified MDSCs of 1:1 or 1:2 in the presence of 2 μg mL^{-1} soluble anti-CD3 mAb and 1 μg mL^{-1} soluble anti-CD28 mAb in RPMI 1640 medium containing 10% FBS in a 96-well flat-bottom plate for 72 h. CD4-APC antibody staining was used to detect the proliferation of CD4$^+$ T cells by flow cytometry.

2.7. ROS detection

Reactive oxygen species (ROS) production was measured by the oxidation-sensitive dye DCFDA. Cells were incubated at 37 °C in Roswell Park Memorial Institute (RPMI) medium in the presence of 5 μM DCFDA for 20 min. Then cells were washed with PBS and measured by the flow cytometry.

2.8. MDSC differentiation assay

Bone marrow cells were obtained from the tibia and fibula of mice. After filtration and erythrocyte lysis, cells were cultured in the presence of 40 ng/mL GM-CSF and IL-6 in RPMI 1640 medium containing 10% FBS for 4 days. At the same time, TLR7 agonist R848 (100 ng/mL), INK128 (50 nM), rapamycin (50 nM), and metformin (2 mM) were added to the culture medium. After the incubation periods, the cell phenotypes were determined by flow cytometry analysis.

2.9. siRNA transfection

MDSCs were transfected with FoxO1 small interfering RNA (siRNA) and NC siRNA and supplemented with RFect^{SP} siRNA transfection reagent following the manufacturer's protocols. The cells were incubated for 4 days and determined by flow cytometry analysis.

2.10. Western blot

Total proteins were separated by SDS-PAGE and electro-transferred to PVDF membranes. Then, membranes were blocked in 5% BSA dissolved in TBST (50 mM Tris/HCL, pH 7.6, 150 mM NaCl and 0.1% Tween-20) for 2 h at room temperature and then incubated with indicated primary antibody overnight at 4 °C, followed by incubation with appropriate HRP-linked secondary antibody for 2 h at room temperature. Protein bands were visualized using ECL plus Western blotting detection reagents (Millipore, Bedford, MA, USA). The gray values were analyzed by Image J gel analysis software. In our studies, GAPDH was used as an internal control.

2.11. Statistical analysis

All statistical calculations were performed using commercially

available statistical software GraphPad Prism (GraphPad, San Diego, CA, USA). Results were expressed as mean ± SEM of three independent experiments, and each experiment included triplicate sets. Data were statistically evaluated by one-way ANOVA followed by Dunnett's test. Statistical significance was defined as equal to or more than 95% confidence interval or $P \leq 0.05$.

3. Results

3.1. Accumulation of MDSCs and their weakened immunosuppressive function in pristane-induced DPH

In our previous studies, we found that MDSCs contribute to SLE by regulating differentiation of Th17 cells and Tregs and participating in the pathogenesis of lupus nephritis [34,35]. In addition, we found that MDSCs accumulate in large amounts in the abdominal cavity of DPH mice [36]. This study further explored the changes in the proportion and function of MDSCs in the lungs from murine DPH model. First, we successfully established an animal model of DPH by injecting pristane, which was sacrificed two weeks later. Mice developed diffuse pulmonary hemorrhage. According to the degree of inflammatory cell infiltration, the degree of basement membrane destruction, and the degree of red blood cell leakage, DPH was divided into complete, partial, and non-diffuse pulmonary hemorrhage (Fig. 1A). The results showed that about 80% of the mice developed DPH (Fig. 1B), which was almost in agreement with the report [37].

We analyzed the changes in the proportion of MDSCs in the BALF together with peritoneal cavity when pristane-induced DPH developed to two weeks by flow cytometry (Fig. 1C, S1A). Our results showed a significant increase in the proportion of MDSCs in these two pathological tissues. In order to further clarify the immune function of MDSCs, we detected the changes in the expression of ROS, an immunosuppressive indicator, in BALF and peritoneal MDSCs. The results showed that the expression of ROS was significantly reduced, suggesting that the immunosuppressive function of MDSCs was weakened (Fig. 1D, S1B). This result was confirmed in the experiment of spleen-sorted MDSCs and splenocytes co-cultured to detect the proliferation of CD4+ T cells (Fig. 1E). The above results suggested that MDSCs might play a role in promoting the occurrence and development of lupus-like DPH.

3.2. MDSCs depletion alleviates diffuse pulmonary hemorrhage in mice with lupus

To further explore the importance of MDSCs involved in lupus-like diffuse pulmonary hemorrhage, we used Gr-1 neutralizing antibody to remove MDSCs in mice; the antibody was injected into the tail vein three days before the induction of pristane and then every three days for two weeks (Fig. 2A). The proportion of MDSCs in the spleen was significantly reduced in the Gr-1 antibody injection group compared to the DPH group (MDSCs undeleted group) without treatment, which indicated that the model for removing MDSCs was successfully established (Fig. 2B). We examined the incidence of lupus-like diffuse pulmonary hemorrhage in the MDSCs undeleted group and the MDSCs deletion group. We found that compared with the control group, the proportion of mice with complete DPH was obviously reduced in the MDSCs deletion group. And the proportion of mice with non-DPH significantly increased (Fig. 2C). These results suggested that MDSCs deletion could significantly alleviate the symptoms of diffuse pulmonary hemorrhage. Moreover, the spleen swelling was obviously reduced in the MDSCs deletion group (Fig. 2D). This suggested that MDSCs might have an important role in the development of lupus-like DPH.

3.3. The mTOR signaling is activated in DPH MDSCs

Next, we wanted to explore the signaling pathways through which MDSCs affect the pathogenesis of DPH. In our previous study, it was
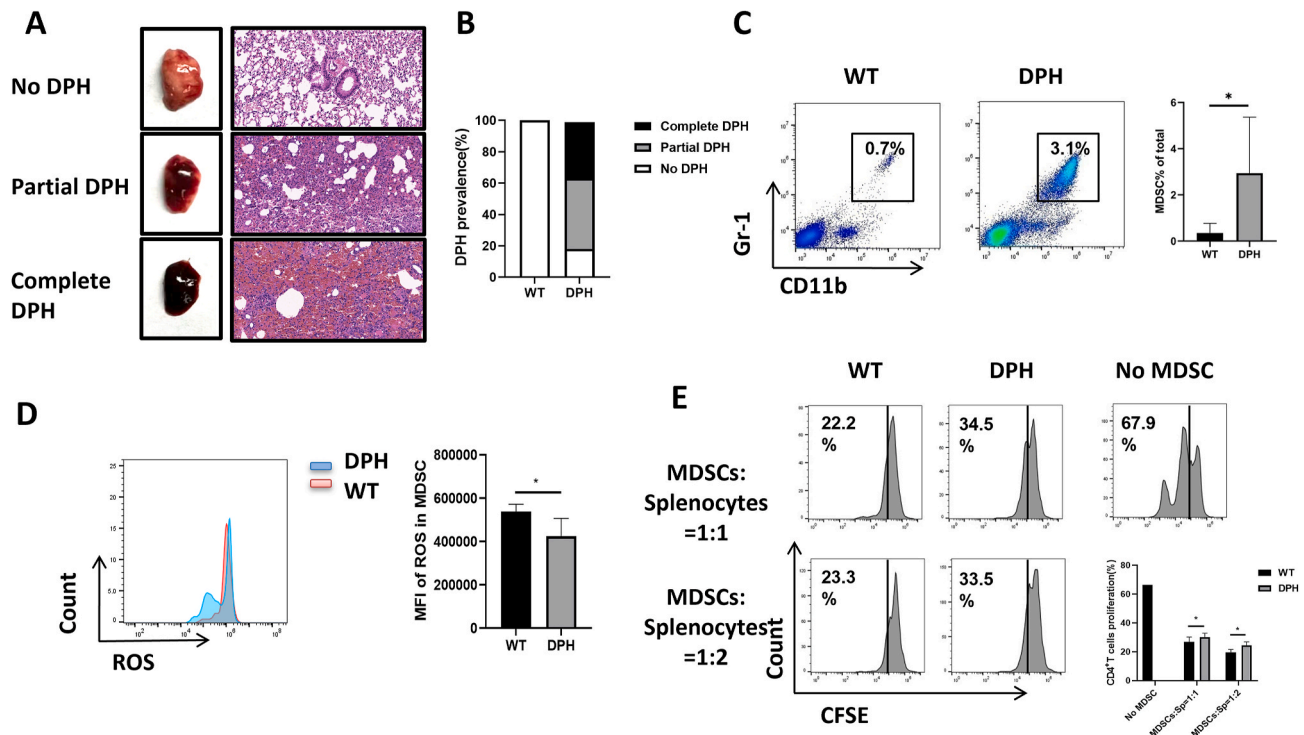
Fig. 1. Changes of MDSCs in DPH model induced by pristane (A, B) The lung tissues were collected to observe the symptoms of diffuse pulmonary hemorrhage; mice were divided into a complete diffuse pulmonary hemorrhage, partial diffuse pulmonary hemorrhage, and no diffuse pulmonary hemorrhage. Statistical analysis of the proportion of three types of pulmonary hemorrhage symptoms in each group. **(C)** Changes in the proportion of MDSCs in BALF. **(D)** Changes in the expression of ROS in MDSCs. **(E)** Spleen sorting MDSC and normal spleen cells were co-cultured with CFSE to detect the proliferation of CD4+ T cells. MDSCs represent myeloid-derived suppressor cells. Data represent the mean scores ±SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 11 animals per group.
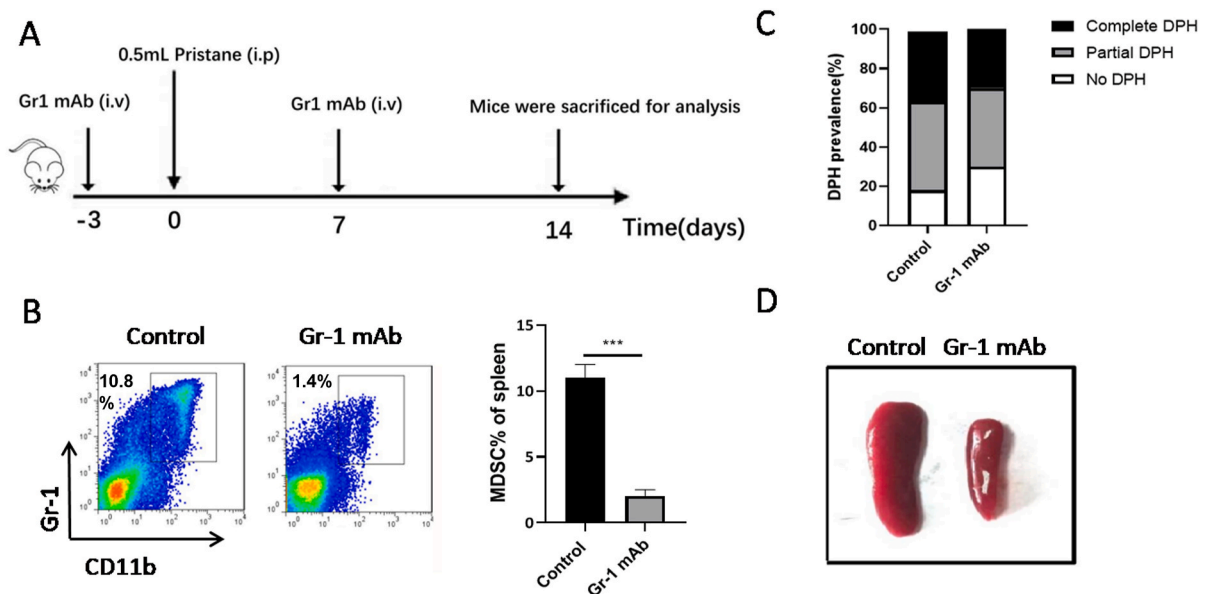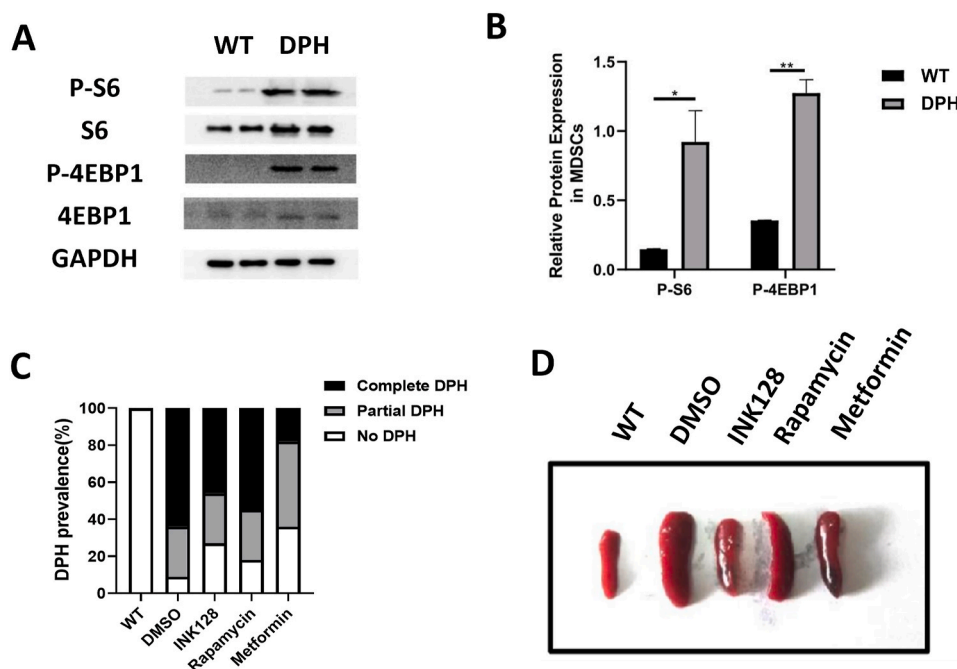
Fig. 2. The effect MDSCs on diffuse pulmonary hemorrhage caused by lupus (A) C57BL/6 mice (6–8 weeks old) received a single intraperitoneal injection of 0.5 ml pristane for 14 days. The neutralizing antibody of Gr-1 was injected into the tail vein three days before the induction of pristane and every three days during the induction process. **(B)** The spleens of mice in different groups were collected, and the proportion of MDSCs in the spleen was detected by flow cytometry. **(C)** The lung tissues of different groups of mice were collected to observe the symptoms of diffuse pulmonary hemorrhage; mice were divided into complete diffuse pulmonary hemorrhage, partial diffuse pulmonary hemorrhage, and no diffuse pulmonary hemorrhage. Statistical analysis of the proportion of three types of pulmonary hemorrhage symptoms in each group. **(D)** Morphological analysis of spleens of mice in different groups. MDSCs represent myeloid-derived suppressor cells. Data represent the mean scores ±SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 11 animals per group.

found that the AMPK/mTOR signaling pathway was involved in the regulation of M-MDSC expansion and involved in the occurrence and development of SLE in a 7-month pristane-induced lupus model [38]. In order to test whether the mTOR pathway was also activated in DPH MDSCs, we established a pristane-induced pulmonary hemorrhage model and isolated MDSCs from spleen. Western blot results showed that the mTOR downstream molecules p-S6 and p-4EBP1 were abnormally activated in MDSCs derived from DPH mice compared with those derived from control mice (Fig. 3A and B).

Many studies have shown that the mTOR signaling pathway

participates in lupus by affecting autophagy, Ca^{2+} fluxing, and other pathways [18,22,25]. Nevertheless, whether mTOR is involved in DPH is still inconclusive. Therefore, to investigate whether the AMPK/mTOR signal is involved in the pathogenesis of DPH, the DPH mice model was treated with metformin (the AMPK agonist), or INK128 and rapamycin (mTOR inhibitors), respectively. We calculated the incidence of diffuse pulmonary hemorrhage in different treatment groups. We found that in the DMSO-treated mice, mice with complete DPH accounted for 64% of the entire group of mice. Compared with the DMSO-treated mice, the number of mice with complete DPH dropped to 46%, 55%, and 18%


Fig. 3. Activation of mTOR signaling pathway in MDSCs is involved in the occurrence of DPH (A) C57BL/6 mice (6–8 weeks old)(n = 11/group) received a single intraperitoneal injection of 0.5 ml pristane for 14 days to establish the DPH mouse model. Expression of p-4EBP, 4EBP, p-S6, and S6 in MDSCs isolated from control and DPH mice spleen was detected by Western blot. **(B)** The density quantification of band intensities. **(C)** C57BL/6 mice (6–8 weeks old) (n = 11/group) received a single intraperitoneal injection of 0.5 ml pristane for 14 days, and metformin, INK128, and Rapamycin during induction. The lung tissues were observed for evaluating the symptoms of diffuse pulmonary hemorrhage, and statistical analysis of the proportion of three types of pulmonary hemorrhage symptoms in each group. **(D)** Morphological analysis of spleens of mice in different groups.

when treated with INK128, rapamycin and metformin, respectively, while the mice with no DPH increased (Fig. 3C). Meanwhile, we analyzed the spleen morphology of different groups and found that the spleen mice treated with INK128, rapamycin and metformin were relieved compared to DPH mice without treatment (Fig. 3D). These data suggested that the AMPK/mTOR signaling pathway has an important

role in lupus-like pulmonary hemorrhage symptoms.

3.4. mTOR signaling pathway inhibitors are related to decrease in M-MDSCs and M1 and promotion of G-MDSCs and M2

To further clarify the role of mTOR signaling pathway of MDSC in
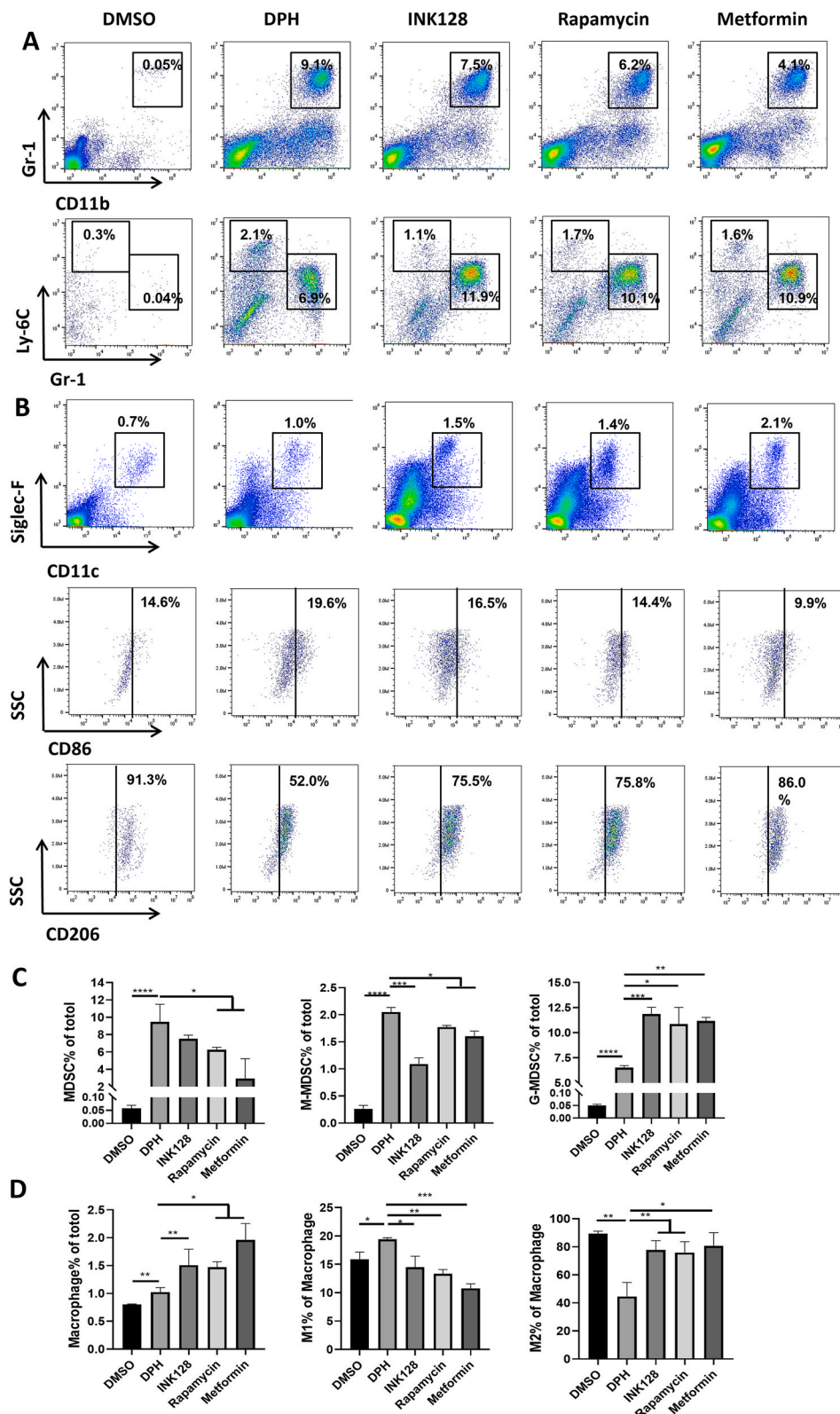
Fig. 4. Inhibition of AMPK/mTOR signaling decreased M-MDSCs and M1, promoted G-MDSCs and M2 Mice were treated as described in Fig. 3. **(A)** The frequency of MDSCs was determined by FACS in BALF. **(B)** The frequency of macrophages was determined by FACS in BALF. **(C)** The statistical results of the frequency of MDSCs. **(D)** The statistical results of the frequency of macrophages. MDSCs represent myeloid-derived suppressor cells. FACS represents fluorescent-activated cell sorting. Data represent the mean scores ±SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 11 animals per group.

DPH, MDSCs and their subpopulations were examined in the DPH mouse model after mTOR signaling pathway inhibition. The cells were collected in the BALF together with peritoneal cavity were examined with flow cytometry. The percentages of MDSCs and M-MDSCs were significantly decreased in the AMPK/mTOR inhibitor treatment groups, and G-MDSCs were significantly increased (Fig. 4A, C; S2A, C). These results are consistent with the previous conclusions (see above), which further confirmed that MDSCs have an important role in the pathogenesis of DPH through the mTOR signaling pathway.

Studies have reported that M-MDSCs have a tendency to differentiate into macrophages under various conditions such as inhibiting STAT3 or PIR-B signal pathway [39,40]. Furthermore, recent studies have found that the activation of TLR7/8 can direct the myeloid-derived suppressor cells to M1-macrophages [41]. Therefore, we hypothesized that MDSC is involved in disease progression by regulating M1/M2 differentiation. We examined BALF and peritoneal cells that were closely associated with pristane-induced lupus-like diffuse pulmonary hemorrhage. The proportion of total macrophages and anti-inflammatory M2 was increased after the treatment with metformin, INK128, and rapamycin. However, the proportion of inflammatory M1 was reduced (Fig. 4B, D; S2B, D). Therefore, we speculated that suppression of the mTOR signaling pathway might also inhibit the differentiation of M-MDSCs into pro-inflammatory M1 and promote the proliferation of anti-inflammatory M2 to play a therapeutic role in the disease.

3.5. The differentiation of M-MDSC/macrophage induced by mTOR inhibition was related to FoxO1

To further explore the effect of the AMPK/mTOR signal on M-MDSC differentiation, bone marrow cells cultured in a medium supplemented with 40 ng/mL IL-6 and 40 ng/mL GM-CSF for 4 days. We used R848 to simulate the lupus environment [42]. When the AMPK/mTOR inhibitor INK128, metformin or rapamycin was added to the culture, the percentage of M-MDSCs and M1 decreased, while G-MDSCs and M2 increased (Fig. 5A–D). The in vitro phenomenon was consistent with the change trend of immune cells under DPH pathological condition in vivo. These results further indicated that the AMPK/mTOR signal pathway regulated the TLR7-induced differentiation of M-MDSCs into macrophages in vitro.

Studies have shown that inhibition of mTORC2 promotes peritoneal resident macrophage generation by increasing FoxO1 expression [43]. We examined the effect of mTOR signaling pathway inhibition on FoxO1 expression in lupus. R848 stimulation reduced FoxO1 expression in MDSCs and macrophages, which was reversed by further blockade of AMPK/mTOR signaling pathway (Fig. 6A–C). These results suggest that FoxO1, as an important downstream target of mTOR signaling pathway, is involved in the pathological process of lupus.

In order to further explore the important role of FoxO1 in the mTOR signaling pathway in regulating the differentiation of MDSCs into macrophages, we tried to detect the proportion changes of immune cells in the culture system by reversing the up-regulation of FoxO1 expression after mTOR inhibitor administered under the background of R848. SiRNA transfection effectively induced FoxO1 expression down-regulation in bone marrow cells (Fig. 7A). After siRNA transfection, although we did not see a uniform trend in total MDSCs, G-MDSCs and total macrophages, the proportion of M-MDSCs and M1 was increased, while the proportion of M2 was down-regulated (Fig. 7B–E). The results showed that the reduced expression of FoxO1 effectively blocked the induction of mTOR signal inhibitor on M-MDSCs to M2 differentiation, but promoted the transformation of M-MDSCs into M1.

3.6. Inhibition of mTOR signaling pathway in DPH mice promotes the differentiation of M-MDSCs into M2 by down-regulating FoxO1 expression

To verify the results in vitro, FoxO1 expression in different immune cell populations of DPH mice treated with mTOR signaling inhibitors was examined. Results showed that FoxO1 upregulation after INK128, rapamycin and metformin administration was mainly concentrated in M-MDSCs, not G-MDSCs, and no significant changes in FoxO1 expression were observed in overall MDSCs. In macrophages, both M1 and M2, FoxO1 expression is significantly up-regulated due to inhibition of mTOR signaling pathway (Fig. 8A–C, S3A-C). The above results suggest that the inhibition of mTOR signaling pathway in M-MDSCs can promote the differentiation into M2 and inhibit their phenotypic transformation into M1 by increasing the expression of FoxO1.

We explored the relationship between MDSCs and DPH in lupus, and found that MDSCs were involved in the pathogenesis of DPH. In lupus mice, mTOR in MDSCs was activated, which promoted the proliferation and differentiation of MDSCs, leading to the occurrence of DPH. When the mTOR signal was suppressed (using INK128, rapamycin or metformin), the number of MDSCs decreased and the DPH was alleviated. It is worth noting that suppression of the mTOR signaling pathway inhibits the differentiation to pro-inflammatory M1 and promotes the differentiation to anti-inflammatory M2 by increasing the expression of FoxO1 in M-MDSCs to play a therapeutic role in the disease (Fig. 9).

4. Discussion

MDSCs have an important role in autoimmune diseases, including lupus, rheumatoid syndrome, and Sjogren's syndrome. Our study found abnormal accumulation of MDSCs in the lungs and abdominal cavity of DPH mice, accompanied by decreased immunosuppressive function. This suggests the promoting role of MDSCs in the pathogenesis of DPH. The relief of DPH symptoms after depletion of MDSCs further supported our hypothesis. MDSCs depleted mice had reduced morbidity and improved splenomegaly, inflammatory infiltration, and erythrocyte leakage compared with controls. These results suggest that MDSCs do play an important role in the pathogenesis of DPH, which is consistent with the previously established role of MDSCs in other autoimmune diseases.

The abnormal activation of the mTOR signal is associated with the pathogenesis of multiple diseases, including cancer, diabetes, rheumatoid arthritis, and SLE [44–46]. mTOR signaling has been reported to function in a wide variety of immune cells. Significantly increased mTORC1 activity has been found in T cells from patients with systemic lupus erythematosus [47]. We found that the mTOR pathway was activated in the MDSCs of SLE mice, which was consistent with the report in SLE patients. Subsequently, we found a significant upregulation of the protein levels of key factors of mTOR pathway including mTOR downstream molecules p-S6 and p-4EBP1 in MDSCs selected from the spleen of DPH mice, which suggests that the mTOR pathway was also activated in MDSCs of DPH mouse model. Subsequently, we established a DPH model of mTOR pathway inhibition by treating mice with INK128, rapamycin or metformin. Inhibition of the mTOR signaling pathway significantly reduced the proportion of MDSCs and alleviated DPH symptoms in mice, suggesting that MDSCs were indeed involved in the pathogenesis of DPH through mTOR pathway.

As bone marrow-derived monocyte precursors, MDSCs are highly plastic. Studies have shown that in response to different environmental factors, M-MDSCs can differentiate into macrophages or dendritic cells [11]. We found that M-MDSCs and M1 were significantly decreased in DPH mice treated with INK128, rapamycin or metformin, while G-MDSCs and M2 were significantly increased. The results suggest that mTOR signaling inhibition of MDSCs may alleviate DPH by inhibiting M1 differentiation and promoting M2 differentiation. In order to verify this hypothesis, INK128, rapamycin or metformin were administered in vitro, and it was found that the blocking of mTOR signaling pathway under R848 stimulation significantly inhibited the differentiation of MDSCs into M1 and promoted its differentiation into M2. These results suggest that the activation of mTOR signaling pathway can participate in DPH disease progression by promoting the differentiation of MDSCs into pro-inflammatory M1 and inhibiting the differentiation into
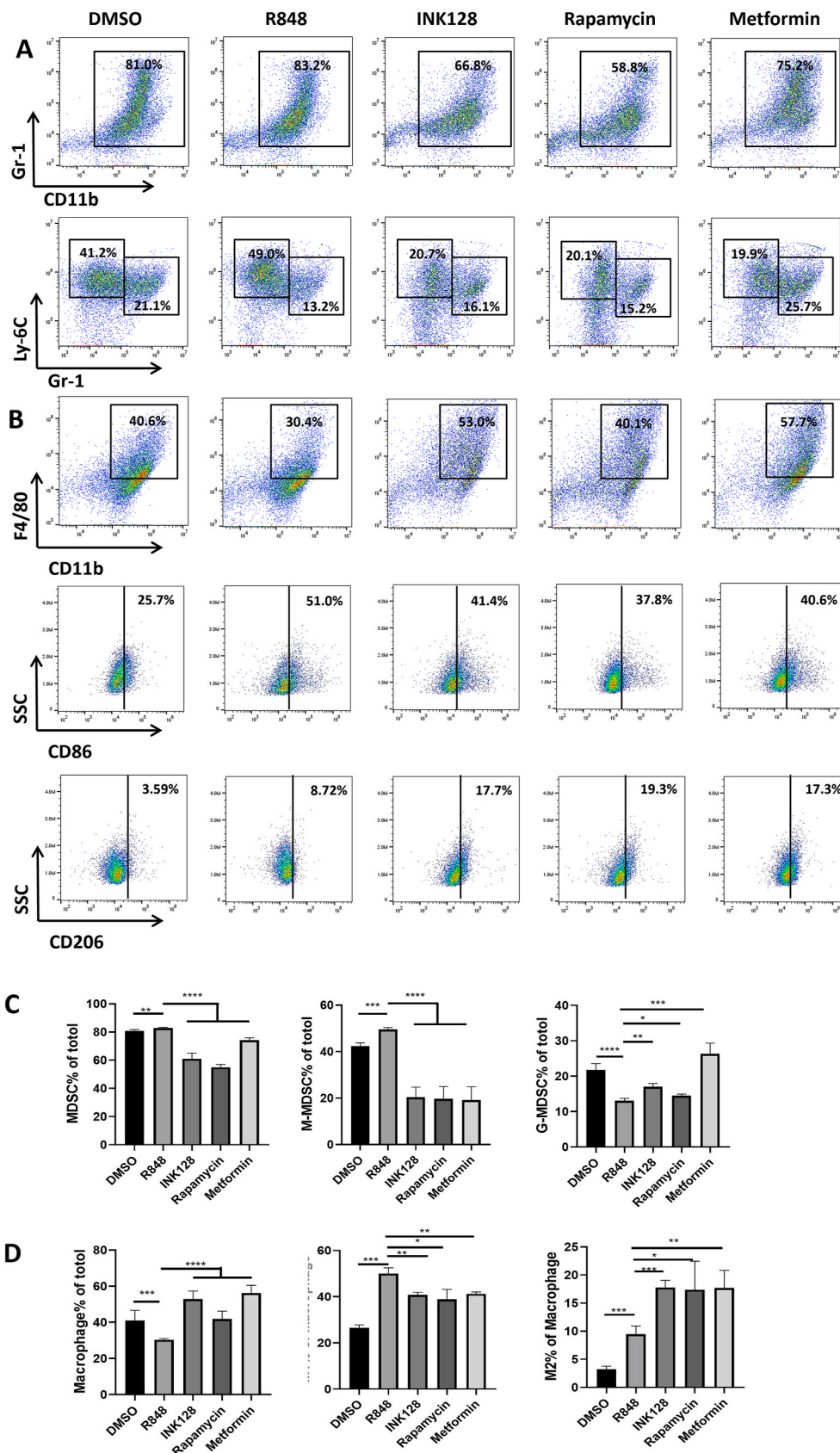
Fig. 5. Inhibition of AMPK/mTOR signaling decreased M-MDSCs and M1, promoted G-MDSCs and M2 under R848 stimulation in vitro Bone marrow cells cultured in a medium supplemented with 40 ng/mL IL-6 and 40 ng/mL GM-CSF for 4 days. R848 (100 ng/mL) is used to simulate lupus in vitro. INK128 (50 nM), rapamycin (50 nM) and metformin (2 mM) are used to block the mTOR signaling pathway. **(A)** The frequency of MDSCs was determined by FACS. **(B)** The frequency of Macrophages was determined by FACS. **(C)** The statistical results of the frequency of MDSCs. **(D)** The statistical results of the frequency of macrophages. MDSCs represent myeloid-derived suppressor cells. FACS represents fluorescent-activated cell sorting. Data represent the mean scores ±SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
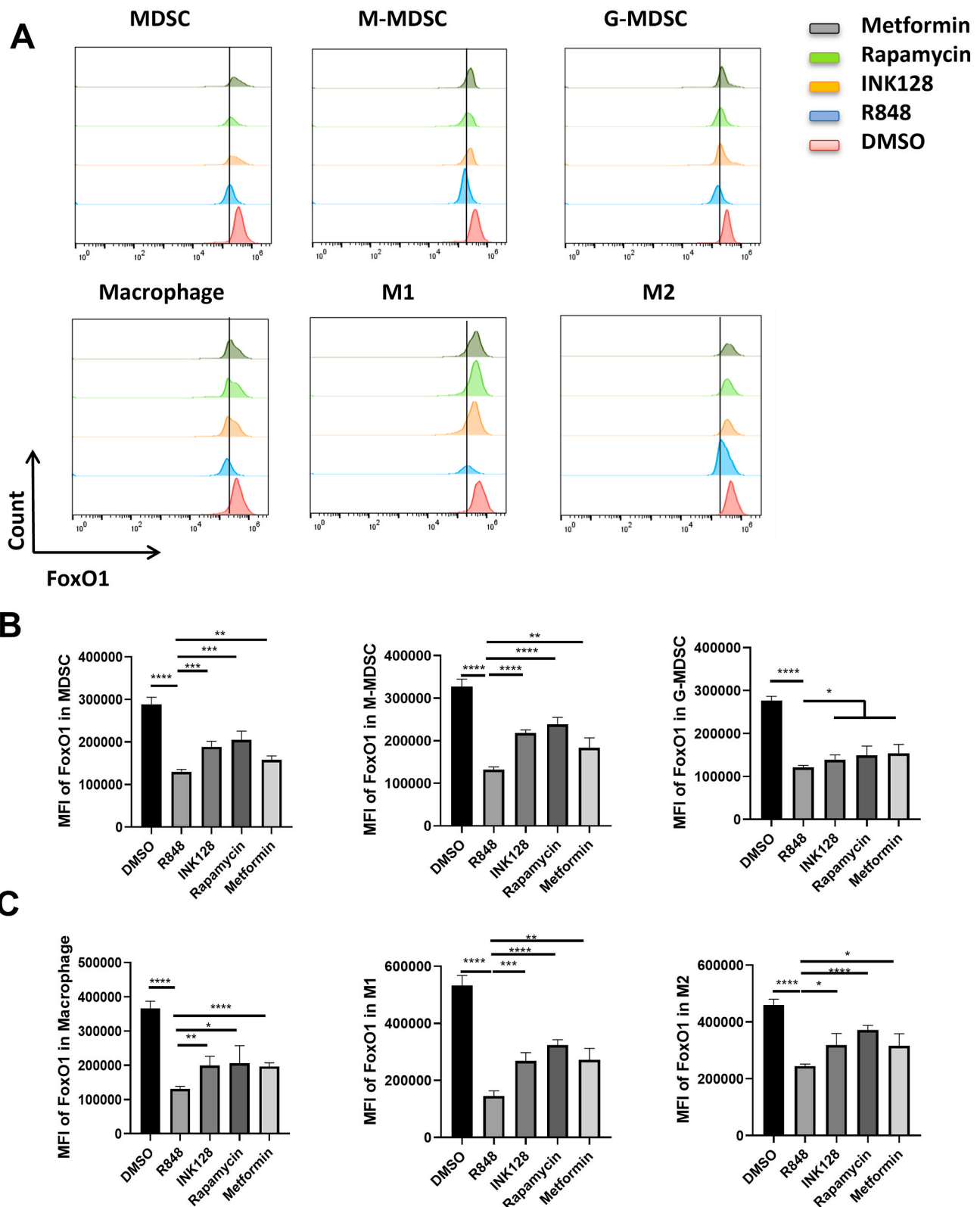
Fig. 6. Inhibition of the mTOR signaling pathway reversed the down-regulation of FoxO1 expression in MDSCs and macrophages stimulated by R848 Bone marrow cells cultured in a medium supplemented with 40 ng/mL IL-6 and 40 ng/mL GM-CSF for 4 days. R848 is used to simulate lupus in vitro. INK128, rapamycin and metformin are used to block the mTOR signaling pathway. **(A)** The median fluorescence intensity of FoxO1 in different cell subpopulations was determined by FACS. **(B)** The statistical results of the median fluorescence intensity of MDSCs. **(C)** The statistical results of the median fluorescence intensity of macrophages. MDSCs represent myeloid-derived suppressor cells. FACS represents fluorescent-activated cell sorting. Data represent the mean scores ±SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
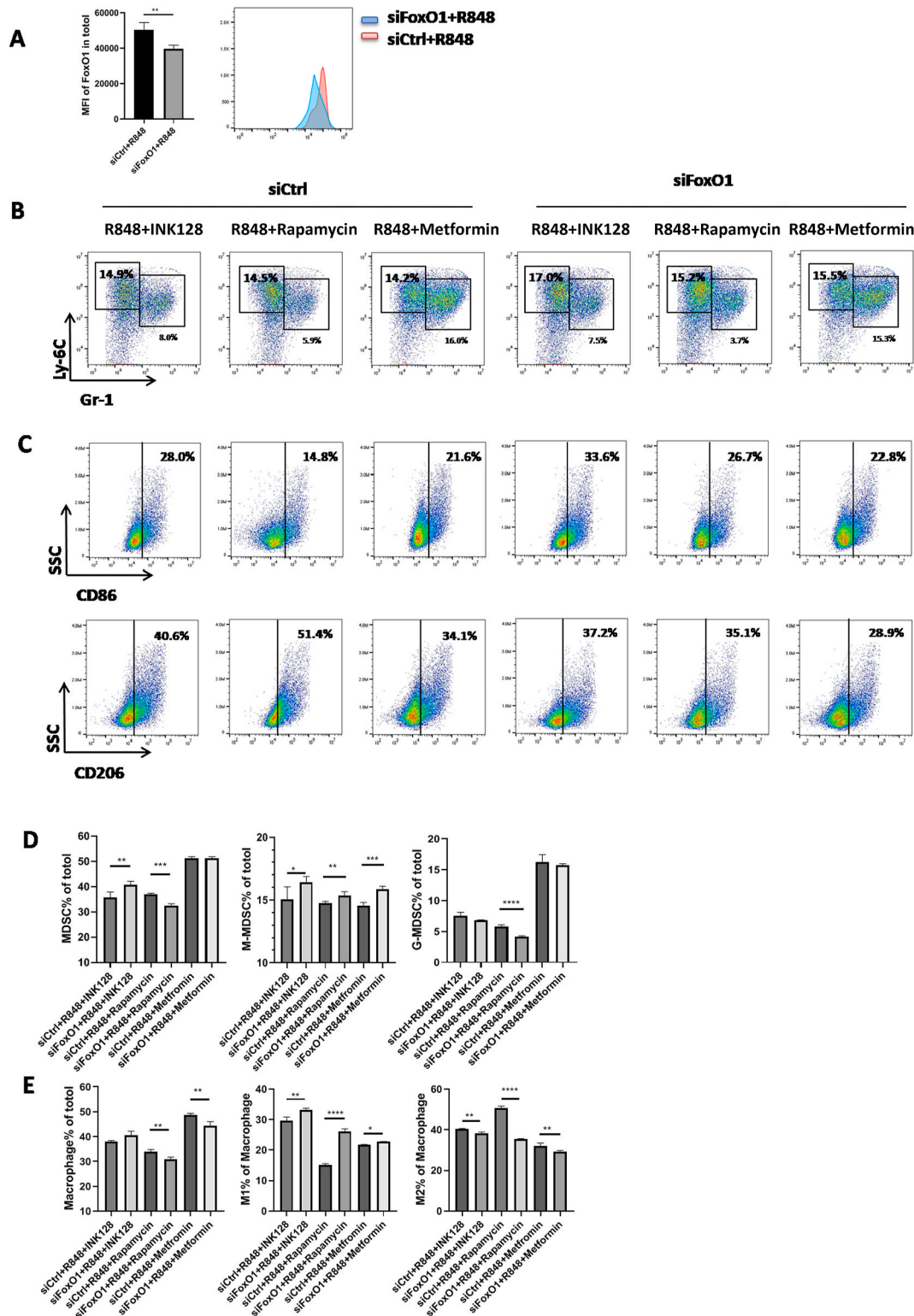
Fig. 7. FoxO1 downregulation reversed the induction of M-MDSCs into M2 differentiation by mTOR signaling inhibition Bone marrow cells cultured in a medium supplemented with 40 ng/mL IL-6 and 40 ng/mL GM-CSF for 4 days. R848 is used to simulate lupus in vitro. INK128, rapamycin and metformin are used to block the mTOR signaling pathway. siRNA transfection was used to down-regulate FoxO1. **(A)** The median fluorescence intensity of FoxO1 in bone marrow cells after siRNA transfection was determined by FACS. **(B)** The frequency of M-MDSCs and G-MDSCs were determined by FACS in bone marrow cells. **(C)** The frequency of M1 and M2 were determined by FACS in bone marrow cells. **(D)** The statistical results of the frequency of MDSCs. **(E)** The statistical results of the frequency of macrophages. MDSCs represent myeloid-derived suppressor cells. FACS represents fluorescent-activated cell sorting. Data represent the mean scores ±SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
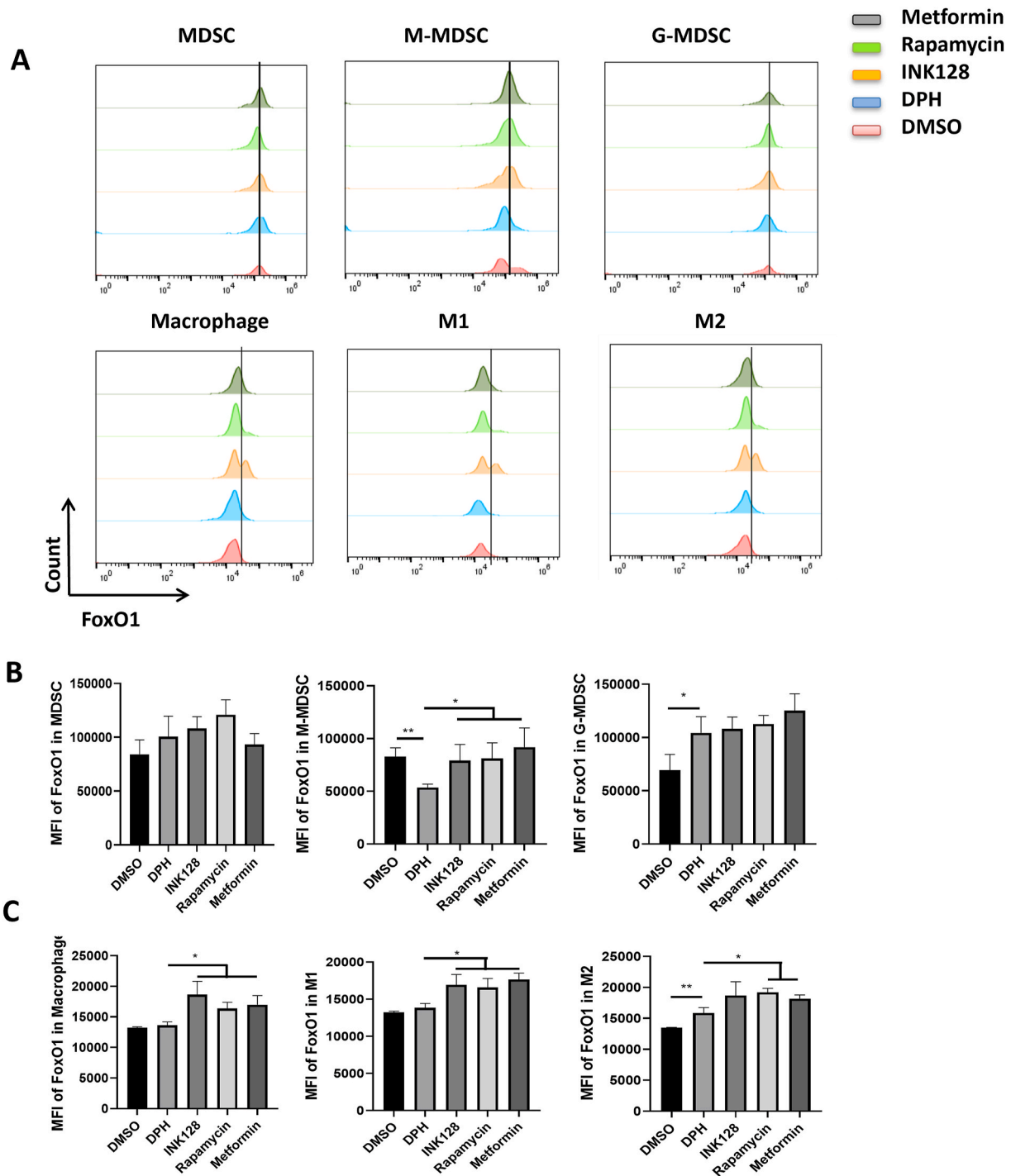
Fig. 8. Inhibition of AMPK/mTOR signaling increased FoxO1 expression in M-MDSCs, M1 and M2 Mice were treated as described in Fig. 3. **(A)** The median fluorescence intensity of FoxO1 in different cell subpopulations was determined by FACS in BALF. **(B)** The statistical results of the median fluorescence intensity of MDSCs. **(C)** The statistical results of the median fluorescence intensity of macrophages. MDSCs represent myeloid-derived suppressor cells. FACS represents fluorescent-activated cell sorting. Data represent the mean scores ±SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 11 animals per group.

anti-inflammatory M2.

FoxO1 is the earliest and most representative transcription factor found in the FoxO subfamily. FoxO1 regulates a number of targets, such as genes involved in apoptosis and autophagy, cell cycle arrest, and metabolic and immune regulators [48]. In SLE, cytoplasmic-predominant FoxO1 (CytoFOX) B cells are significantly increased in patients as compared to healthy controls [49]. Foxo1 transcription level in peripheral blood mononuclear cells (PBMCs) of SLE patients is significantly lower than that of normal controls [50], and

elevated Mir-873 in PBMCs promotes Th17 cell differentiation by down-regulating Foxo1 [51]. Previous studies in our lab found that the loss of Dectin-3 can promote the reduction of M-MDSCs and alleviate lupus by inducing FoxO1 nuclear transfer [52]. As one of the transcription factors closely related to inflammatory response, FoxO1 expression can be regulated by the mTOR signaling pathway [53,54], and a study demonstrated that inhibition of mTORC2 promoted the generation of tissue-resident macrophages by increasing FoxO1 expression [43]. FoxO1 inhibition has been shown to promote the
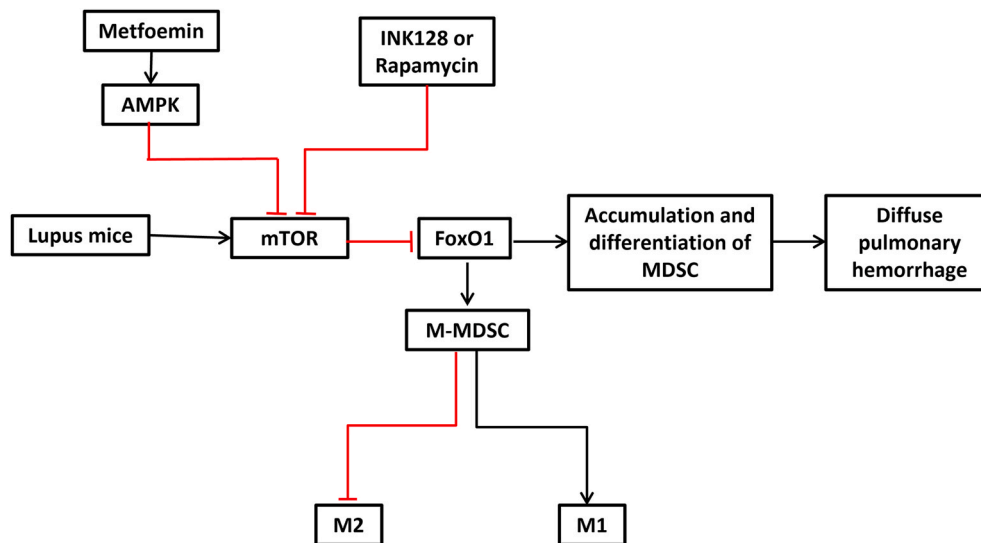
Fig. 9. Schematic diagram of MDSCs affecting DPH.

expression of CD86 and down-regulate the expression of CD206 in vitro [55,56]. Under DPH pathology, it has not been reported whether abnormal amplification of MDSCs is related to mTOR and FoxO1 signaling pathway.

Our study showed that inhibition of the mTOR signaling pathway significantly increased FoxO1 expression in M-MDSCs and macrophages, both in the pathological environment of DPH in vivo and in the simulated lupus environment in vitro. Down-regulation of FoxO1 reduced the induction of M2 and increased the ratio of M-MDSCs and M1 by blockade of mTOR signaling pathway under R848 stimulation. The above research results indicate that the activation of mTOR signaling pathway can induce M-MDSCs differentiation into M1, and inhibit their differentiation into M2 by inhibiting the expression of FoxO1, thereby promoting the progression of DPH.

5. Conclusion

Our study indicated that MDSCs affect the process of diffuse pulmonary hemorrhage through activation of the mTOR signaling pathway. This may be achieved by promoting the accumulation of MDSCs and inducing the differentiation of M-MDSCs into pro-inflammatory M1 by down-regulating FoxO1.

CRediT authorship contribution statement

Liping Tan: Data curation, Conceptualization, Methodology. **Guoping Shi:** Methodology, Investigation. **Junyu Zhao:** Methodology, Software. **Xiaoyu Xia:** Data curation, Writing - original draft. **Dan Li:** Formal analysis. **Saiwen Wang:** Methodology. **Jun Liang:** Writing - review & editing. **Yayi Hou:** Conceptualization, Funding acquisition. **Huan Dou:** Writing - review & editing, Funding acquisition.

Conflictofinterest

All the Authors have no conflict of interest.

Funding

Declaration of competing interest

The authors declare that they have no known competing financialinterestsor personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101351.

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