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Methylglyoxal Potentiates AMPK-Mediated Polarization of M2d-Like Tumor-Associated Macrophages

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***Abstract*—Methylglyoxal (MGO) is a widely-used compound in food industry, which is also produced by glycolytic metabolism. It is known to be associated with multiple chronic diseases, such as type 2 diabetes, cardiovascular diseases, and cancers. Although our immune system is actively removing mutated cells, recent findings indicated that tumor-associated macrophages (TAMs) could induce immunosuppression and promote cancer cell proliferation in tumor microenvironment, the polarization of which was regulated by cell metabolism. Since the MGO levels can be increased by the accelerated glycolysis in tumor microenvironment, the impacts of MGO on TAM polarization should be examined to reveal its potential immunotoxicity. In the present study, the polarization of THP-1-derived macrophages to M2d-like TAMs was found to be promoted by MGO at 50 to 200 μM. The M2d phenotypes and the immunosuppressive functions were found to be induced by MGO during polarization. MGO was also found to increase AMPK phosphorylation in the M2d-like TAM model and the MGO-promoted polarization was also found to be reversed by an AMPK inhibitor. Collectively, these results not only demonstrated that MGO could potentiate AMPK-mediated polarization of M2d-like TAMs, but also indicated that the AMPK pathway might be utilized as a therapeutic target for MGO-induced immunotoxicity.**

Keywords—methylglyoxal; tumor-associated macrophages; AMPK; M2d polarization; immunosuppression; tumor microenvironment

I. INTRODUCTION

Methylglyoxal (MGO) is widely used in food industry and leather tanning, which is also known as the major antibacterial compound and grade determinant in manuka honey [1]. On the other hand, MGO can be generated as a side product in various metabolic pathways in different types of cells, and glyoxalase 1 (Glo-1) is the rate-limiting enzyme for MGO removal [2]. It is a potent inducer of advanced glycation end products (AGEs), which is toxic to different types of cells [3]. Previous studies indicated that MGO was closely associated with multiple diseases, such as diabetes, cardiovascular complications, Alzheimer's disease and cancers [4,5]. The onset and progression of these diseases are also closely associated with the imbalance and dysfunction of immune system. However, the exact effects induced by MGO on immune cells are controversial. While some evidences indicated that MGO induced proinflammatory and autoimmune responses, one recent study showed that MGO could paralyze anti-cancer immunity in tumor microenvironment [6-8]. Based on its critical roles in disease development and immune regulation, it is essential to identify the alterations induced by MGO in different types of immune cells to verify its immunotoxicity.

The special metabolic patterns in tumor tissues are the foundations to form a unique tumor microenvironment [9]. MGO can be overproduced in the tumor microenvironment as the result of high glycolysis level [10]. Tumor-associated macrophages (TAMs) are the most abundant immune cells serving as the stromal components of tumor tissues, which represent a homogenous population of both classically activated M1 macrophages and alternatively activated M2 macrophages [11]. Most of the tumor-infiltrating macrophages were identified as the M2d-polarized immunosuppressive cells with highly expressed Arginase 1 (Arg-1), IL-10 and VEGF, which could support disease progression and therapeutic resistance [12]. This kind of polarization can also be triggered in in vitro models by the synergistic effect of TLR4 agonists and adenosines [13]. Two important M2 cell surface markers, CD163 (scavenger receptor) and CD206 (mannose receptor), were also found to be significantly increased in TAMs [14]. Metabolism is known as one of the key regulators of macrophage functions and polarizations, and the AMP-

activated protein kinase (AMPK) activation has already been identified as the primary mediator of M2 polarization [15,16]. Previous studies revealed that MGO was a potent activator of AMPK pathway in neurons and endothelial cells, which is very likely to be involved in the M2d polarization of macrophages in tumor microenvironment [17,18].

Our previous studies indicated that the production of MGO could be stimulated by increased glycolytic metabolism induced by formaldehyde, a ubiquitous environmental carcinogen [19,20]. Based on the high bioactivity and immunomodulatory roles of MGO, it is important to identify the possible immunosuppression induced by MGO in tumor microenvironment. In the present study, a THP-1-derived in vitro TAM model was utilized to evaluate the effects of MGO on M2d polarization. The phosphorylation of AMPK was also analyzed to reveal the molecular basis of MGO-mediated M2d polarization, which was also confirmed using compound C, a potent AMPK inhibitor. As far as we know, it is the first report illustrating the MGO induced effects on TAM polarization.

II. MATERIAL AND METHODS

A. Chemicals and reagents

MGO (~40% in H2O), 5'-(N-Ethylcarboxamido)adenosine (NECA), Dorsomorphin (Compound C), phorbol-12-myristate-13-acetate (PMA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The endotoxin levels in the MGO, NECA, compound C and PMA were determined by endpoint chromogenic LAL assays after preparation of the stock solutions in DMSO to exclude the endotoxin-induced effects. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Lipopolysaccharide (LPS, E. coli 055:B5), CCK-8 assay kit, 100× penicillin-streptomycin were purchased from Yeasen Biotechnology (Shanghai, China). RIPA lysis solution, BCA protein assay kit, BeyoECL Moon reagent, anti-VEGF, anti-AMPKα1/AMPKα2 and anti-phospho-AMPKα(Thr172) rabbit polyclonal antibodies, HRP-labeled goat anti-rabbit IgG, HRP-labeled goat anti-mouse IgG were purchased from Beyotime (Shanghai, China). Human VEGF, IL-10 and TNF-α ELISA kits were purchased from Abcam (Cambridge, UK). Anti-IL-10, anti-TNF-α, anti-Glo-1, anti-PIK3CA, and anti-inducible Nitric Oxide Synthase (iNOS) rabbit polyclonal antibodies, anti-β-actin and anti-Arg-1 mouse monoclonal antibody were purchased from Sangon Biotech (Shanghai, China). PE mouse anti-human CD206 antibody was purchased from BD Biosciences (San Diego, CA, USA). APC anti-human CD163 antibody was purchased from Biolegend (San Diego, CA, USA).

B. Macrophage differentiation and polarization

The human monocyte cell line THP-1 was kindly provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in cell culture medium (RPMI 1640 + 10% FBS + 1× penicillin-streptomycin) and the THP-1-derived macrophages were prepared as we previously described [21]. Basically, THP-1 monocytes were treated with 200 nM PMA for 3 d, followed by a 5 d resting step in fresh cell culture medium to be differentiated into M0 macrophages. M2d polarization was induced by stimulating the M0 macrophages with 100 ng LPS and 5 μM NECA for 24 h [22]. Cells were also treated with MGO and compound C during the stimulation to examine their effects on M2d polarization and cellular functions.

C. Phenotyping by flow cytometry

After the treatments, macrophages were resuspended in 100 μL ice cold DPBS buffer in a flow cytometry tube and 1 μg of CD163 and CD206 antibodies were added to the cells. After 30 min incubation at 4 ℃ in dark, cells were washed twice with ice cold DPBS and immediately analyzed on a BD AccuriC6 Plus flow cytometer. For each individual sample, 10000 events were collected and the data was analyzed with FlowJo v10.6.1 (BD Life Sciences). The gates for CD163 and CD206 positive staining were set up with the unstained, unstimulated, and 0 μM MGO controls (Fig. 1A).

D. Protein expression analysis by Western Blot

After the MGO and compound C treatments, whole cell lysates were prepared with RIPA buffer and the lysates were loaded on polyacrylamide gels (20 μg protein/well, determined by BCA assay) in a Bio-Rad Mini-PROTEAN tetra cell contained with SDS-PAGE buffer. The power supplier was set at 120 constant voltages. After the electrophoresis, proteins were transferred onto PVDF membranes using a Bio-Rad Trans-Blot semi-dry electrophoretic transfer cell. Skim milk was prepared in TBST and used for blocking. Primary antibodies of the VEGF, IL-10 and TNF-α, PIK3CA, iNOS, Arg-1, Glo-1, AMPKα, phospho-AMPKα and β-actin were prepared in TBST and loaded onto the membranes. After incubation, the respective mouse or rabbit secondary antibodies were added to the membranes, and ECL detection reagent was the applied to develop the protein bands. The image was captured using a Bio-Rad ChemiDoc XRS+ system. ImageJ software (v1.8.0, download link: https://imagej.nih.gov/ij/download.html) was used to obtain the band intensity for statistical analysis.

E. Wound healing assay

The HCT-116 colorectal cancer cell line was kindly provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). After treating M2d cells with MGO and compound C for 24 h, cells were washed and cultured with fresh medium without MGO or compound C for another 24 h. The conditioned medium was then harvested and centrifuged at 2000 ×g for 5 min. The supernatant was used as the conditioned medium for the wound healing assay. After the HCT-116 cells formed a confluent monolayer, a straight scratch was created with a 200 μL pipette tip. The cells were washed twice with DPBS and the conditioned medium was added to the cell culture plates. Images were captured on an XD-202 inverted microscope (Jiangnan Novel Optics) at 0 h and 24 h time points. Cell migration was calculated from the changes of the scratch area acquired with ImageJ software at the two time points [23].

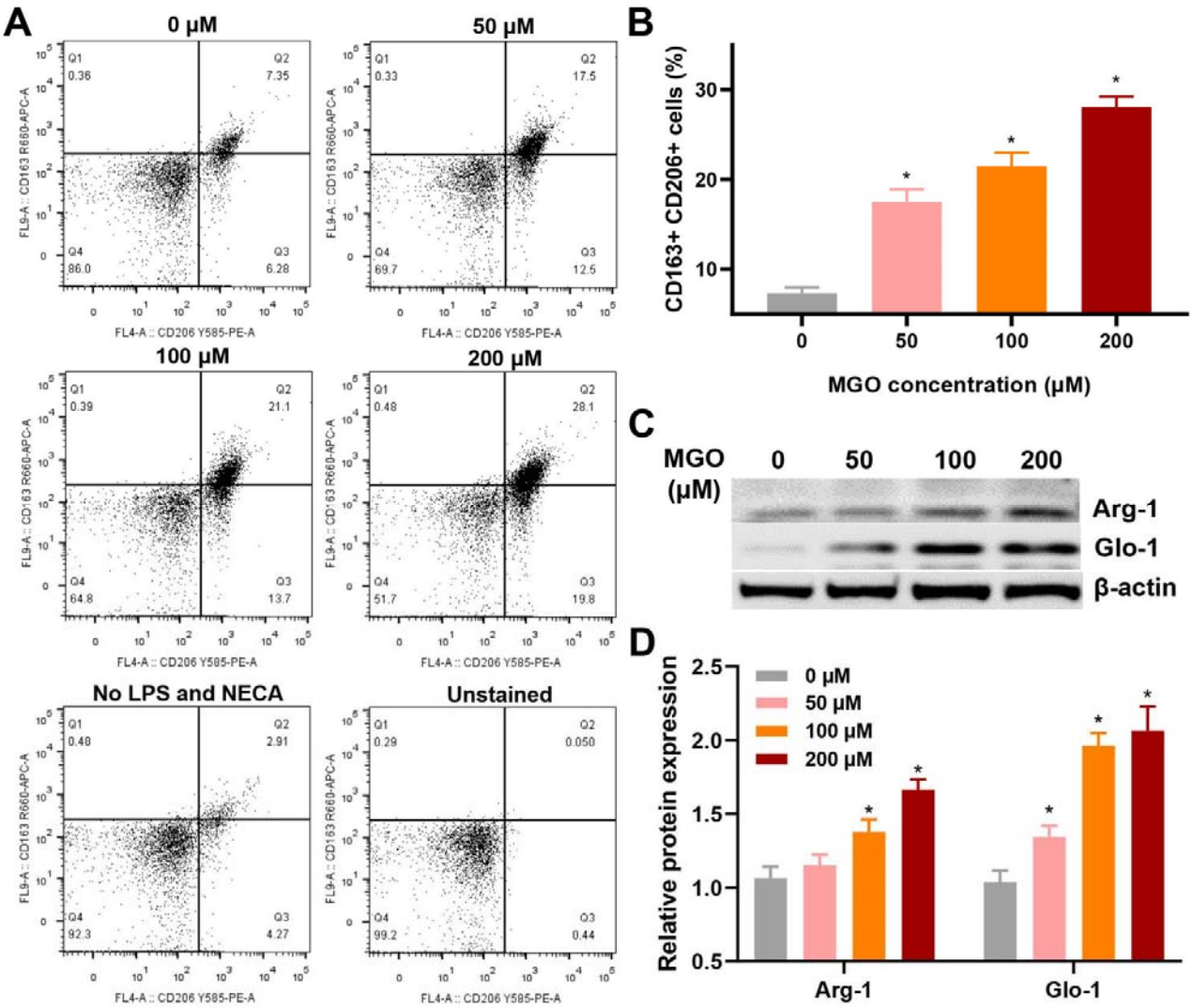

Fig. 1. MGO promoted the phenotypic polarization of THP-1-derived macrophages to M2d-like TAMs. THP-1 cells were treated with 200 nM PMA for 3 d, followed by a 5 d resting step in fresh cell culture medium to be differentiated into M0 macrophages. M2d polarization was induced by stimulating the M0 macrophages with 100 ng LPS and 5 μM NECA for 24 h. Noncytotoxic doses of MGO was also added during the polarization, and flow cytometry and WB assays were used to explore the effects induced by MGO on M2d polarization. A, representative flow cytometry plots for each dose of MGO. B, statistical analysis of the CD163+ CD206+ double positive population. C, representative blots from three independent WB experiments of Arg-1 and Glo-1. D, relative Arg-1 and Glo-1 protein expressions. Results are mean ± SD. *Significantly different from the 0 μM MGO group ($p < 0.05$).

F. Statistical analysis

All data was analyzed with Excel 2016 and GraphPad Prism v8.0.1. Three independent experiments were performed and analyzed for each individual dose of MGO, compound C and the combined treatment. One-way analysis of variance (ANOVA) and Dunnett's t test were applied to determine statistical differences between groups.

III. RESULTS

A. MGO promoted the phenotypic polarization of THP-1-derived macrophages to M2d-like TAMs

Our recent findings showed the accelerated glycolytic rate could increase the cellular production of MGO in tumor and immune cells by up to 100 μM [19,20]. However, the biological roles of MGO in tumor microenvironment are still not clear. Since TAMs are the most abundant immune cells in tumor microenvironment, the THP-1-dereived *in vitro* model of M2d-like macrophages were developed by LPS and NECA stimulation and utilized to reveal the effects induced by 50 to

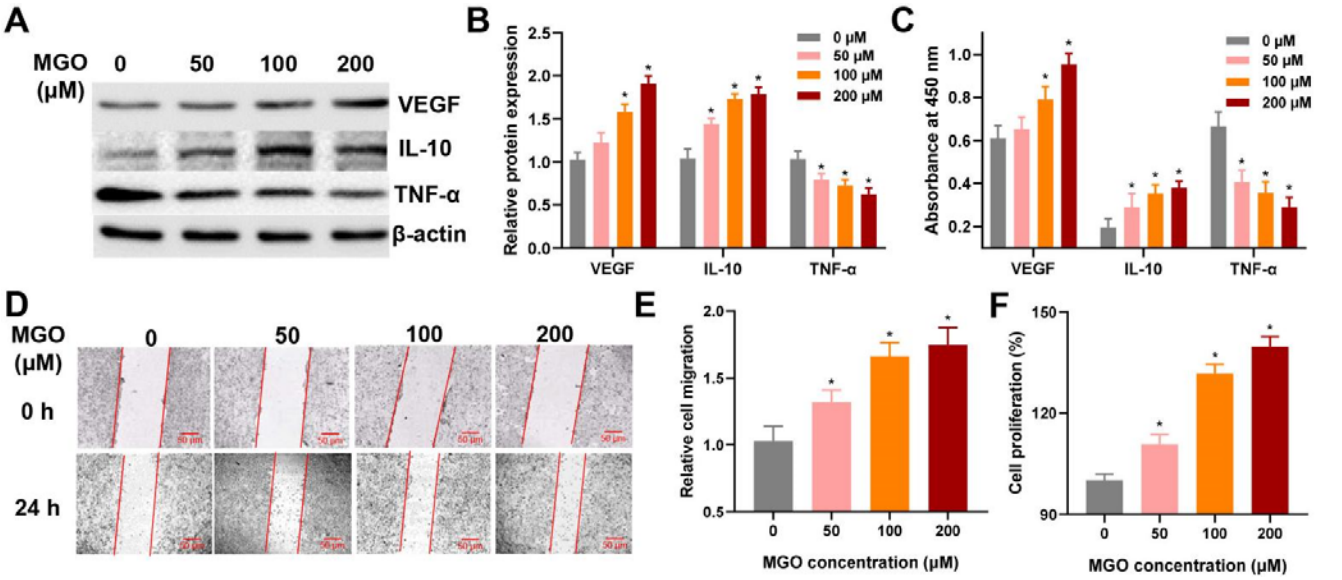

Fig. 2. MGO enhanced M2d-associated functions in THP-1-derived macrophages. THP-1 cells were treated with 200 nM PMA for 3 d, followed by a 5 d resting step in fresh cell culture medium to be differentiated into M0 macrophages. M2d polarization was induced by stimulating the M0 macrophages with 100 ng LPS and 5 μM NECA for 24 h. Noncytotoxic doses of MGO was also added during the polarization. Cytokine releases were analyzed by WB and ELISA assays, and conditioned medium was also prepared to perform wound healing and cell proliferation assays with HCT-116 cells. A, representative blots from three independent WB experiments of VEGF, IL-10 and TNF-α. B, relative VEGF, IL-10 and TNF-α protein expressions. C, absorbance at 450 nm indicating relative VEGF, IL-10 and TNF-α protein expressions in ELISA assays. D, representative images of the wound healing assays with conditioned medium collected from the polarized M2d cells treated with various concentrations of MGO. E, relative cell migration calculated from the images. F, cell proliferation measured by CCK-8 assays with the conditioned medium. Results are mean ± SD. *Significantly different from the 0 μM MGO group ($p < 0.05$).

200 μM MGO on TAM polarization. Dose-dependent increases of CD163+ CD206+ double positive population were observed in the polarized macrophages treated with MGO, indicating that MGO promoted the differentiation of TAMs (Fig. 1A and B). The expressions of Arg-1 and Glo-1 were also stimulated by MGO treatments M2d-like TAM models (Fig. 1C and D). These results not only demonstrated that MGO was able to promote the M2d polarization of macrophages at 50 to 200 μM in vitro, but also indicated that the cells were trying to eliminate MGO to alleviate the effects.

B. MGO reinforced M2d-associated functions

M2d cells are known for their immunosuppressive and tumor-promoting roles. VEGF, the key mediator of angiogenesis, and IL-10, an anti-inflammatory cytokine, are highly expressed in M2d cells [14]. Productions of both cytokines were found to be stimulated by MGO treatments in M2d cells, which were measured by WB and ELISA assays (Fig. 2A, B and C). On the contrary, TNF-α, the typical pro-inflammatory cytokine expressed by M1 macrophages, was found to be suppressed by MGO treatment in the M2d models (Fig. 2A, B and C). The wound healing assay, which measures the directional tumor cell migration in vitro, was utilized to evaluate the tumor-promoting ability of the conditioned medium collected from polarized M2d cells treated with MGO. The conditioned medium collected from MGO-treated M2d cells significantly increased cell migration, which was also confirmed by the cell proliferation assay (Fig. 2C, D and E). Together, these results indicated that MGO treatment could enhance the immunosuppressive and tumor-promoting functions of M2d cells.

C. AMPK-mediated M2d polarization was enhanced by MGO and reversed by compound C

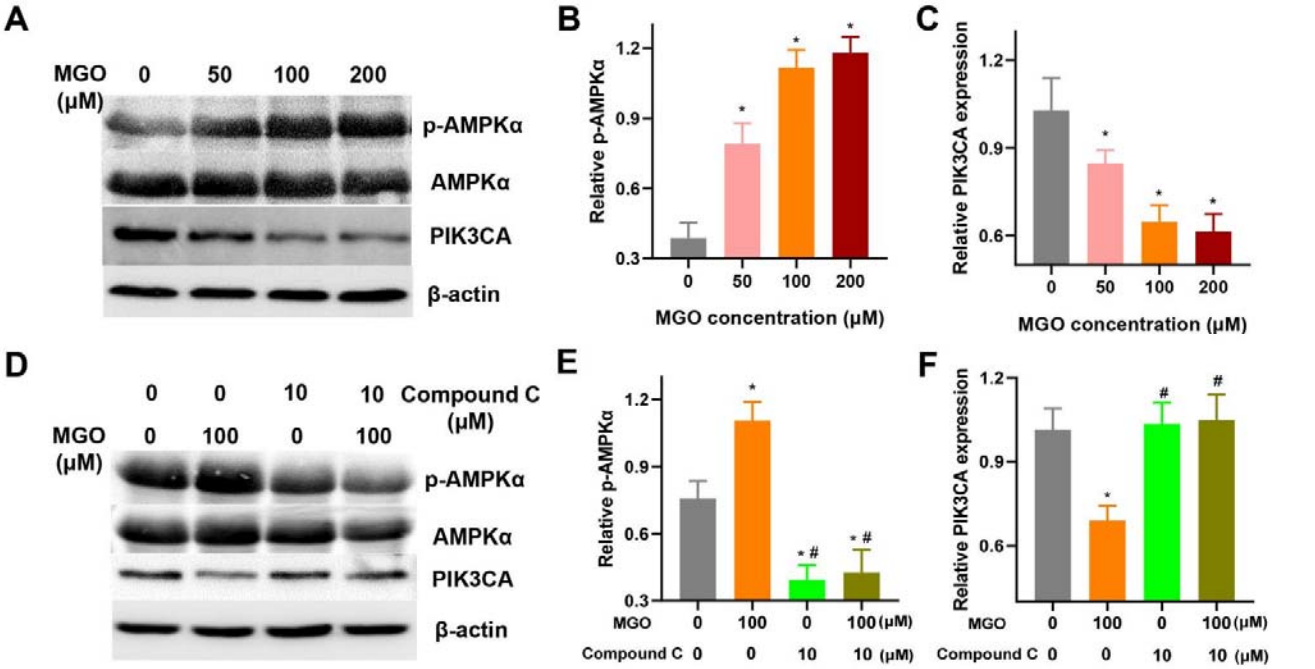

Fig. 3. Induction of AMPK phosphorylation by MGO in THP-1 derived macrophages. THP-1 cells were treated with 200 nM PMA for 3 d, followed by a 5 d resting step in fresh cell culture medium to be differentiated into M0 macrophages. M2d polarization was induced by stimulating the M0 macrophages with 100 ng LPS and 5 μM NECA for 24 h. Noncytotoxic doses of MGO and compound C were also added during the polarization. A, representative blots from three independent WB experiments of p-AMPKα, AMPKα and PIK3CA. B, relative AMPKα phosphorylation. C, relative PIK3CA expression. D, representative blots from three independent WB experiments of p-AMPKα, AMPKα and PIK3CA. E, relative AMPKα phosphorylation. F, relative PIK3CA expression. Results are mean ± SD. *Significantly different from the 0 μM MGO group ($p < 0.05$). #Significantly different from the 100 μM MGO + 0 μM compound C group ($p < 0.05$).

Since the M2d polarization was known to be mediated by AMPK signaling, and MGO was found to induce AMPK phosphorylation, the MGO-induced M2d polarization was very likely to be regulated by AMPK phosphorylation. Therefore, the phosphorylation of the α subunit of AMPK was analyzed in MGO-treated M2d cells, and significant increase of AMPKα phosphorylation was observed in cells treated with 50 to 200 μM MGO (Fig. 3A and B). The phosphatidylinositol 3-kinase (PI3K) activation was found to counteract AMPK phosphorylation, which can be measured by the expression of its catalytic subunit α (PIK3CA) [24]. Decreased PIK3CA expressions were observed in MGO-treated M2d cells, which confirmed with the increased AMPKα phosphorylation (Fig. 3A and C). A potent and widely-used AMPK inhibitor, compound C, was used to verify the AMPK stimulation induced by 100 μM MGO in M2d cells. Compound C reversed the MGO-induced AMPKα phosphorylation to a relatively low level, which was also confirmed by the restored PIK3CA expression (Fig. 3D, E and F). In addition, the phenotypic alterations induced by 100 μM MGO was also reversed by 10 μM compound C, indicating that the MGO-triggered polarization could be attenuated by the AMPK inhibitor (Fig. 4A, B and C). The M2d-associated functions, including the stimulation of tumor cell migration, accelerated proliferation, and cytokine expression, were also reversed by compound C in 100 μM MGO treated M2d cells (Fig. 4D, E and F). Collectively, these results clearly demonstrated that the MGO-enhanced M2d polarization was mediated by AMPK, which could be reversed by compound C, a potent AMPK inhibitor.

IV. DISCUSSION

Warburg effect, which is defined as the enhanced glycolysis level even when oxygen is abundant in tumor microenvironment, is known as a universal metabolic alteration in carcinogenesis. MGO is one of the most active side products in glycolysis, which is overproduced by the accelerated glycolytic metabolism. Therefore, MGO concentration in tumor microenvironment can be significantly higher than the normal tissues. In addition, MGO concentration is also known to be higher in patients in diabetic conditions [25]. Previous reports indicated that patients with type 2 diabetes were at higher risk of developing cancer as the result of the mutations induced by hyperglycemia-associated AGEs formation and MGO-related stress, which could be caused by the high MGO level [3,26]. Besides the increased probability of mutagenesis, the breakdown of immunosurveillance is another vital step in carcinogenesis. Therefore, the immunotoxicity induced by MGO on immune cells should be analyzed to reveal its impact on tumor microenvironment and immunosurveillance, which will help us to understand process of tumor onset and progression triggered by metabolism-related carcinogens and hyperglycemic conditions.

Based on previous studies, TAMs are known to play critical roles in tumor progression and immune escape. Besides their immunosuppressive roles, these alternatively polarized macrophages can also inhibit phagocytosis and tumor immunity through the expression of PD-1 [27]. Therefore, we utilized an in vitro THP-1-derived macrophage model to analyze the MGO-enhanced polarization of M2d, the most

abundant population of TAMs. The elevated secretions of IL-10 and VEGF were defined as the major markers for the identification of M2d [12]. However, as one subtype of the M2 macrophages, the expressions of other M2 markers, such as CD163, CD206 and Arg-1, were also analyzed in our study to evaluate the alterations induced by MGO on M2d polarization. Interestingly, these immunosuppression-related M2 markers were also found to be induced by MGO treatments in the M2d model, indicating that MGO might also enhance the polarization of other subtypes of M2 macrophages, which may potentially engender beneficial effects in wound healing and other conditions. In order to reveal the complete biological effects of MGO, the alterations induced by MGO on the polarization of other M2 subtypes should be confirmed in future studies.

The AMPK pathway is one of the central regulators of cellular metabolism, which is closely associated with many metabolism-associated diseases, such as type 2 diabetes, cardiovascular diseases and various types of cancers [28]. It is also known as a therapeutic target for cancers and diabetic complications [29,30]. Since AMPK activation was found to mediate the M2 polarization, we analyzed the phosphorylation of AMPKα induced by MGO in the M2d polarization model. The elevated AMPKα phosphorylation and reduced PIK3CA expression induced by MGO clearly demonstrated that MGO could activate the AMPK pathway, which was also confirmed by cellular ATP levels. However, there are many different regulators of AMPK phosphorylation, which should be

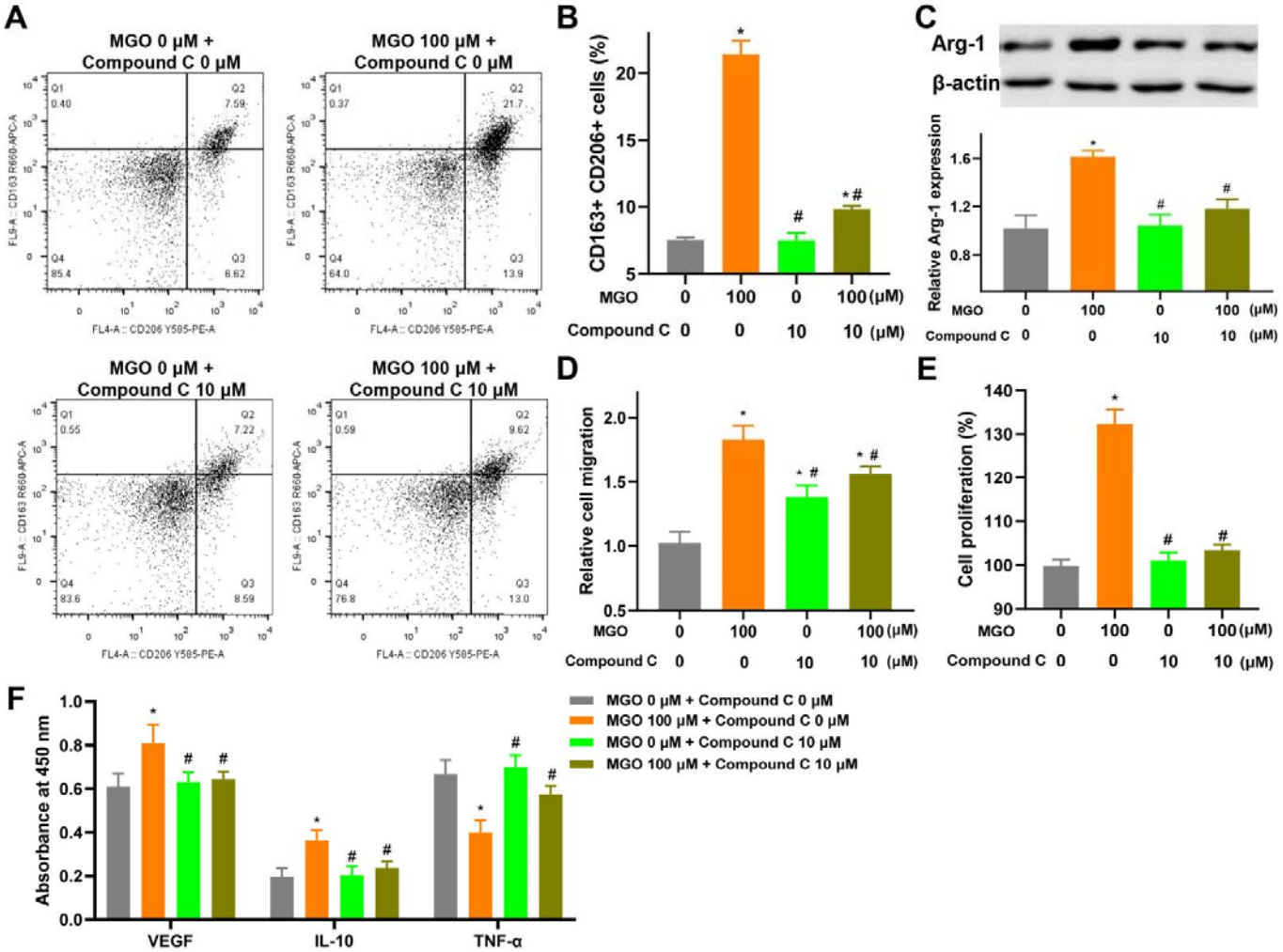

Fig. 4. Reversal of the potentiated polarization and functions by compound C. THP-1 cells were treated with 200 nM PMA for 3 d, followed by a 5 d resting step in fresh cell culture medium to be differentiated into M0 macrophages. M2d polarization was induced by stimulating the M0 macrophages with 100 ng LPS and 5 μM NECA for 24 h. Noncytotoxic doses of MGO and compound C were also added during the polarization, and flow cytometry and WB assays were used to explore the alterations induced by the two compounds on M2d polarization. A, representative flow cytometry plots for the MGO and compound C treatments. B, statistical analysis of the CD163+ CD206+ double positive population. C, representative blots from three independent WB experiments of Arg-1 and the statistical analysis of relative Arg-1 expression. D, relative cell migration calculated from the images of the wound healing assays with conditioned medium. E, cell proliferation measured by CCK-8 assays with the conditioned medium. F, absorbance at 450 nm indicating relative VEGF, IL-10 and TNF-α protein expressions in ELISA assays. Results are mean ± SD. *Significantly different from the 0 μM MGO group ($p < 0.05$). #Significantly different from the 100 μM MGO + 0 μM compound C group ($p < 0.05$).

examined in future studies to identify the other targets of MGO in cellular metabolism. On the other hand, compound C, a potent AMPK inhibitor, was found to reverse the MGO-induced M2d polarization in our in vitro model, which not only confirmed the molecular mechanism of AMPK-mediated polarization, but also indicated that the M2d model might be utilized to perform drug screening for modulatory compound of AMPK-mediated macrophage polarization in tumor microenvironment.

In summary, the polarization of THP-1-derived macrophages to M2d-like TAMs was found to be stimulated by MGO at 50 to 200 μM. Both typical phenotypes and immunosuppressive functions of M2d were found to be induced by the MGO treatments. The enhancement of the AMPK-mediated M2d polarization triggered by MGO was found to be reversed by compound C, an AMPK inhibitor, which not only confirmed the molecular basis of the MGO-induced polarization, but also indicated that the AMPK pathway could be the potential target for the reversal of MGO-induced immunotoxicity.

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