mTOR Inhibitor INK128 Promotes Wound Healing by Regulating MDSCs

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Abstract

Background: Skin wounds in diabetic patients are hardly to recover. Accumulating evidence has shown that mammalian target of rapamycin (mTOR) pathway and myeloid-derived suppressor cells (MDSCs) are involved in inflammatory-related response. INK128 is a novel mTOR kinase inhibitor in clinical development. However, the exact roles of MDSCs and INK128 in healing wound of diabetic patients are unclear.

Methods: Mice models of normal, diabetic, and diabetic+INK128 were constructed. Bone marrow (BM)-derived macrophages and RAW264.7 cell line co-cultured with MDSCs, which were induced at different conditions. Flow cytometry, western blot, quantitative real-time PCR, and immunohistochemical analysis were performed.

Results: Diabetic mice (DM) had a slower recovery rate, thinner epidermal and dermal, and less blood vessels than those of normal mice. MDSCs were abnormally accumulated in DM and mTOR was activated in MDSCs of DM and the cells treat with high glucose. Moreover, mTOR signaling inhibitor INK128 could promote wound healing through reducing the MDSCs. MDSCs function was disordered in DM and high glucose environments, while INK128 could help retrieve their function. Furthermore, high glucose and other factors in DM could promote M-MDSCs differentiation to M1 pro-inflammatory macrophage cells, thus inhibiting wound healing. The differentiation, which was dependent on mTOR signaling, could be reversed by INK128.

Conclusion: INK128 is potential to be developed as a clinical strategy to promote wound healing of diabetic patients.

Background

The skin of diabetic patients is prone to damage, prolonged and unhealed to form refractory ulcers. Diabetic ulcer is one of the common complications of diabetic patients, in which foot ulcerations, account for 15% of diabetic patients, is an important cause of non-traumatic amputation (1), which seriously affects the health and quality of life of diabetic patients. Therefore, finding effective ways to accelerate wound healing in diabetic patients has become an important research direction. Wang et al. (2) reported that hyaluronic acid could be a useful method to heal diabetic wound. Topical simvastatin could accelerate wound healing in diabetic patients by enhancing angiogenesis and lymphangiogenesis (3). Moreover, the pbFGF-loaded electrospun fibrous was reported might have the function of accelerating skin healing of diabetic patients (4). Despite this, more therapies were demanded for diabetic patients to promote wound healing.

Immune cells are of vital importance in the process of wound healing, especially myeloid cells (5). Myeloid-derived suppressor cells (MDSCs) are a group of immature and heterogeneous cell populations, which can be characterized by the expression of CD11b and Gr-1 in mice (6). It can be further divided into granulocyte-like MDSCs (G-MDSC) and monocyte-like MDSCs (M-MDSC) (7). Increased numbers of

MDSCs have been observed under pathological conditions such as tumors, chronic inflammation and obesity (7–9). Studies have shown that the proportion of MDSCs in peripheral blood of type I diabetic patients is significantly higher than that of normal people, while the proportion of M-MDSCs decreases (10). The frequency of $CD33^{+}HLA^{-}DR^{-/low}$ MDSCs is higher in patients with type 2 diabetic mellitus (DM2) comparing with non-DM2 individuals(11). Similar conclusion was drawn by Whitfield-Larry et al. (12), MDSCs are unexpectedly enriched in peripheral blood of both mice and patients with autoimmune diabetes. However the immunosuppressive function of native T1D MDSCs was impaired. As reported by Shi et al. (6), arctigenin was able to ameliorate inflammation through accumulating G-MDSCs, and enhancing the immunosuppressive function of MDSCs. This provides us with ideas that the changes both in number and function of MDSCs may be crucial for the development of inflammation state of diabetic mellitus and maladjustment of the wound healing process. Nonetheless, it is still not clear which factors contribute to the changes of MDSCs in diadetic mellitus.

The mammalian target of rapamycin (mTOR) signaling pathway has been widely recognized to control cell survival, metabolism and proliferation (13). It forms the catalytic subunit of two distinct protein complexes, known as mTOR Complex 1 (mTORC1) and 2 (mTORC2) (14). It has been reported that the mTOR inhibitor INK128 is an oral, highly effective and selective adenosine triphosphate (ATP) competitor that inhibits mTORC1 and mTORC2 (15, 16). According to the study of Shi et al. (17), INK128 as a second generation mTOR inhibitor, had a good therapeutic action on lupus development by regulating MDSCs. However, the effects of mTOR on diabetes are complexed, with the anti- and pro-diabetic effects. Activating of mTOR in β cells can can stimulate their proliferation, but the mTOR activation in immune cells may exacerbate β cells dysfunction (18). Accordingly, we hypothesis that INK128 can affect the course of wound healing in diabetic mice by adjusting MDSCs.

To verify the role of mTOR in MDSCs on regulating the diabetic skin wound healing, we constructed streptozotocin (STZ) –induced diabetic mice and used different concentration of glucose to mimic the MDSCs living environment. By focusing on mTOR signaling, we found that high glucose environment activated mTOR signaling in MDSCs, which resulting in aberrant accumulation and differentiation of MDSCs. Moreover, we explored whether in vivo and in vitro treatment with INK128 can accelerate the wound closure of diabatic mice.

Methods

Antibodies and reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11b mAb, Allophycocyanin (APC)-coonjugated anti-mouse Gr-1 mAb, Allophycocyanin (APC)-coonjugated anti-mouse F4/80 mAb, P-phycoerythrin (PE)-conjugated anti-mouse Ly6G mAb and Allophycocyanin (APC)-coonjugated anti-mouse Ly6C mAb were purchased from Biolegend, and β-Tubulin (2144), p-S6 (4858S), S6 (2217S), p-4EBP-1 (2855S) and 4EBP-1 (9644T) were got from cell Signal Technology Inc. mTOR inhibitor INK128 was purchased from Selleckchem. Trizol Reagent and SYBR green dye were bought from Invitrogen.

Recombinant mouse IL-6, GM-CSF and MDSC Isolation Kit obtained from Miltenyi Biotec. Glucose and streptozotocin (STZ) were perchased from Sigma-Aldrich.

Mice model construction

Male C57/B6 mice (6-8 weeks old) were obtained from the Model Animal Research Center of Nanjing University. They were kept under pathogene-free conditions in 12 h:12 h light and dark cycle. All procedures involving mice were approved by the Medical School for Animal Use and Care Committee of Nanjing University in accordance with guidelines of the US NIH. Diabetic mice were induced by low-dose injections of STZ. Mice were fasted for 5 h and then injected with vehicle or STZ (intra-peritoneal (i.p.) injection, 50mg/kg per day, pH4, dissolved in 0.1 M sodium citrate buffer) for 5 consecutive days. After blood glucose level keeps steadily over 16.6 mM for 3 weeks, two full-thickness wounds of 5 mm in diameter were made on the dorsal surface of mice. To evaluate the effects of INK128, one month after STZ injection, diabetic mice were received daily intraperitoneal injection of 1mg/kg INK128 for another 45 days, then two full-thickness wounds of 5 mm in diameter were made on the dorsal surface.

Generation and isolation of MDSCs

BM cells were isolated from mice by flushing tibiae and femurs as described previously(19). Spleen-derived MDSCs were purified from control and diabetic mice using Myeloid-Derived Suppressor Cell Isolation Kit. BM cells isolated from mice were cultured in culture medium (sugar-free RPMI 1640 with 10% FBS (Gibco)) supplemented with 40ng/ml murine IL-6 and 40ng/ml GM-CSF for 4 days, and added 0, 5, 10, 20, 30, 60, and 120mM glucose. Moreover, BM cells were supplemented with 0, 25, 50, and 100nM INK128.

Macrophage differentiation assay

BM cells were cultured in the presence of 40ng/ml murine IL-6 and 40ng/ml GM-CSF and added 5mM glucose, 30mM glucose, 30mM glucose+50nM INK128 for 4 days. After the different incubation periods, MDSCs were collected and co-cultured with BMDM and RAW264.7 seperately. phenotypes of BMDM and RAW264.7 were determined by flow cytometry analysis.

Flow cytometry analysis

BM cells, splenocytes and PBMCs from mice were prepared as single-cell suspensions. To detect mouse MDSC subsets, cells were pre-incubated with FITC-conjugated anti-mouse CD11b mAb and APC-conjugated anti-mouse Gr-1 mAb, then they were stained for 20 min at room temperature in the dark. For the detection of macrophages, cells were labeled with FITC-conjugated anti-mouse CD11b mAb and APC-conjugated anti-mouse F4/80 mAb, and then incubated for 20 min at room temperature in the dark. For the detection of M-MDSC and G-MDSC subsets, cells were labeled with FITC-conjugated anti-mouse CD11b mAb, APC-conjugated anti-mouse Ly6C mAb and PE-conjugated anti-mouse Ly6G mAb, then cells were incubated for 20 min at room temperature in the dark. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences). The data was analyzed by the FlowJo software.

Western blot analysis

The protein expression levels of p-S6, S6, p-4EBP-1, 4EBP-1, on MDSCs were evaluated. β-Tubulin was used as an internal control in our study. Proteins were extracted on a normal way (20), and the western blot analysis was performed according to Wang et al.(21). Protein bands were visualized using ECL Plus Western blotting detection reagents (Millipore, Bedford, MA, USA).

RNA extraction and quantitative real-time PCR

Total RNA of MDSCs were isolated with Trizol Reagent according to the manufacturer's instructions. Quantitative real-time PCR experiment was performed using SYBR green dye on Step One sequence detection system (Applied Biosystems, Waltham, MA, USA). Relative expression of genes was calculated using $2^{-\Delta\Delta CT}$ method, with GAPDH as internal control. Primers sequences are as shown in Table 1.

Table 1

Gene	Forward primers	Reverse primers
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
p47phox	ACACCTTCATTCGCCATATTGC	CCTGCCACTTAACCAGGAACA
gp91phox	AGTGCGTGTTGCTCGACAA	GCGGTGTGCAGTGCTATCAT
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	GGAGCTGTCATTAGGGACATCA
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
CD206	CTCTGTTCAGCTATTGGACGC	TGGCACTCCCAAACATAATTTGA
IL-10	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGG
IGF-1	CACATCATGTCGTCTTCACACC	GGAAGCAACACTCATCCACAATG
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
S100A8	AAATCACCATGCCCTCTACAAG	CCCACTTTTATCACCATCGCAA
S100A9	GCACAGTTGGCAACCTTTATG	TGATTGTCCTGGTTTGTGTCC

Histologic and immunohistochemical analyses

Epidermal and dermal and blood vessels of wound skin tissue were obtained from paraffin-embedded tissue, fixed in formalin and stained with HE, Masson, and CD31, as well as DAPI.

Statistics analysis

Results were expressed as mean±SEM of three independent experiments and each experiment were tripled. Data between two groups were statistically evaluated by Student's t-test. P <0.05 was presents as statistically significant difference.

Results

Skin wound recovery of diabetic mice is slow after injury

to evaluate the wound closure rate of control mice and diabetic mice a 5mm full-thickness round cut on the back was made, which could be normally healed within 11 days. As shown in the images in **Figure 1A** and **1B**, the wound closure of diabetic mice was continuously slower than that of control group. The wound area of DM was significantly larger than that of CON on days of 3 ($P < 0.05$), 5 ($P < 0.01$), 7 ($P < 0.05$), 9 ($P < 0.05$), and 11 ($P < 0.01$). On the 11^{th} day of healing, the wound of CON was completely healed, while the DM still had obvious wounds. Then, the thicknesses of epidermal and dermal were assessed. The epidermal thickness of DM exhibited a significant reduction over 60% than CM wound ($P < 0.01$, **Figure 1B**). DM group displayed an ~35% reduction in dermal thickness compared with CON group ($P < 0.05$, **Figure 1C**). Moreover, we also calculated the number of endothelium blood vessels in DM and CON. **Figure 1D** showed that the number of endothelium blood vessels in DM was also significantly less than that of normal mice ($P < 0.01$) at 7-days after injury. Taken together, the DM exibited a slower recovery rate, thinner epidermis and dermis, and less regeneration of blood vessels than normal mice.

mTOR inhibitor INK128 promotes skin wound healing and angiogenesis in diabetic mice

The wound recovery rate of DM and DM+INK128 was assessed. As shown in **Figure 2A** that the wound healing rate in DM+INK128 did significantly increased than DM. The wound area of DM was significantly larger than that of DM+INK128 on days of 5 ($P < 0.05$), 7 ($P < 0.01$), 9 ($P < 0.05$), and 11 ($P < 0.05$). The epidermal thickness of DM was similar with that of DM+INK128 ($P > 0.05$, **Figure 2B**), whereas DM displayed a significant reduction in dermal thickness compared to DM+INK128 ($P < 0.05$, **Figure 2C**). **Figure 2D** showed that the number of endothelium blood vessels in DM was also significantly less than that of DM+INK128 ($P < 0.01$) at day 7, the proliferative phase after injury. These results suggested that INK128 could promote diabetic skin wound healing.

The percentage of MDSCs is increased in high glucose environment

The key for proper wound healing is whether the various reactions in the inflammatory can transition to proliferative phases appropriately (22). As reported that the MDSCs in peripheral blood mononuclear cell (PBMC) of type I diabetic patients are significantly accumulated than healthy human (10), we doubted that whether the slow recovery of wound related to the accumulation of MDSCs. Thus, the amount of MDSCs at inflammatory (3 days after injury) and proliferative (7 days after injury) phases in BM, spleen, and PBMC were detected.

We found that the percentage of MDSCs in BM, PBMC and spleen were significantly higher in DM than that in control group both at inflammatory and proliferative phases after injury (**Figure 3A-F**; $P < 0.05$). In diabetic mice, the infiltration of MDSCs cells in the skin tissue around the wound increased on inflammatory and proliferative phases after injury through immunofluorescence staining Gr-1 of skin tissue (**Figure 3G, H**). These results indicate the persistence of inflammation during wound healing process in diabetic mice.

To investigate whether the increased glucose concentration in DM contributed to the accumulation of MDSCs, the BM cells were isolated, treated with IL-6, GM-CSF and graded glucose of 5mM, 10mM, 20mM, 30mM, 60mM, and 120mM for 4 days to generate MDSCs. Under the gradient addition of glucose in BM cells, the percentage of MDSCs exhibited an increasing trend (**Figure 3I**). Then we chose 30mM glucose concentration for further in vitro experiments, for 25-35mM is commonly recognized range in hyperglycemic study. Taken together, the results showed that MDSCs were abnormally accumulated in DM and high glucose promoted the increase of MDSCs proportion.

High glucose promotes the activation of mTOR signaling in MDSCs

To confirm the role of mTOR signaling on accumulation of MDSCs in high glucose. The activation of mTOR pathway were assessed by evaluating the protein expression of downstream signaling molecules phosphorylated mammalian target of rapamycin (p-4EBP1), 4EBP1, phosphorylated ribosomal protein S6 (p-S6) and S6, with β-Tublin as the reference. Our results showed that p-4EBP1 and p-S6 were significantly highly expressed in MDSCs isolated from diabetic mice than control mice (**Figure 4A**). Moreover, the protein expression of p-4EBP1 and p-S6 in BM-derived MDSCs under the presence of 5mM and 30mM glucose were also evaluated. The results showed that p-4EBP1 and p-S6 were highly expressed in 30mM than that in 5mM (**Figure 4B**). These results indicated that mTOR signaling in MDSCs was activated in high glucose microenvironment.

mTOR inhibitor INK128 inhibist the accumulation of MDSCs in high glucose

To explore the role of mTOR on MDSCs expansion, gradient doses (0nM, 25nM, 50nM, and 100nM) of INK128 was added to BM in the process of generating MDSCs. **Figure 5A** showed that the percentage of MDSCs under addition of 25mM, 50mM and 100mM INK128 was significantly decreased compared to control, which demonstrated that INK128 could suppress BM cells differentiate into MDSCs. BM cells under treatments of 5mM glucose, 30mM glucose, and 30mM glucose + 50nM INK128 showed that high glucose promoted MDSCs expansion, which can be inhibited by INK128 (**Figure 5B**).

To further confirm INK128 inhibit the accumulation of glusoce-induced MDSCs in vivo, STZ-induced diabetic mice were treated with vehicle and 1mg/kg INK128 for 45 days. The percentage of MDSCs in BM, PBMC and spleen was significantly lower in DM+INK128 group than that in DM group both at inflammatory and proliferative phases after injury (**Figure 5C-I**; $P < 0.05$). In INK128 treated diabetic mice, the infiltration of MDSCs in the skin tissue around the wound significantly decreased at inflammatory and proliferative phases after injury through immunofluorescence staining Gr-1 of skin tissue (**Figure 5J, K**).

Taken together, these results suggested high glucose caused the accumulation of MDSCs on mTOR-dependent manner and INK128 inhibited the expansion of MDSCs in vitro and in vivo.

INK128 suppresses functional gene expression of high glucose-induced MDSCs

To examine whether the function of MDSC cells was changed in diabetic mice, the MDSCs were isolated from spleens and the expression of several functional molecules including p47phox, gp91phox, arginase-1 (Arg-1), and inducible nitric oxide synthase (iNOS) were detected. The results showed that the expression levels of them were significantly higher in diabetic mice than that in control mice ($P < 0.05$; **Figure 6A-C**). Moreover, the effect of INK128 on MDSCs function was also evaluated in vitro. The gene expression levels of Arg-1, iNOS, and IL-6 were assessed in MDSCs supplemented with glucose of 5mM, 30mM, and 30mM+INK128. These genes presented a higher expression level in 30mM glucose than that of 5mM and INK128 suppressed the elevation of gene expression in 30mM group ($P < 0.05$; **Figure 6D-F**). Together, the results demonstrated that the MDSCs function were disordered in DM and high glucose environments, which could explain for the slow wound healing. Moreover, INK128 could help retrieve their function, thus promote wound healing.

INK128 inhibits high glucose-induced differentiation of MDSCs into macrophages

Macrophages are considered as the primary effector cells in regulating wound healing, unregulated macrophage activation represent a source of excessive inflammation, leading to aberrant wound healing (23-25). MDSCs have the potential to differentiate to macrophages in chronic inflammation (26). Diabetes presents a systemic inflammatory state. It is unclear whether high glucose promotes macrophage development and mTOR signaling is involved. Therefore, the amount of macrophage in BM and spleen of CON, DM, DM+INK128 were detected. **Figure 7A-D** showed that the percentage of $CD11b^{+}F4/80^{+}$ macrophages increased in diabetic mice compared with control group and INK128 reduced macrophages in DM ($P < 0.05$). Moreover, in the skin tissue around the wound of diabetic mice showed massive macrophage infiltration which was mitigated by INK128 treatment (**Figure 7E, F**).

To explore whether mTOR signaling was involved in differentiation of MDSC cells into macrophages in high glucose in vitro, BM cells were incubated with IL-6, GM-CSF as well as 5mM glucose, 30mM glucose, and 30mM glucose + INK128 for 4 days. The MDSCs treated with 30mM glucose displayed a higher macrophage amount than that of 5mM glucose and 30mM glucose + INK128 (**Figure 7G**), which demonstrated that high glucose could promote MDSCs differentiated into macrophage, which was on a mTOR-dependent manner. Some studies have demonstrated that S100A8 and S100A9 proteins are directly involved in inhibiting MDSCs maturation (26). Our result showed that INK128 significantly increased the expression levels of S100A8 and S100A9 ($P < 0.05$; **Figure7H**). Taken together, high glucose promoted MDSCs to differentiate into macrophage, and INK128 suppressed the differentiation.

INK128 reduces M-MDSCs differentiated into pro-inflammatory macrophages induced by high glucose

The phenotype of mice MDSCs is $CD11b^{+}Gr\text{-}1^{+}$, which can be further divided into two subtypes, including G-MDSCs and M-MDSCs. It was reported that M-MDSCs is the subtype which can differentiate into macrophages, therefore, we detected the percentage of M-MDSCs. **Figure 8A-D** demonstrated that the percentage of M-MDSCs in DM was significantly higher than that in CON and INK128 reduces the M-MDSC in DM ($P < 0.05$). *In vitro*, the percentage of M-MDSCs decreased within the increace of INK128 (**Figure 8E**). Moreover, the percentage of M-MDSCs in 30mM glucose group was significantly higher than that in 5mM glucose and 30mM glucose + INK128 ($P < 0.01$; **Figure 8F**). In summary, the results suggested the percentage of M-MDSCs increased in diabetes and high glucose environment, which can be inhibited by INK128.

Macrophages can be divided into pro-inflammatory (M1) and anti-inflammatory (M2) types. In the early stage of wound formation, M1 macrophages infiltrate the periwound tissue, swallow pathogens and necrotic tissue, and play a cleaning role. In the later stage, M2 macrophages cells perform repair functions. The continuous presence of M1 type causes persistence of inflammatory state poor wound healing.(27) In our study, inflammatory cell model BMDM and RAW264.7 were co-cultured with MDSCs pretreated with glucose 5mM, 30mM, and 30mM+INK128 (**Figure 8G**). Then, the expression of M1 macrophage markers, i.e., IL-6, and iNOS in BMDM cells, IL-6 and IL-1β in RAW264.7 cells were detected. The expression of M2 macrophage markers, i.e., CD206, IL-10 in BMDM cells, CD206 and IGF-1 in RAW264.7 cells were detected. The result showed that the relative expression levels of CD206 and IL-10 were significantly lower, and IL-6 and iNOS were significantly higher under 30mM glucose treatment in BMDM than that under 5mM glucose and 30mM glucose+INK128 (**Figure 8H**). The relative expression levels of CD206 and IGF-1 were significantly lower, and IL-6 and IL-1β were significantly higher under 30mM glucose treatment in RAW164.7 than that under 5mM glucose and 30mM glucose+INK128 (**Figure 8I**). These results indicated that MDSCs from high glucose environment promoted macrophages differentiate towards M1 type and INK128 suppressed the effect of high glucose. Taken together, these findings demonstrated that high glucose caused M-MDSCs to differentiate into M1 type which can be inhibited by INK128.

Discussion

The skin of diabetic patients is easily damaged and difficult to cure, which troubles diabetic patients and affects their lives and health. Zhang et al. (28) reported that MDSCs ameliorated acute kidney injury and the protective effect was enhanced by mTOR signal inhibition. As MDSCs was significantly increased in type I diabetic patients (10, 29), we wonder whether the MDSCs could promote wound recovery of diabetic patients through mTOR-dependency way. In the present study, we obtained the following conclusions: 1) MDSCs were abnormally accumulated in diabetic mice and high glucose environment; 2) mTOR signaling pathway promotes abnormal accumulation of MDSCs, moreover, the mTOR inhibitor INK128 can alleviate wound healing by regulating the accumulation of MDSCs; 3) the dysfunction of MDSCs in diabetic mice and high glucose environment leading to difficult wound healing; 4) It was the high glucose environment that promoted M-MDSCs to differentiate into pro-inflammatory macrophages,

resulting in difficult wound healing. In the present study, the therapeutic action of mTOR inhibitor INK128 for healing of diabetic wound was covered for the first time. Moreover, these findings provide important theoretical basis for treating diabetic patients with difficult wound healing.

In the study, we found that the diabetic mice had a slower recovery rate, thinner epidermal and dermal, and less blood vessels than comtrol mice. In the previous studies, the healing methods is mainly around increasing angiogenesis, and proliferation of endothelial cells (2, 3). While, in the present study, we investigated the connection of wound healing with MDSCs, and tried to find another molecular method to enhance wound recovery. The key for wound healing is whether the various reactions in the inflammatory and proliferative phases can be completed on time and appropriately (22), and the MDSCs was detected to be highly accumulated at inflammatory and proliferative phases in diabetic mice and cells in high glucose. The result was consistent with that in diabetic patients (29). Moreover, we found that the MDSCs were increased in multiple organs (bone marrow, PBMC, and spleen) of the diabetic mice. Furthermore, we found that the MDSCs amount were correlated positively with the supplementary of glucose, which might indicate that high glucose in diabetic mice is the main factor responsible for MDSCs accumulation.

The increased mTOR activity is related to insulin resistance and short-term treatment with rapamycin can led to an increase of insulin sentitivity, thus ameliorate diabetic mellitus. Therefore, we speculated that mTOR inhibitor treaiment could promote diabetic wound healing by regulating MDSCs. However long-term and chronic mTOR inhibiton by rapamycin or other rapalogs has been associated with glucose intolerance (30). Since the drug failure of rapamycin may due to incomplete mTOR suppression, INK128 was selected in our study for its ability to more effectively inhibit mTORC1, and to inhibit mTORC2 additionally(31). As expected, the results in our present study confirmed that mTOR signaling could promote the MDSCs. When applied the mTOR inhibitor INK128 to diabetic mice and cells in high glucose, the amount of MDSCs was reduce, and the wound healing rate was improved. we demonstrated that the INK128 promoting wound healing through two ways in diabetic mice. Firstly, the function of MDSCs disordered in diabetic mellitus. We found that the level of ROS (up-regulated with P47 and GP91) produced by MDSCs in diabetic mice was significantly higher. The expression of Arg-1 and iNOS in MDSCs also increased significantly. Moreover, the gene expression levels of Arg-1, iNOS, and IL-6 in cells supplemented with high glucose *in vitro* was also significantly highly expressed. Under proinflammatory conditions, human islets produce and release IL-1, resulting in inhibition of β-cell function (32). Besides, stimulation of mTOR in immune cells, such as NKs and CD8 + T cells, amplifies their functions, potentially exacerbating immune-mediated β-cell damage and dysfunction (13, 33). Elevated number of CD8 + T cells are implicated in the pathogenesis of TIDM. In humans and experimental animals with T2DM, migration to and infiltration of pancreatic islets with immune cells, especially macrophages, can be elevated. Secondly, mouse macrophages readily express iNOS in response to LPS and IFN-γ, and for this reason, it is recognized as an M1 macrophage marker in mice (34). In the present study, the expression of iNOS was significantly higher in high glucose cells. M1 macrophages express CD86, and produce high levels of ROS and pro-inflammatory cytokines, including IL-6 (35), which was highly expressed in high glucose environment. Moreover, the M2 macrophages markers, such as CD206, IL-10, and IGF-1, with their expression significantly decreased in high glucose environment (36). In the wound

healing process, M1 pro-inflammatory macrophages was supposed to conversed to M2 anti-inflammatory macrophage. While, in diabetic mice, their function is improperly regulated, caused a prolonged M1 macrophage presence and inefficient transition to the M2 phenotype, with diabetic mice retained pro-inflammatory characteristics at day 10 after injury (37). Notably, as the gene expression in 30mM + INK128 group have the consistent level with low glucose group, we suspected that the INK128 might promete macrophages into M2 pro-inflammatory phenotype, moreover, it might promote the transition from M1 to M2 macrophage, thus promoting wound healing. Moreover, mTOR suppression influenced the differentiation of MDSCs, which further confirmed that mTOR-dependency way of MDSCs to regulete wound healing of diabetic mice. Some studies have shown that S100A8 and S100A9 proteins are directly involved in inhibiting the maturation of MDSCs (17), thus our study indicated that mTOR inhibitor INK128 have obvious effect on suppressing MDSCs to M1 macrophages. Together, the result revealed that injury caused M-MDSCs accumulation in in diabetic mice, which differentiated in M1 macrophages, thus suppressing wound healing. Conversely, mTOR inhibitor INK128 could decrease the percentage of M-MDSCs, recover the function of MDSCs, and inhibit M-MDSCs differentiated to M1 macrophage thus achieving the purpose of promoting wound healing of diabetic mice.

Conclusion

In summary, we demonstrated that the number and function of MDSCs in diabetic mice was disorder. MDSCs in high glucose were abnormally accumulated and tend to differentiate into M1 macrophages, thus inhibited wound healing in an mTOR-dependent way. mTOR inhibitor INK128 could reverse high glucose induced changes of MDSCs, thus accelerate wound healing process. Taken together, these findings highlight that INK128 is a potential therapeutic strategy to promote diabetic wound healing.

Abbreviations

HE
Hematoxylin-eosin staining; iNOS:Inducible nitric oxide synthase; IL-6:Interleukin-6; IL-10:Interleukin-10; IL-1β:Interleukin-1β; IGF-1:Insulin like growth factor-1; TNF-α:Tumor necrosis factor-α; TGF-β:Transforming growth factor-β; ROS:Reactive oxygen species; Arg-1:Arginase-1; GM-CSF:Granulogyte-macrophage colony-stimulating factor

Declarations

Acknowledgements

Not applicable

Authors' contributions

Yi Li, Yujun Xu and Xinghen Liu conducted experiments, Xin Yan and Yue Lin participated in the interpretation and writing of the manuscript, Qian Tan and Yayi Hou designed the experiments, reviewed

research data and interpreted the results.

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Available of data and materials

All data generated or analysed during study are included in the article.

Ethics approcal and consent to participate

All experiments were carried out in accordance with the national guidelines. The animal care protocols was approved by Nanjing University Animal Care Committee following the ‘Guide for the Care and Use of Laboratory’. Animals as published by the Chinese National Institutes of Health. The permit number of the experimental animal was SCXK (Jiangsu) 2019-0056.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

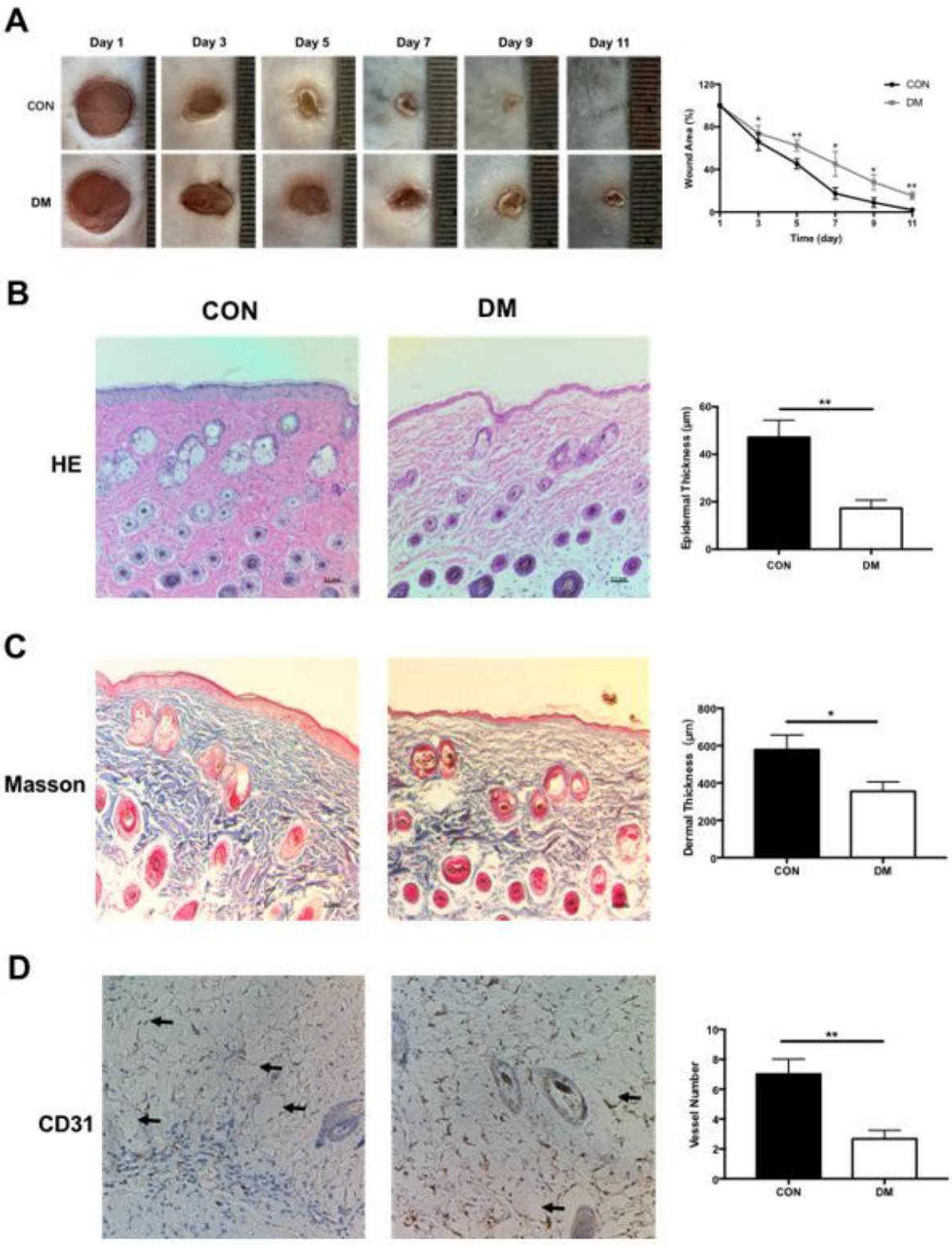

Figure 1

Skin recovery and evaluation of CON and DM after injury. (A) Image of representative wound and area of wound on different days. (B) HE staining showed the thickness of epidermis, dermis in control mouse and diabetic mouse. (C) Masson staining showed the thickness of epidermis, demis in control mouse and diabetic mouse. (D) immunohistochemical staining of CD31 showed the number of endothelium blood

vessels of the normal wound and DM wound at 7-days after healing. All data were expressed as the mean ± SEM. n = 3, * P < 0.05; **P < 0.01. DM, diabetic mice; CON, control mice; HE, hematoxylin eosin.

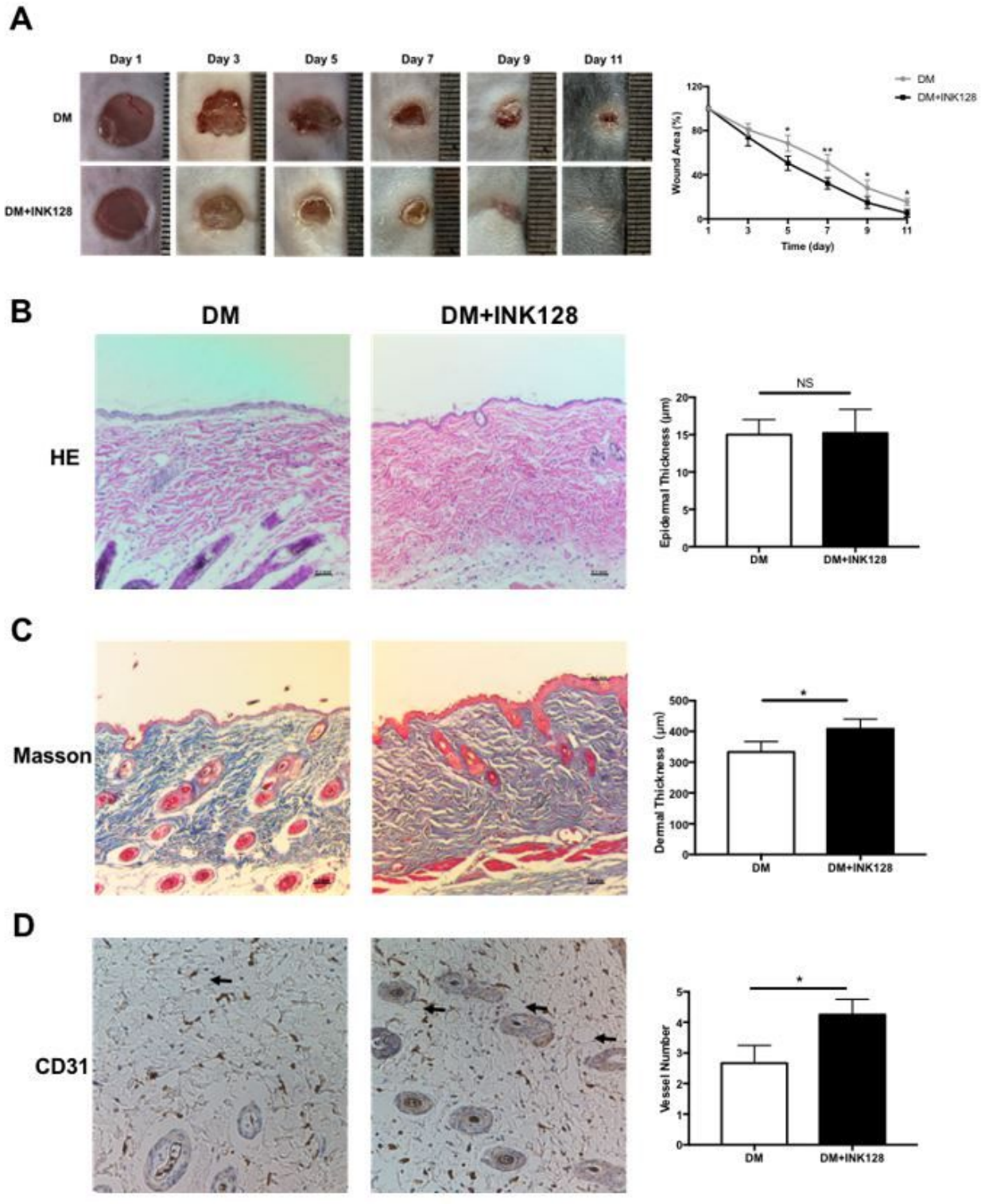

Figure 2

mTOR inhibitor INK128 promotes skin recovery of DM after injury. (A) INK128 decreased the area of wound on different days. (B) HE staining showed the effect of INK128 on the thickness of epidermis in DM. (C) Masson staining showed INK128 promoted the thickness of dermis in DM. (D)

Immunohistochemical staining of CD31 showed that INK128 promoted the number of endothelium blood vessels of DM wound at day 7 after injury. All data were expressed as the mean ± SEM. n = 3, * P < 0.05; **P < 0.01. DM, diabetic mice; DM+INK128, the diabetic mice treated with INK128.

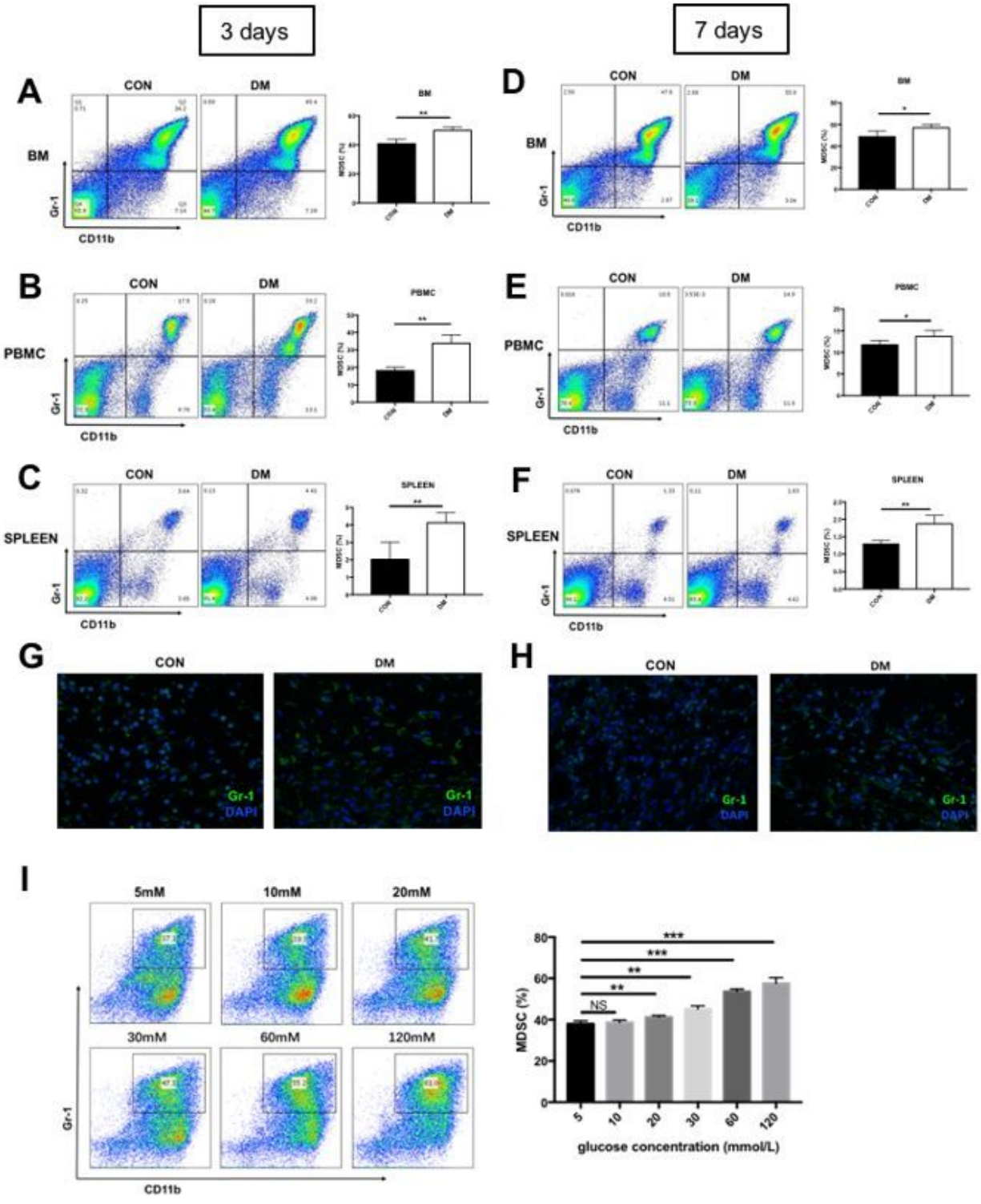

Figure 3

Accumulation of MDSCs in DM and promotion of high glucose on MDSCs. (A-C) The accumulation of MDSCs was showed by flow cytometry in bone marrow, PBMC and spleen of DM and CON at day 3 after

injury. (D-F) The accumulation of MDSCs in bone marrow, PBMC and spleen at day 7 after injury was showed by flow cytometry. (G, H) MDSCs of skin tissue around the wound at day 3 and day 7 after injury were investigated by immunofluorescence staining. (I) The percentage of MDSCs was analyzed by flow cytometry in bone marrow cells from CM treated with the graded concentration of glucose in vitro. MDSCs, myeloid-derived suppressor cells; BM, bone marrow; CON, control mice; DM, diabetic mice; PBMC, peripheral blood mononuclear cell.

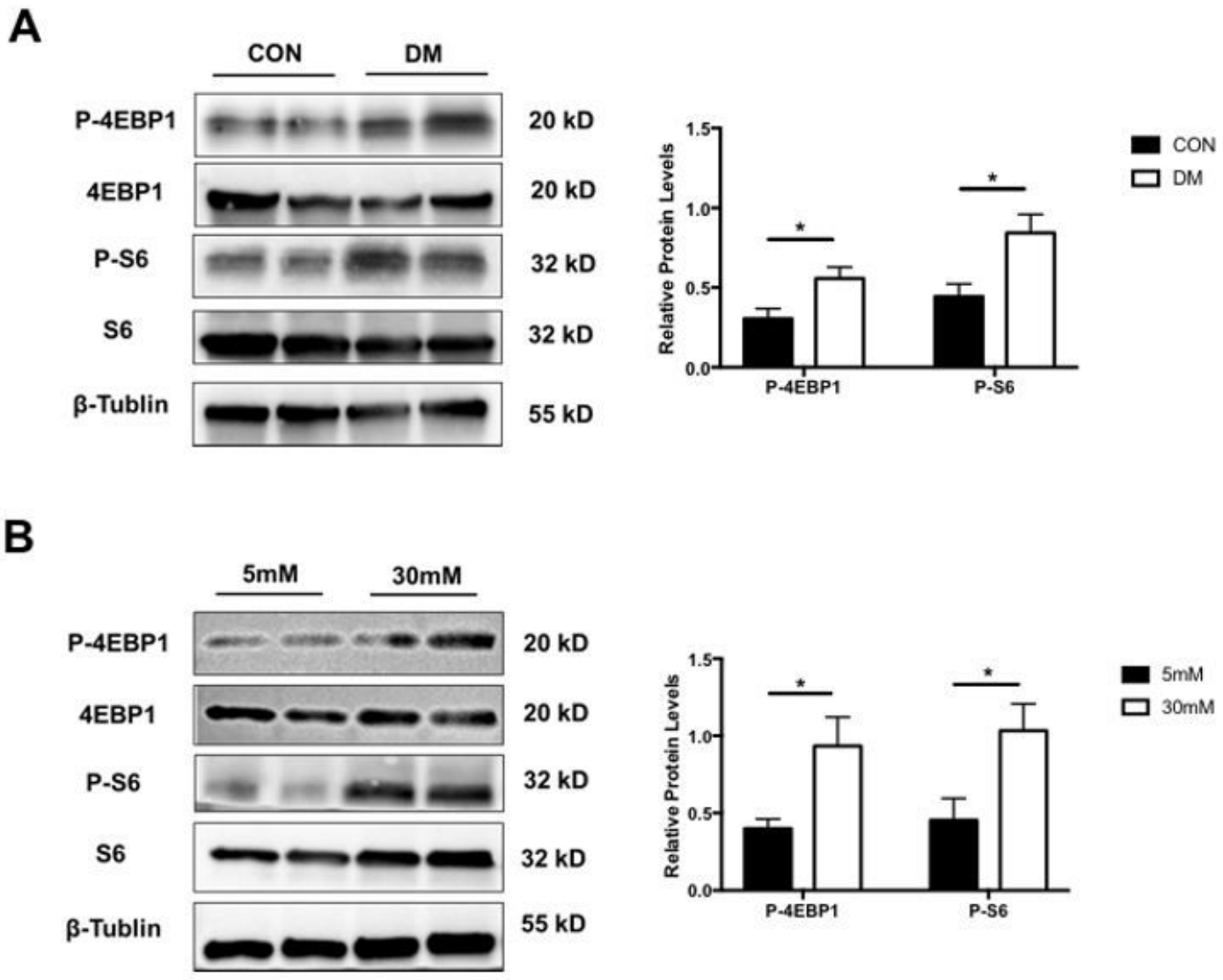

Figure 4

The expressions of the 4EBP1 and S6 along with p-4EBP1 and p-S6 proteins in MDSCs. (A) The expression of 4EBP1, S6, P-4EBP1 and P-S6 was analyzed by Western blot in MDSCs from spleen of CON and DM. (B) The expression of 4EBP1, S6, P-4EBP1 and P-S6 was analyzed by Western blot in MDSCs from bone marrow under presence of 5mM and 30Mm glucoses, respectively. MDSCs, myeloid-derived suppressor cells; CON, control mice; DM, diabetic mice; P-4EBP1, phosphorylated mammalian target of rapamycin; P-S6, phosphorylated ribosomal protein S6. The experiments were tripled. β-Tublin was used as the reference.

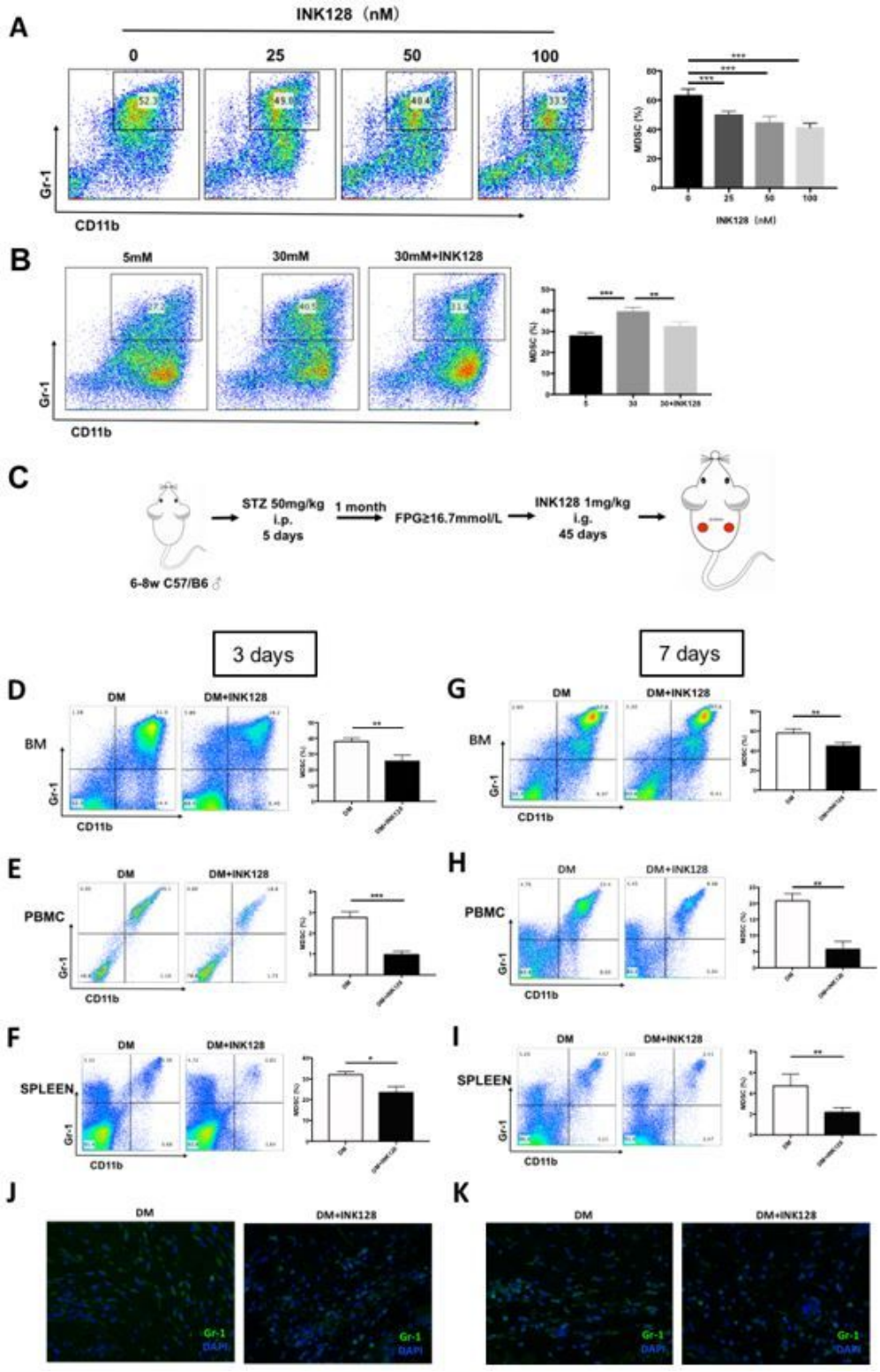

Figure 5

mTOR inhibitor INK128 inhibits accumulation of MDSCs. (A) INK128 inhibited the proliferation of MDSCs in vitro. (B) INK128 decreased the accumulation of glucoses-induced MDSCs in vitro. (C) The construction process of DM and DM+INK128 models. (D-F) INK128 inhibited the accumulation of MDSCs in marrow, PBMC and spleen of DM at day 3 after injury. (G-I) INK128 inhibited the accumulation of MDSCs in marrow, PBMC and spleen of DM at day 7 after injury. (J, K) Immunofluorescence staining

showed that INK128 decreased the accumulation of Gr-1+ cells in skin tissue around the wound of DM at day 3 and day 7 after injury. MDSCs, myeloid-derived suppressor cells; PBMC, peripheral blood mononuclear cell; DM, diabetic mice.

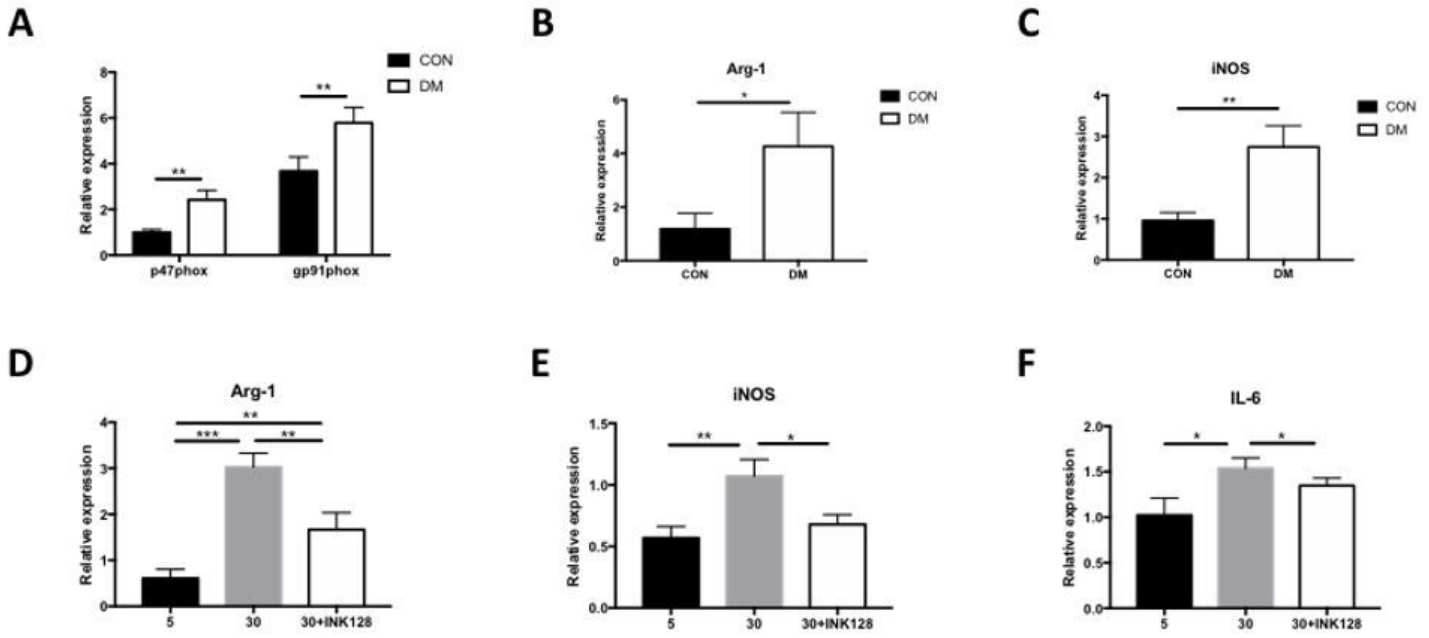

Figure 6

mTOR inhibitor INK128 suppresses the expression of genes related to MDSC functions. (A-C) The expressions of p47phox, gp91phox, Arg-1, iNOS, mRNA were increased in MDSCs purified from the spleen of DM by qRT-PCR analysis. (D-F) INK128 decreased the expression of Arg-1, iNOS, and IL-6 in glucose-induced MDSCs in vitro. * P < 0.05; **P < 0.01. CON, control mice; DM, diabetic mice.

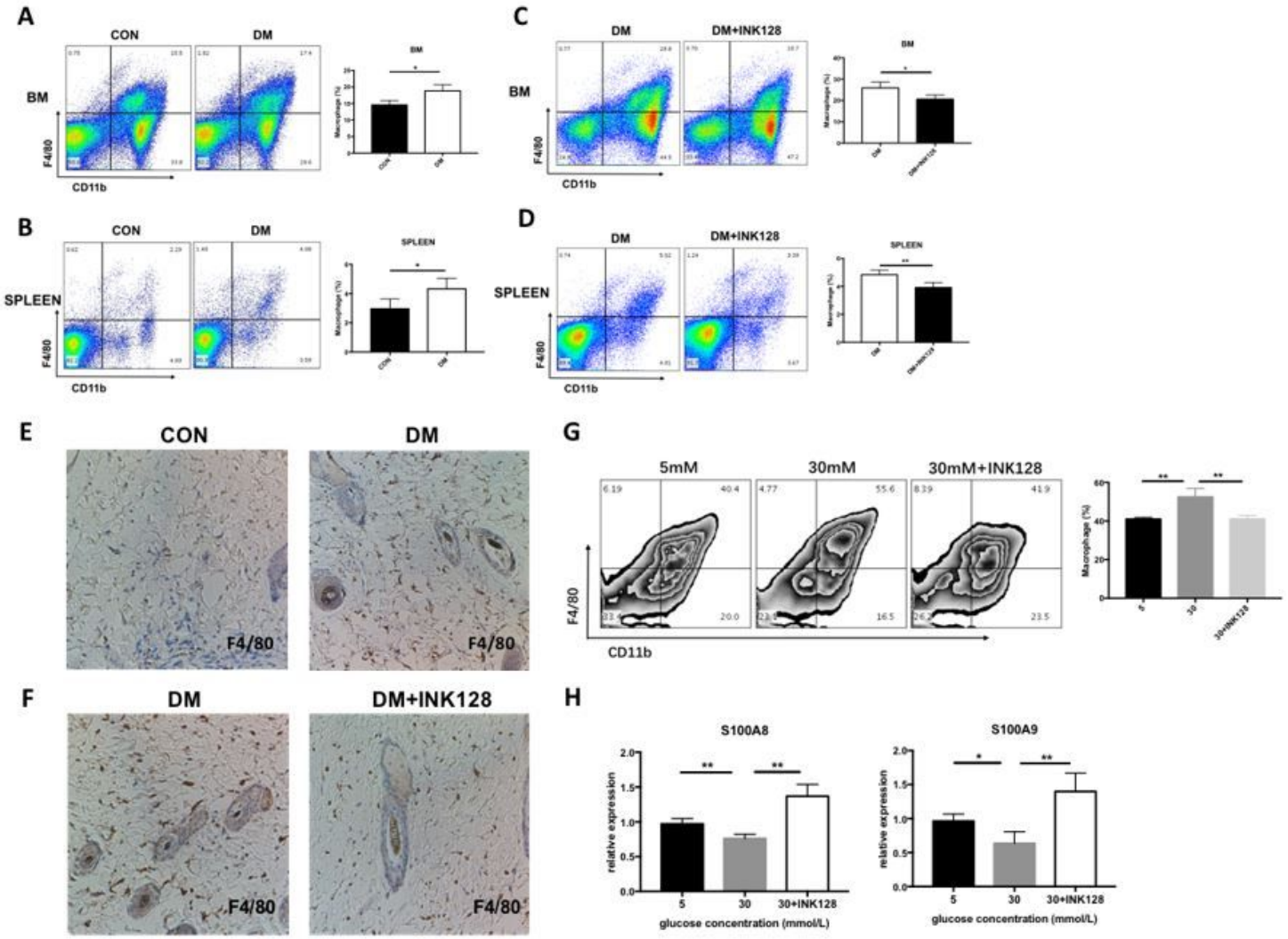

Figure 7

INK128 inhibits differentiation of MDSCs into macrophage in bone marrow and spleen of DM as well as high glucose environment. (A-B) The percentage of macrophage was increased in bone marrow and spleen of DM. (C-D) INK128 decreased the percentage of macrophage in bone marrow and spleen of DM. (E) Immunofluorescence staining showed that macrophage was increased in skin of DM. (F) INK128 decreased macrophage in skin of DM. (G) the percentage of macrophage under glucose treatments of 5mM, 30mM, and 30mM+INK128 in vitro. (H) INK128 promoted the gene expression of S100A8 and S100A9 with glucose treatments. * $P < 0.05$; **$P < 0.01$. CON, control mice; DM, diabetic mice; DM+INK128, the diabetic mice treated with INK128.

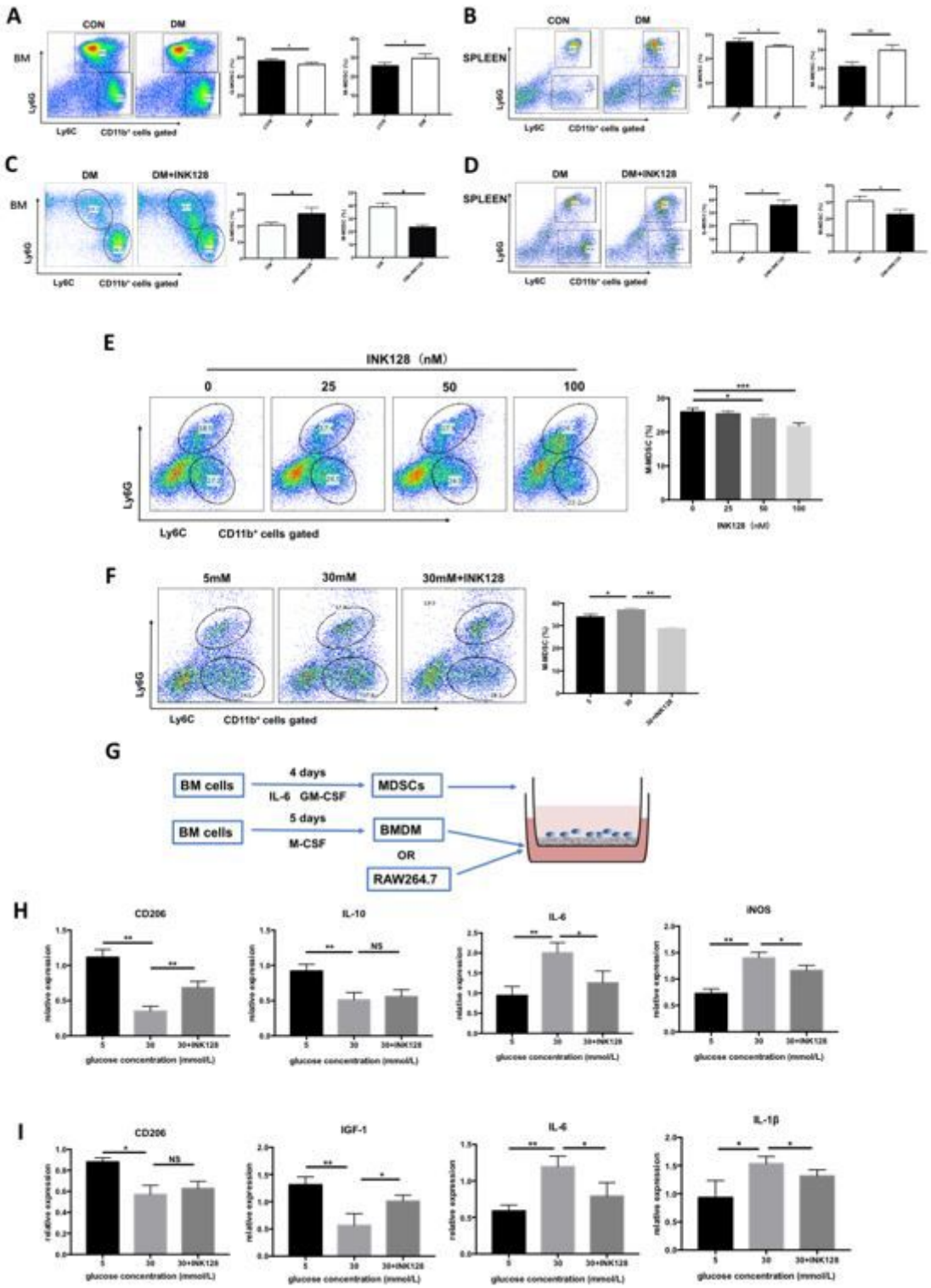

Figure 8

INK128 inhibits the differentiation of M-MDSCs into M1 macrophages in MD. (A-B) The MDSCs differentiated into M-MDSCs in bone marrow and spleen of MD. (C-D) INK128 decreased the MDSCs to differentiate into M-MDSCs in bone marrow and spleen of MD. (E-F) INK128 inhibited the differentiation of glucose-induced MDSCs into M-MDSCs in vitro. (G) The construction process in differentiation of MDSCs/M-MDSCs into macrophage models. (H-I) INK128 inhibited mainly the differentiation of glucose-

induced M-MDSCs into M1 macrophage. * P < 0.05; **P < 0.01. M-MDSCs, M-myeloid-derived suppressor cells; CON, control mice; DM, diabetic mice; DM+INK128, the diabetic mice treated with INK128.