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SIRT1 Limits the Function and Fate of Myeloid-Derived Suppressor Cells in Tumors by Orchestrating HIF-1α–Dependent Glycolysis

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Abstract

Myeloid-derived suppressor cells (MDSC) display an immature phenotype that may assume a classically activated (M1) or alternatively activated phenotype (M2) in tumors. In this study, we investigated metabolic mechanisms underlying the differentiation of MDSCs into M1 or M2 myeloid lineage and their effect on cancer pathophysiology. We found that SIRT1 deficiency in MDSCs directs a specific switch to M1 lineage when cells enter the periphery from bone marrow, decreasing the suppressive function in favor of a proinflammatory M1 phenotype associated with tumor cell attack. Glycolytic activation through the mTOR–hypoxia-inducible factor-1α (HIF-1α) pathway was required for differentiation to the M1 phenotype, which conferred protection against tumors. Our results define the essential nature of a SIRT1–mTOR/HIF-1α glycolytic pathway in determining MDSC differentiation, with implications for metabolic reprogramming as a cancer therapeutic approach. *Cancer Res; 74(3); 727–37. ©2013 AACR.*

Introduction

Myeloid-derived suppressor cells (MDSC) are one of the major components of the immunosuppressive network responsible for immune cell tolerance in cancer (1–6). Polarized MDSC lineages can be distinguished as M1 and M2 cells. M2 can be induced by interleukin (IL)-4 or IL-13, and produce arginase 1 and anti-inflammatory cytokines, eventually converging to facilitate tumorgenesis (5–9). In marked contrast, M1 could be induced by lipopolysaccharide (LPS) or/and IFN-γ and produce inducible nitric oxide synthase (iNOS), nitric oxide (NO), and proinflammatory cytokines, leading to their antitumor effects (1, 2, 7). Although the roles of a distinct lineage of MDSCs have long been extensively known, how M1- and M2-MDSC lineage differentiates remains elusive.

SIRT1, a leading family member of the human orthology of yeast *Sir2*, has marked anti-inflammtory effects in several systems (10, 11). SIRT1 may limit the inflammatory process by inhibiting the expression of proinflammatory cytokines such as TNF-α (11, 12). In addition, SIRT1 could function as an anergic factor to maintain peripheral $CD4^+$ T-cell tolerance (13), prolong allograft survival (14), and inhibit Th2 response in airway allergy (15). However, basic regulatory roles of SIRT1 on the myeloid cell differentiation remain unclear.

A defining feature of immune cell activation is that it is highly anabolic and demonstrates a striking increase in glycolysis, as well as an increase in glucose and amino acid uptake (16, 17). In support of this notion, proper regulation of glucose metabolism is required for the development of immune responses (18, 19). Although a role for the metabolic pathways in immune cell activation and responses is beginning to be appreciated (20–22), little information exists about whether the basic metabolic machinery is actively regulated and contributes to MDSC differentiation.

In the present study, we reported that SIRT1 is a key factor in the regulation of MDSC differentiation into M1 and M2 phenotypes through hypoxia-inducible factor-1α (HIF-1α)–induced glycolytic metabolic reprogramming and has an impact on MDSC functions in both immune suppression and promotion of tumor progression.

Materials and Methods

Mice, chimeras, and tumor model

C57BL/6 $SIRT1^{fl/fl}$ and Lysm-Cre and $HIF\text{-}1\alpha^{fl/fl}$ mice were obtained from The Jackson Laboratory. All mice used were ages 6 to 12 weeks. Reconstitution of lethally irradiated $CD45.1^+$ congenic mice was carried out with $CD45.2^+$ bone marrow cells from SIRT1-deficient ($SIRT1^{flox/flox}$; $Cre^{+/-}$) or wild-type (WT; $SIRT1^{flox/flox}$; $Cre^{-/-}$) mice (23–25). To establish subcutaneous tumors, 5×10^5 EL-4 or B16.F10 tumor cells were injected into C57BL/6 mice. These cells formed a tumor of 1 to 2 cm diameter within 2 to 3 weeks of injection. Experimental

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protocols were approved by the Animal Ethics Committee of Fudan University (Shanghai, China).

Monoclonal antibody and flow cytometry

Flow cytometry was used to perform the analysis of cell surface markers, phosphorylated or intracellular signaling proteins, as described previously (25). Flow cytometry data were acquired on a FACSCalibur (Becton Dickinson), and data were analyzed with FlowJo (TreeStar).

MDSC cell isolation

Single-cell suspensions were prepared from spleens and bone marrow. MDSCs were isolated by cell sorting on a FACSAria (BD Biosciences) after cell staining with anti-Gr1-allophycocyanin and anti-CD11b-phycoerythrin. Tumors were dissected and digested with 0.7 mg/mL of collagenase XI (Sigma-Aldrich) and 30 mg/mL of type IV bovine pancreatic DNase (Sigma-Aldrich) for 45 minutes at 37°C. $Gr1^+$ cells were isolated by using a biotinylated anti-Gr1 antibody and streptavidin microbeads with MiniMACS columns (Miltenyi Biotech). MDSCs were isolated from tumor tissues as described previously (26).

Arginase activity and NO production

Arginase activity was measured in cell lysates; arginine hydrolysis was conducted by incubating the lysate with L-arginine. For NO production, culture supernatant or serum was incubated with Greiss reagent, as described previously (24, 27).

Real-time PCR and immunoblot analysis

RNA was extracted with the RNeasy Kit (Qiagen), and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). An ABI 7900 real-time PCR (RT-PCR) system was used for quantitative PCR (qPCR). The expression of each target gene is presented as the "fold change" relative to that of WT, as described previously (23, 28). Immunoblot analysis was performed as described (25) using anti-HIF-1α (Cayman Chemicals), anti-acetyl–NF-κB (Cell Signaling Technology), and anti-NF-κB (Cell Signaling Technology) antibody.

MDSC suppression assay

For the *in vitro* suppression assay, sorted $CD11b^+Gr1^+$ cells were added in the mixed lymphocyte reaction system for 96 hours in a 96-well plate, as described previously (8, 25). Eighteen hours before harvesting, cells were pulsed with ^{3}H-thymidine (GE Healthcare). ^{3}H-thymidine uptake was counted using a liquid scintillation counter and expressed as counts per minute (cpm).

ELISA

Cytokine concentrations in culture supernatants were measured with mouse IL-10, TGF-β1, TNF-α, and IL-12 ELISA kits (eBioscience) as per the manufacturer's instructions.

Tumor-killing activity

Sorted MDSCs were isolated from tumor or spleen and coincubated with tumor cells (target cells) at 20:1, 10:1, and 5:1 for 4 hours. Tumor-killing activities were measured as a percentage of tumor cell death.

Glycolysis flux assay

Glycolysis of MDSCs was determined by measuring the detritiation of [3-^{3}H]-glucose. In brief, the assay was initiated by adding 1 μCi [3-^{3}H]-glucose (PerkinElmer) and, 2 hours later, the medium was transferred to microcentrifuge tubes containing 50 μL 5N HCL. The microcentrifuge tubes were then placed in 20 mL scintillation vials containing 0.5 mL water, and the vials were capped and sealed. ^3H_2O was separated from unmetabolized [3-^{3}H]-glucose by evaporation diffusion for 24 hours at room temperature.

Statistical analysis

All data are presented as the mean ± SD. The Student unpaired *t* test was used for the comparison of means between groups. A comparison of the survival curves was performed using the log-rank (Mantel–Cox) test. A *P* value (α) of less than 0.05 was considered statistically significant.

Results

Characterization of SIRT1 in MDSCs

To dissect the potential intrinsic role of SIRT1 in hematopoietic differentiation, we evaluated its expression throughout myeloid development in highly purified cell populations from bone marrow cells. Myeloid progenitors originate from hematopoietic stem cells (HSC). According to the FcγRII/III (CD16/32) and CD34 expression levels in gated $Lin^-Sca1^-c\text{-}Kit^+$ cells, megakaryocyte erythroid progenitors ($CD34^{low}CD16/32^{low}$), CMPs ($CD34^{hi}CD16/32^{low}$), and GMPs ($CD34^{hi}CD16/32^{hi}$) could be distinguished (23). As MDSC differentiation proceeds in bone marrow, $CD11b^+Gr1^+$ MDSCs migrate and release into peripheral blood to the spleen, and then to the tumor local site, where the expression of SIRT1 steadily increases, whereas HSCs and progenitor cells express relatively low levels of SIRT1 (Fig. 1A). The level of SIRT1 expression was highest in tumor-infiltrating local MDSCs than in splenic MDSCs and CMPs.

Differentiation of MDSCs could be differently regulated by a lineage differentiation factor. TLR4 ligands, LPS, and/or IFN-γ are generally considered to be potent and effective for inducing M1 differentiation, whereas IL-4 or IL-13 could induce M2 differentiation. Therefore, we compare the SIRT1 activity stimulated by LPS or IL-4 *in vitro*. Because splenic MDSCs are the representative characteristic cells, we examined signaling pathways in WT splenic MDSCs. LPS and IL-4 stimulation *in vitro* led to different activities of SIRT1 expression. SIRT1 is promptly downregulated in a time-dependent manner following LPS stimulation. In contrast, it is easily upregulated by IL-4 stimulation (Fig. 1A). Tumor-infiltrating MDSCs also showed a similar tendency (data not shown). These observations, showing that the expression of SIRT1 progressively increased with MDSC differentiation from progenitor cells in a highly lineage-specific pattern, prompted us to propose that SIRT1 might be an important intrinsic modulator for MDSC differentiation and functional activity.

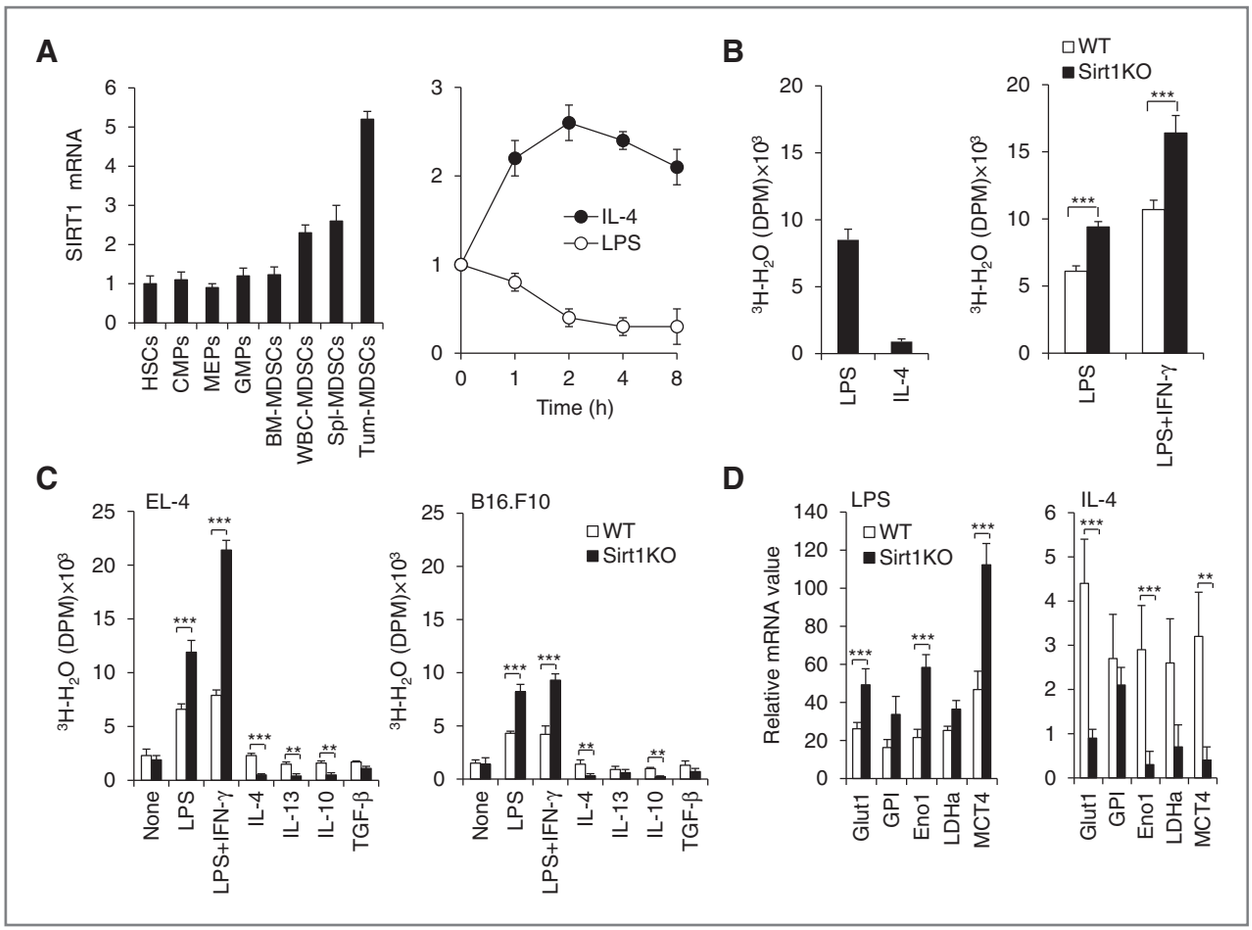

Figure 1. MDSC polarizing differentiation condition alters SIRT1-dependent glycolytic activity. A, qPCR analysis of SIRT1 expressions during MDSC development and differentiation from HSCs. The expression of SIRT1 on the MDSCs stimulated by 10 ng/mL LPS and 5 ng/mL IL-4 was assayed by RT-PCR (level in unstimulated MDSCs was set to 1). B, sorted splenic MDSCs ($CD11b^+Gr1^+$) from tumor-bearing mice were stimulated with LPS and IL-4 for 10 to 12 hours. Right, sorted splenic MDSCs from tumor-bearing WT or SIRT1KO mice were stimulated with LPS or LPS + IFN-γ for 10 to 12 hours. The glycolytic activity of these cells was measured by the generation of ^3H-labeled H_2O from [3-^3H]-glucose. C, MDSCs isolated from mouse solid tumors in WT or SIRT1KO mice were stimulated by the indicated innate stimuli and the glycolytic activity of these cells was measured by the generation of ^3H-labeled H_2O from [3-^3H]-glucose. All tumor tissues were subjected to the same enzymatic digestions. Two indicated tumor models were used. D, RNA was isolated from EL-4 tumor MDSCs in WT or SIRT1KO mice and these cells stimulated by LPS or IL-4 for 10 to 12 hours, and used for RT-PCR analyses of glycolytic molecules. Data are representative of three to four independent experiments ($n = 4$). **, $P < 0.01$; ***, $P < 0.001$, compared with the indicated groups.

MDSC polarizing conditions alter SIRT1-dependent glycolytic activity

Although it has been well appreciated that immune cell activation is accompanied by a metabolic switch to glycolysis, how glycolytic activity is regulated during MDSC differentiation is poorly understood. We found that the MDSC polarizing factor LPS and IL-4 could induce a different glycolytic activity. WT splenic MDSCs were isolated and activated under the M1 (LPS) and M2 (IL-4) polarizing condition *in vitro* with selective expression of IL-12 and IL-10, respectively. The glycolytic activity of differentiated cells was measured by the generation of ^3H-labeled H_2O from [3-^3H]-glucose. Results showed that M1 cells contained much higher glycolytic activity than M2 cells in splenic (Fig. 1B) and tumor-infiltrating MDSCs (Supplementary Fig. S1A). Glucose utilization depends on a chain of reactions catalyzed by multiple enzymes, eventually leading to the generation of lactate and net production of two ATP molecules as the energy source. Transcriptional mRNA expression of glucose transport 1 (Glut1) and LDH-α (lactate dehydrogenase) in splenic MDSCs stimulated by LPS or IL-4 showed consistent results (Supplementary Fig. S1B). Together, these data indicate strong upregulation of glucose metabolism in proinflammatory M1-MDSCs.

We next examine whether SIRT1 contributes to the differential activity of glycolysis during MDSC differentiation. To directly test this possibility, we used mice with conditional SIRT1 deletion. SIRT1 flox mice were crossed with LysM-Cre mice and SIRT1 specifically deleted in myeloid cells (called SIRT1KO mice hereafter). RT-PCR analysis indicated efficient deletion of the *SIRT1* gene in $CD11b^+Gr1^+$ MDSC cells (Supplementary Fig. S2A). SIRT1KO mice contained similar number and distribution of peripheral $CD11b^+Gr1^+$ MDSC cells

(Supplementary Fig. S2B). Also, cell proliferation and apoptosis in response to LPS stimulation were comparable between WT and SIRT1KO MDSCs (Supplementary Fig. S3). To examine whether SIRT1 controls glycolytic activity in the M1 condition, we purified splenic MDSCs from WT and SIRT1KO mice and differentiated and stimulated them by LPS or/and IFN-γ. Deficiency in SIRT1 resulted in greatly unregulated glycolytic activity in splenic MDSCs (Fig. 1B). Therefore, these data indicate an important role for SIRT1 to promote glucose metabolism in M1-MDSC differentiation.

To ascertain the important significance of SIRT1-dependent glycolytic activity in regulating MDSCs, we directly compared MDSCs from the solid tumor site in several models. EL-4 thymoma and B16.F10 melanoma were established as subcutaneous tumors. In these two models, tumor tissues were digested and the glycolytic activity of MDSCs was measured by the generation of ^{3}H-labeled H_2O from [3-^{3}H]-glucose in both WT and SIRT1KO mice. Under the M1 polarizing condition, the expression of iNOS and arginase I was confirmed by RT-PCR (Supplementary Fig. S4). SIRT1KO MDSCs showed a much more glycolytic activity than that of WT MDSCs in both tumor models (Fig. 1C). This finding indicated that SIRT1-dependent glycolytic activity is probably associated with MDSC polarizing differentiation.

Glucose-associated multiple enzymes are important to control the generation of lactate and ATP molecules as the energy source. Consistently, transcriptional mRNA analyses of Glut1, glucose-6-phosphate isomerase, Eno1 (enolase 1), LDH-α, and monocarboxylic acid transporter member 4 in WT cells undergoing M1 polarization revealed marked upregulation of genes encoding various molecules involved in glycolysis. In contrast, under the M2 polarizing condition (IL-4), the above glycolytic pathway molecules were significantly downregulated (Fig. 1D and Supplementary Fig. S4). Taken together, the SIRT1-dependent glycolytic pathway is associated with MDSC programming.

Deficiency of SIRT1 programs splenic M1-type MDSC and potentiates glycolytic activity

Changes in SIRT1-dependent glycolytic activity in MDSC programming prompted us to investigate whether SIRT1 could direct the MDSC polarization. We investigate the changes in the MDSC phenotype in EL-4 tumor–bearing WT and SIRT1KO mice. CD11b^{+}Gr1^{+} cells were isolated from bone marrow cells and spleen in tumor-bearing mice, and the suppressive activity was determined by ^{3}H-TdR incorporation analysis. CD11b^{+}Gr1^{+} cells isolated from the spleen, not from bone marrow cells, show significantly diminished immunosuppressive activity on allogeneic CD4^{+} T-cell proliferation *in vitro* coculture assays than that of WT in a dose-dependent manner (Fig. 2A). This finding suggests that MDSC function and differentiation could be regulated by SIRT1 after MDSC migration from bone marrow into peripherial lymphoid organs.

Moreover, SIRT1KO splenic MDSCs showed higher expression of the M1 markers IFN-γR, MHC I, and CD86, whereas the expression of the M2 markers SR-A, IL-4R, PD-L1, Tie2, and IL-10R was significantly diminished compared with that of WT control (Fig. 2B). Furthermore, SIRT1KO MDSCs exhibited increased production of NO, TNF-α, and IL-12, whereas they showed less arginase activity, and IL-10 and TGF-β1 secretion (Fig. 2C), which indicates that MDSCs preferentially differentiate into M1-type phenotypes. However, these differences could not be observed in bone marrow MDSCs (Supplementary Fig. S5).

Consistently, higher glycolytic activities were induced by splenic MDSCs in SIRT1KO compared with those of WT (Fig. 2D). However, glycolytic activities induced by bone marrow–derived MDSCs were comparable between SIRT1KO and WT (data not shown). Taken together, these data reveal that SIRT1 deficiency in MDSCs directs splenic M1-MDSC differentiation and potentiates glycolytic activity.

Deficiency of SIRT1 directs tumor M1-type MDSCs against tumor growth and potentiates glycolytic activity

To investigate the biologic significance of SIRT1, we observed changes in tumor growth in SIRT1KO and WT mice. The rate of tumor growth was significantly slower in SIRT1KO than in WT mice (Fig. 3A). To examine whether this effect is associated with changes in the MDSC phenotype in SIRT1KO mice, we directly isolated MDSCs from these tumors. Tumor-infiltrating MDSCs showed a significantly diminished suppressive activity in SIRT1KO MDSCs compared with WT control (Fig. 3B). Moreover, significant higher iNOS, TNF-α, and IL-12 expressions, M1-MDSC phenotypes, were observed in SIRT1KO than in WT mice (Fig. 3B). Consistently, higher glycolytic activities were induced by tumor-infiltrating MDSCs in SIRT1KO compared with those of WT (Fig. 3C). These findings indicate that tumor-infiltrating M1-MDSCs probably contribute to delay tumor growth and upregulates glycolytic activity.

To ascertain the intrinsic regulatory effects of SIRT1 on MDSC differentiation and function against tumor and ensure that SIRT1 deletion is confined only to hematopoietic cells, we used bone marrow reconstitution of the WT recipient. Bone marrow cells from CD45.2^{+} SIRT1KO or control mice were used to reconstitute lethally irradiated CD45.1 congeneic mice. Bone marrow progenitors from SIRT1KO and WT mice showed similar engraftment potential (Supplementary Fig. S6A). Two weeks after bone marrow transfer, mice were inoculated subcutaneously with EL-4 tumor cells. In both tumor-bearing groups, tumor grew progressively during the first 4 weeks. However, thereafter, mice reconstituted with SIRT1KO bone marrow demonstrated a substantial delay in tumor growth (Supplementary Fig. S6B). Four weeks after tumor injection, myeloid cells were evaluated with flow cytometry. Significant CD11b^{+}Gr1^{+} MDSC expansion was observed in spleen and tumor, but no significant differences were observed in SIRT1KO and WT mice (data not shown). However, isolated MDSCs exhibited a diminished suppressive activity (Supplementary Fig. S6C), M1-type characters, less arginase I and IL-10 (Supplementary Fig. S6D), more iNOS, TNF-α and IL-12 production (Supplementary Fig. S6E), and upregulated glycolytic activities (Supplementary Fig. S6F) in the SIRT1KO compartment compared with that of WT. Therefore, SIRT1 deficiency

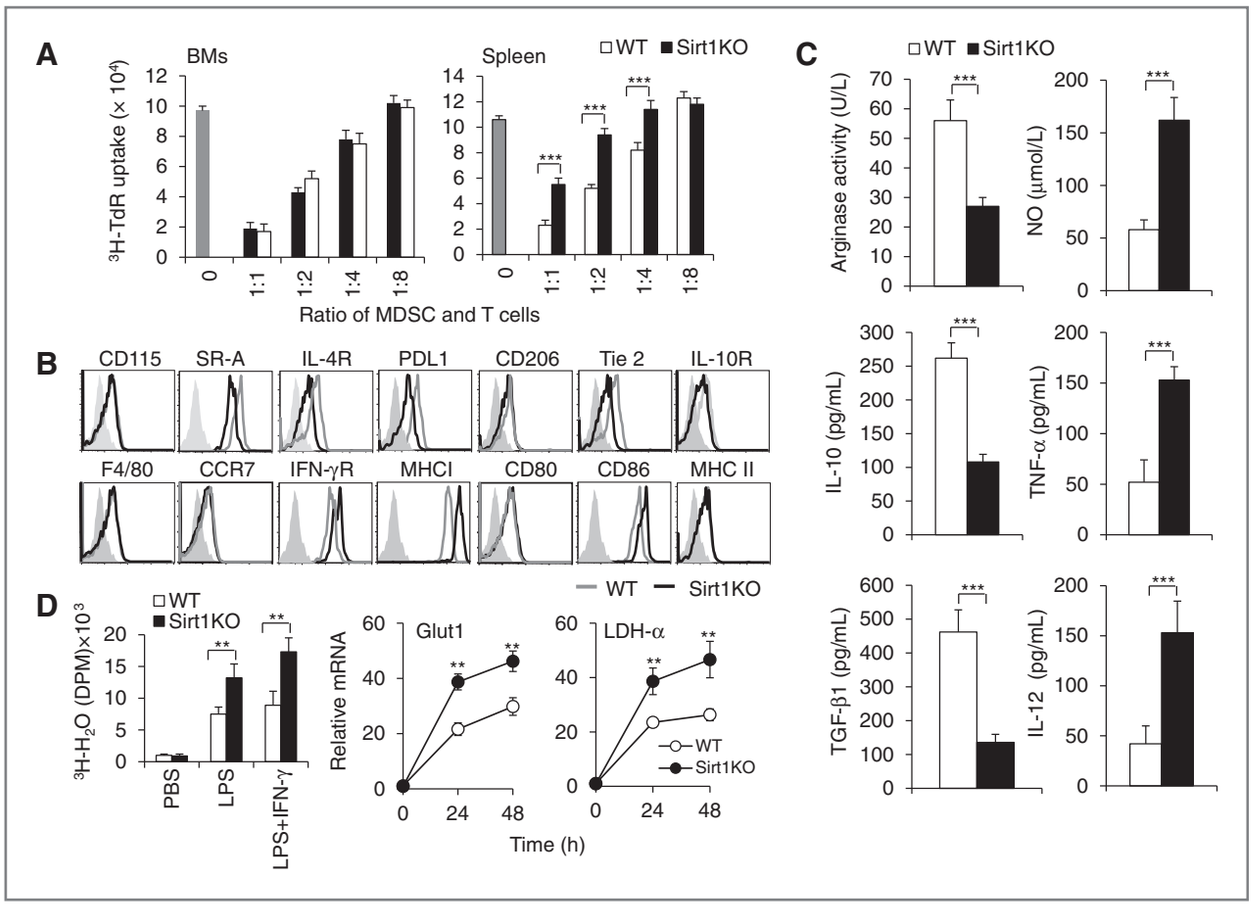

Figure 2. SIRT1KO splenic MDSCs but not bone marrow (BM)–derived MDSCs exhibit an M1 phenotype. A, suppressive assay with various ratios of MDSCs from tumor-bearing WT and SIRT1KO bone marrow (left) and spleen (right). Coculture with allogeneic $CD4^+$ T cells is shown. Proliferation cpm ×1,000. B, purified splenic MDSCs were stained for CD115, scavenger receptor A (SR-A), mannose receptor (CD206), IL-4R, Tie2, PDL1, IL-10R, F4/80, CCR7, IFN-γR, MHCI, CD80, CD86, and MHC class II. Expression was evaluated in the $CD11b^+Gr1^+$ cell population. C, SIRT1KO splenic MDSCs exhibit an M1 phenotype. Splenic MDSCs from WT or SIRT1KO tumor-bearing mice were cultured in culture media in the absence of any stimulation for 24 hours. Arginase activity and IL-10, TGF-β1, IL-12, NO, and TNF-α production in the culture supernatant were measured. D. SIRT1KO splenic MDSCs exhibit higher glycolytic activities. Splenic MDSCs from WT or SIRT1KO tumor-bearing mice were cultured in culture media in the absence of stimulation or stimulated by LPS or LPS + IFN-γ for 24 hours; the cells were measured by the generation of ^3H-labeled H_2O from [3-^3H]-glucose. Right, the cells were stimulated by LPS for the indicated time and RNA was isolated and used for RT-PCR analyses of glycolytic molecules. Data are representative of three (A–C) or four (D) independent experiments ($n = 3$–5). **, $P < 0.01$; ***, $P < 0.001$, compared with the indicated groups.

intrinsically directs M1-MDSC differentiation and upregulates glycolytic activity while protecting against tumor.

To directly assess the biologic role of the tumor-infiltrating MDSC activities, we directly observed the MDSC-killing activity and sorted tumor MDSC *in vitro*. MDSCs have potentiated killing activity accompanying higher glycolytic activity against tumor in SIRT1KO than in WT mice (Fig. 3D and Supplementary Fig. S7). Consistently, SIRT1KO $CD11b^+Gr1^+$ MDSCs transfer significantly delayed the tumor growth and contributed to the mouse-bearing tumor survival *in vivo* (Fig. 3E and F). Importantly, $CD11b^+Gr1^+Ly6C^{hi}CD115^+$ (monocytic) MDSCs, but not $CD11b^+Gr1^+CD115^-Ly6G^{hi}$ (neutrophil) MDSCs, in SIRT1KO mice showed stronger tumor-killing activities (Supplementary Fig. S8) and, more significantly, delayed the tumor growth (data not shown). Therefore, SIRT1-deficient–induced M1-monocytic MDSCs have the therapeutic potential.

Deficiency of SIRT1 impairs tumor M2-type MDSC differentiation accompanying diminished glycolytic activity

SIRT1-directing M1-MDSC differentiation prompted us to investigate whether SIRT1 controls M2-MDSC differentiation. To this end, we observed the M2 phenotype alteration of tumor-infiltrating MDSCs isolated from SIRT1KO and WT mice. As expected, SIRT1KO MDSCs showed lower arginase activity and IL-10 and TGF-β1 expression compared with WT (Fig. 4A and B). Furthermore, the M2 markers, SR-A, IL-4R, PD-L1, Tie2, and IL-10R, except CD206, are significantly diminished in SIRT1KO MDSCs compared with those of WT (Fig. 4C). Therefore, these data indicate that the deficiency of SIRT1 probably inhibits the tumor M2-MDSC differentiation. To ascertain the intrinsic regulatory effects of SIRT1 on MDSC differentiation, we set up a mouse mixed chimera model. $CD45.1^+$ lethally irradiated recipients were reconstituted with

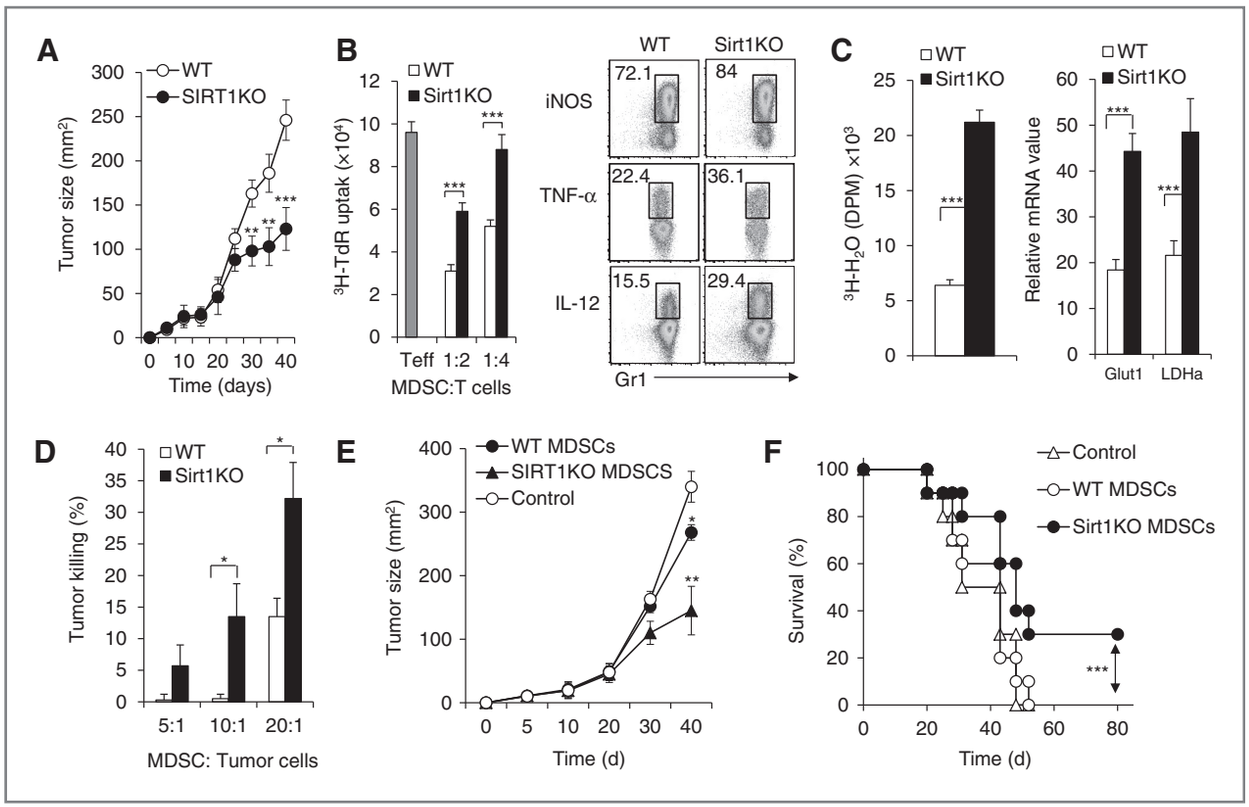

Figure 3. SIRT1KO tumor environment gives rise to M1-polarized MDSCs in protecting against tumor growth. A, EL-4 tumor cells were implanted subcutaneously in WT and SIRT1KO mice ($n = 10$) and tumor size was measured every 5 days for 40 days. B, suppressive assay of tumor-infiltrating MDSCs from tumor-bearing WT and SIRT1KO mice. Coculture with allogeneic $CD4^+$ T cells is shown. Right, purified tumor MDSCs were stained for iNOS, TNF-α, and IL-12 with intracellular staining by flow cytometry. The typical fluorescence-activated cell sorting analysis is shown. C, tumor MDSCs from WT or SIRT1KO tumor-bearing mice were stimulated by LPS for 24 hours; the cells were measured by the generation of ^3H-labeled H_2O from [3-^3H]-glucose. RNA was isolated from the stimulated cells and used for RT-PCR analyses of glycolytic molecules. D, tumor cytotoxicity mediated by MDSCs. Tumor-killing activities were measured as a percentage of tumor cell death. E, effects of adoptive cell transfer on subcutaneous EL-4 tumor growth. F, effects of adoptive transfer on subcutaneous EL-4 tumor's long-term survival rate of treated mice. Log-rank (Mantel–Cox) test was performed in comparison with the indicated group. Data are representative of four (A–C) or two (D–F) independent experiments ($n = 5$–10). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with the indicated groups.

bone marrow cells from SIRT1KO or WT $CD45.2^+$ mice. Two weeks later, mice were inoculated subcutaneously with 5×10^5 EL-4 tumor cells. Three weeks later, $CD45.2^+$ SIRT1KO or WT MDSCs were isolated from the spleen of tumor-bearing mice and then transferred into ascites of congenic $CD45.1^+$ mice. $CD45.2^+Gr1^+$ donor cells were isolated and used in the subsequent experiments. Interestingly, the arginase 1, IL-10, and TGF-β1 showed sustained and strong downregulation in SIRT1 MDSCs compared with WT (Fig. 4D). Importantly, glycolytic activities and glucose-associated enzyme molecules are significantly diminished in SIRT1KO MDSCs compared with WT (Fig. 4E), indicating that SIRT1-dependent glycolytic activities are significantly required for deciding the reciprocal differentiation of the M1- and M2-MDSC lineage.

Blocking glycolysis reciprocally reduced M1- but promoted M2-type MDSC differentiation

To directly test the importance of the SIRT1-dependent glycolytic activity in programming MDSC differentiation, we cultured splenic MDSCs isolated from tumor-bearing mice under M1-polarizing conditions in the presence or absence of 2-DG, a prototypical inhibitor of the glycolytic pathway via blocking hexokinase, the first rate-limiting enzyme of glycolysis and rapamycin, and mTOR inhibitor, which can block the mTOR-dependent metabolic pathway, including glycolysis, as a control. As expected, an optimized dose of 2-DG (1 mmol/L) of MDSCs, similar to rapamycin treatment, significantly reduced the glycolytic activity (Fig. 5A). Importantly, treatment of MDSCs with 2-DG or rapamycin resulted in increased suppressive activity and recovered the increased production of NO and TNF-α in SIRT1KO cells (Fig. 5B and C). Of note, because the cells were treated with the pharmacologic inhibitors during their differentiation but not at the terminal mature stage, a specific effect of glycolytic inhibition reflected on the MDSC differentiation.

To ascertain the effects of 2-DG on the M1-type MDSC cell on tumor, we isolated splenic MDSCs from SIRT1KO and WT mice and expanded them *in vitro* in the presence of 2-DG. These cells exhibited a diminished tumor-killing activity compared with WT (Fig. 5D) accompanying lower glycolytic activity

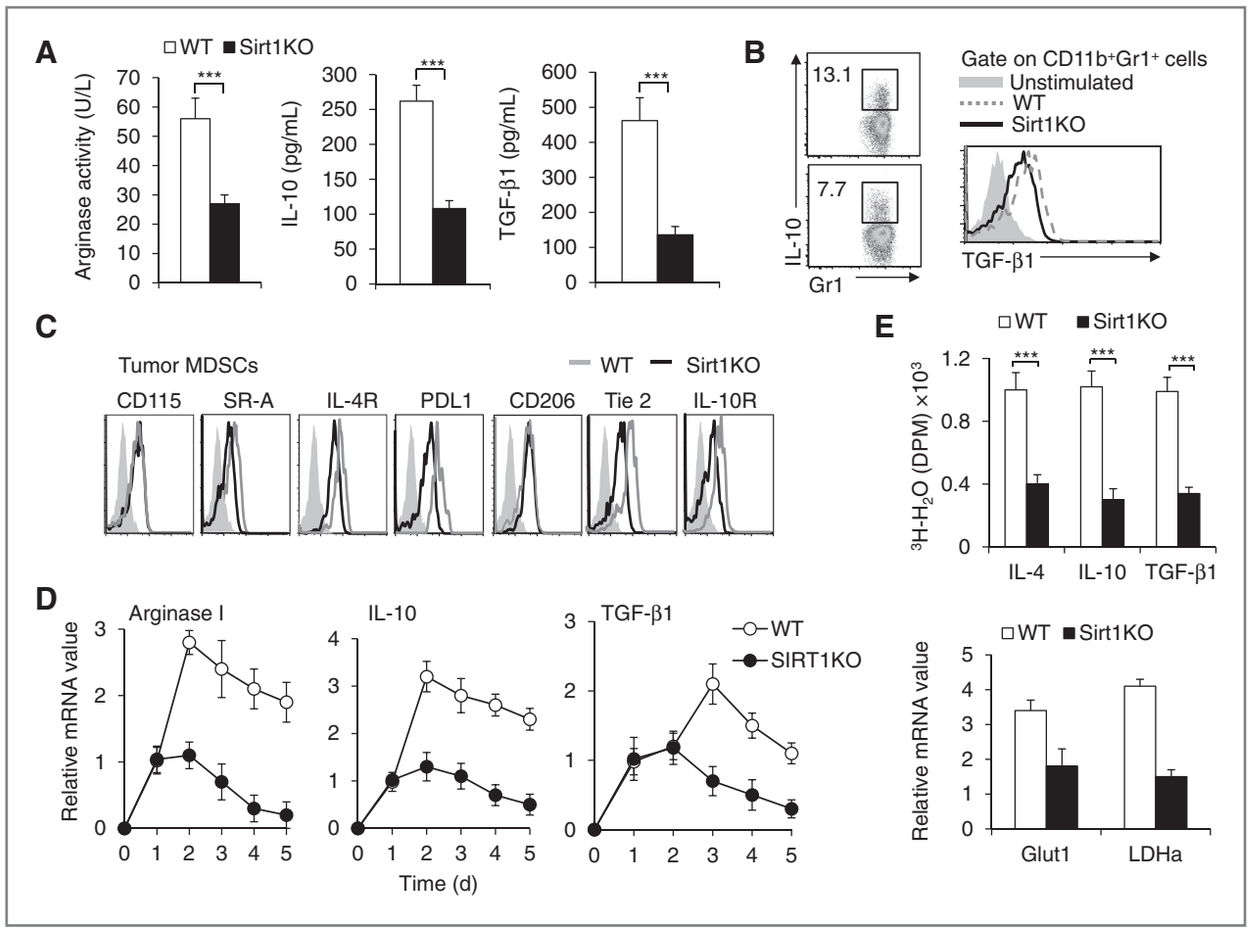

Figure 4. SIRT1KO tumor environment impairs the M2-polarized MDSCs. A, purified EL-4 tumor MDSCs were stimulated by IL-4 for 24 hours. Arginase activity, IL-10, and TGF-β production in the culture supernatant were measured. B, purified EL-4 tumor MDSCs were stimulated by IL-4 for 24 hours. IL-10 and TGF-β production were measured by flow cytometry. C, purified EL-4 tumor MDSCs were stained for CD115, SR-A, CD206, IL-4R, Tie2, PDL1, and IL-10R. Expression was evaluated in the $CD11b^+Gr1^+CD115^+$ cell population. D and E, $CD45.1^+$ lethally irradiated recipients were reconstituted with BM cells from SIRT1KO or WT $CD45.2^+$ mice. Two weeks later, mice were inoculated subcutaneously with 5×10^5 EL-4 tumor cells. Three weeks later, $CD45.2^+$ SIRT1KO or WT MDSC were isolated from the spleen of tumor-bearing mice and then transferred into ascites of congenic $CD45.1^+$ mice. At indicated time point, $CD45.2^+Gr1^+$ donor cells were isolated and used in the subsequent experiments. D, expression of arginase1, IL-10, and TGF-β1 was analyzed during the indicated time point in the MDSCs after adoptive transfer into the tumor milieu. E, the cells were stimulated by IL-4, IL-10, or TGF-β1 for 24 hours and measured by the generation of ^3H-labeled H_2O from [3-^3H]-glucose. RNA was isolated from IL-4–stimulated MDSCs and used for RT-PCR analyses of glycolytic molecules. Data are representative of three (A–C) or two (D and E) independent experiments ($n = 4$–6). ***, $P < 0.001$ compared with the indicated groups.

(Supplementary Fig. S7). When these cells were transferred into C57BL/6 recipients bearing EL-4 tumor, 2-DG–treated SIRT1KO MDSCs exhibited a markedly delayed tumor growth (Fig. 5E) and diminished survival percentage (Fig. 5F) compared with SIRTKO control. Therefore, blocking glycolysis in SIRT1KO mice alters M1- and M2-MDSC differentiation and promotes the development of tumor, indicating that glycolytic activity is required for SIRT1-directed MDSC differentiation while protecting against tumor.

mTOR–HIF-1α activity is required for MDSC differentiation modulation of the SIRT1-dependent glycolytic pathway

How does SIRT1 regulate glycolytic activity in directing MDSC programming? To study the mechanisms that mediate SIRT1 function, splenic MDSCs stimulated by LPS, we assessed the activation of LPS "downstream" pathways, including ErK, c-*jun*–NH2–kinase (JNK), p38, AKT, mTOR, NF-κB, and HIF-1α, by flow cytometry analysis or immunoblot analysis. As expected, LPS activated all pathways in WT MDSCs. In contrast, SIRT1KO MDSC cells activated the ErK, JNK, p38, NF-κB, and AKT pathways like WT cells did but showed a more stronger and sustained activation of phosphorylation S6 ribosomal protein and transcriptional factor HIF-1α (Fig. 6A and Supplementary Fig. S9). Thus, SIRT1 is associated with mTOR–HIF-1α.

We pretreated WT and SIRT1 MDSCs with U0126 (an inhibitor of ErK) and rapamycin (an inhibitor of mTOR), respectively, followed by LPS stimulation. Rapamycin could significantly alter activity of the arginase and NO and

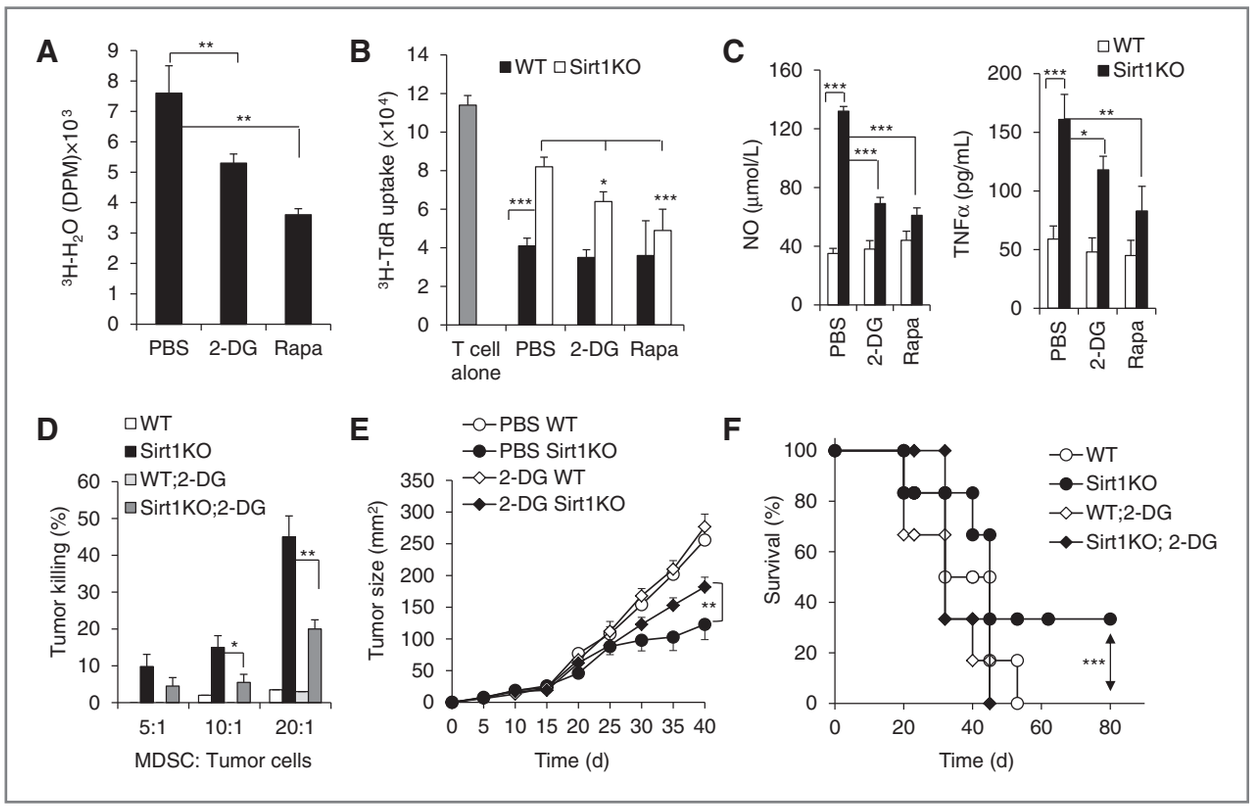

Figure 5. Blocking glycolytic metabolism pathway recovered SIRT1KO-induced MDSC M1 programming. A, splenic MDSCs isolated from tumor-bearing mice were stimulated by LPS for 10 to 12 hours in the presence of vehicle (control), 1 mmol/L 2-DG, or 50 nmol/L rapamycin (Rapa). Glycolytic activity was measured. B, in a separate group of the experiment in A, splenic MDSCs with indicated treatment were extensively washed and incubated with $CD4^+$T cells, whose proliferation was determined with ^{3}H-thymidine incorporation in a MDSC suppression assay. C, splenic MDSCs from WT or SIRT1KO tumor-bearing mice were cultured in culture media in the presence of 2-DG or rapamycin for 24 hours. NO and TNF-α production in the culture supernatant were measured. D, EL-4 tumor cells were implanted subcutaneously in WT and SIRT1KO mice and also were injected intraperitoneally with 2-DG (2 g/kg body weight) or solvent alone (PBS). Of note, 2-DG/PBS was given daily until the day before the mice were euthanized. Splenic MDSCs were isolated and MDSCs-mediated tumor-killing activities were measured as a percentage of tumor cell death. E, EL-4 tumor cells were implanted subcutaneously in WT and SIRT1KO mice and they also were injected intraperitoneally with 2-DG or PBS. Tumor size was measured every 5 days for 40 days. F, SIRT1KO or WT mice were inoculated subcutaneously with 5×10^5 EL-4 tumor cells and also were injected intraperitoneally with 2-DG or PBS daily, respectively. One week later, MDSCs were isolated from spleen of tumor-bearing mice and then transferred into recipient mice and these mice were implanted subcutaneously EL-4 tumor cells on day 1. Long-term survival percentage of recipient tumor-bearing mouse was summarized. Data are representative of two (A, E, and F) or three (B–D) independent experiments ($n = 3$–10). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, compared with the indicated groups.

production of TNF-α and IL-10 (Fig. 6B), by blocking mTOR as indicated (Fig. 6C), indicating that SIRT1 regulates the MDSC differentiation depending on the mTOR. Consistently, the upregulated glycolytic activity in SIRT1 MDSCs could be rescued by rapamycin treatment, not U0126 (Supplementary Fig. S10A). Therefore, SIRT1-dependent MDSC programming is performed through mTOR.

However, to determine whether HIF-1α is involved in this regulation, we used SIRT1 and HIF-1α double knockout mice in this investigation. Interestingly, increased TNF-α and diminished IL-10, and an upregulated ratio between TNF-α and IL-10 in SIRT1KO MDSCs could be significantly rescued in SIRT1–HIF-1α double knockout mice (Fig. 6D). Moreover, the activity of diminished arginase and increased NO could be significantly recovered in SIRT1–HIF-1α double knockout mice (Fig. 6E). Consistently, the increased glycolytic activity in SIRT1KO mice could be significantly recovered in SIRT1–HIF-1α double knockout mice (Supplementary Fig. S10B). Thus, HIF-1α–induced glycolytic modulation is required for SIRT1-dependent MDSC programming. However, the SIRT1KO-induced HIF-1α expression could not be completely blocked by rapamycin, indicating that HIF-1α expression in SIRT1KO MDSCs is not completely determined by mTOR (Fig. 6C). Therefore, mTOR- and HIF-1α–induced glycolytic metabolism modulation is required for SIRT1-dependent MDSC programming.

To further assess the role of SIRT1-dependent MDSC programming as a therapeutic target, we directly investigate the role of the SIRT1 activator and HIF-1α inhibitor on splenic MDSC differentiation *in vitro*. We treated MDSCs derived from the spleen in the tumor-bearing mice with an SIRT1 activator, SRT1720, and observed the polarization alteration. As expected, M2 marker, arginase activity and IL-10, is upregulated, whereas M1 marker, NO, and TNF-α production is diminished in SIRT1720-treated MDSCs, which accompanies the downregulation of glycolytic activities (Supplementary Fig.

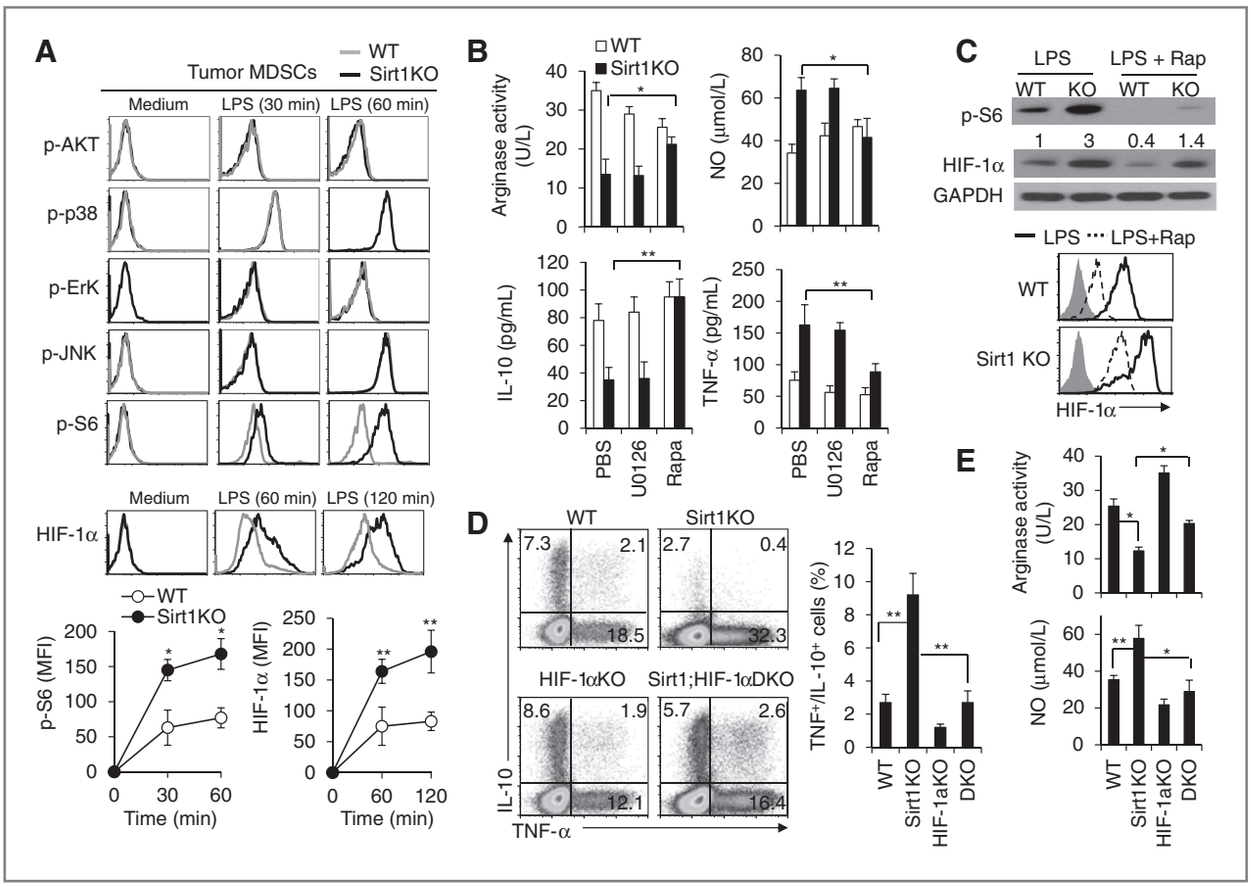

Figure 6. SIRT1KO promotes MDSC M1 polarization through mTOR/HIF-1α–dependent glycolytic pathway. A, activation of downstream signals of TLR4 stimulated by LPS. Intracellular staining of phosphorylation of AKT, ErK, p38, JNK, S6, and expression of splenic MDSC HIF-1α in the tumor-bearing mice. B, splenic MDSCs isolated from WT or SIRT1KO tumor-bearing mice were pretreated with U0126 (10 μmol/L) and rapamycin (100 μmol/L) for 0.5 to 1 hour and then stimulated with LPS for 24 hours. Arginase activity and IL-10, NO, and TNF-α production in the culture supernatant were measured. C, immunoblot analysis (top) and intracellular staining (bottom) of phosphorylation of S6 and expression of HIF-1α treated by LPS alone or LPS + rapamycin are shown. D, purified splenic MDSCs isolated from WT or SIRT1KO tumor-bearing mice were stained for IL-10 and TNF-α, with intracellular staining by flow cytometry. The typical flow cytometry figure is shown. Right, the ratio between $TNF^{+}/IL\text{-}10^{+}$ is summarized. E, splenic MDSCs from WT, SIRT1KO, HIF-1αKO, and SIRT1/HIF-1α double knockout (DKO) tumor-bearing mice were cultured in culture media in the absence of any stimulation for 24 hours. Arginase activity and NO production in the culture supernatant were measured. Data are representative of three independent experiments ($n = 3$–5). *, $P < 0.05$; **, $P < 0.01$, compared with the indicated groups.

S11A and S11B). These indicate that SIRT1 is essential to instruct the M1-MDSC differentiation through a glycolytic activity–associated mechanism. Moreover, in the presence of 2-ME (an inhibitor of HIF-1α), diminished suppressive activities in SIRT1KO MDSCs could significantly be recovered compared with WT. Consistently, the 2-ME treatment partly recovered the diminished arginase activity, increased NO amount and glycolytic activities (Supplementary Fig. S11C and S11E), revealing that HIF-1α is required for SIRT1-dependent glycolytic metabolism–directed MDSC differentiation. Furthermore, combining treatment with Ex527 (an inhibitor of SIRT1) and Cocl2 (an activator of HIF-1α) could effectively promote the M1-MDSC differentiation, and potentiates the tumor-killing and glycolytic activities (Supplementary Fig. S12). Therefore, targeting the SIRT1–mTOR/HIF-1α axis could effectively integrate immune signals and metabolic programming to determine MDSC differentiation for treatment.

Discussion

The differentiation and expansion of MDSCs has been described in cancers and is believed to possess a phenotype of both M1- and M2-like MDSCs (1, 5). Although the phenomenon is well described in recent reports, how MDSCs differentiate into M1- or M2-MDSC remains elusive. Inhibition of the paired immunoglobulin-like receptor B signaling has been shown to promote MDSC differentiation into M1 macrophages in tumors (29). In this study, we reported that MDSCs are immature myeloid precursors, which have the potential to differentiate into both the M1 and M2 (Supplementary Fig. S13). This switch can be regulated through SIRT1-dependent glycolytic metabolic programming as the cells migrated from the bone marrow to peripheral organs and into tumor local sites.

SIRT1 is an important regulator involved in nearly every system of mammalian biology. Some studies have suggested

that SIRT1 attenuates immune responses (10–14, 30, 31). In contrast, our results expand the unappreciated role of SIRT1 in MDSC differentiation and shape the decision about the fate of MDSC lineage. Furthermore, SIRT1 is also an important nutrient-sensing deacetylase in which levels and activity increase with caloric restriction to preserve euglycemia and promote efficient energy utilization (32). In our report, we identified a novel SIRT1-dependent glycolytic metabolism mechanism in shaping MDSC programming in tumors. We demonstrated that SIRT1 probably is a critical "switcher" to direct bone marrow MDSCs into M1- or M2-MDSCs when entering the periphery or tumor local sites, resulting in decreased suppressive function, proinflammatory M1 phenotype, and delayed tumor growth.

HIF-1α is a key transcriptional factor that orchestrates the expression of glycolytic enzymes (20, 33–35). The binding of HIF-1α to many hypoxia response element–containing promoters was shown to regulate the function of macrophages in response to hypoxia, including the shift to anaerobic glycolysis and immune suppression (36–38). Moreover, arginine metabolism was enhanced toward NO production in an HIF-1α–dependent manner upon LPS or IFN-γ stimulation, but NO production was reduced by IL-4 (38, 39). Furthermore, HIF-1α is also induced in normoxia and plays a central role in the proinflammatory cytokine production (39, 40). Recent studies have shown that SIRT1 can directly deacetylates and inactivates HIF-1α at Lys674 through the direct interplay between SIRT1 and HIF-1α in mouse tissue, even in the absence of hypoxia (41–43). Both SIRT1activation and HIF-1α inhibition have been linked to age-associated diseases, which confer a beneficial effect (44). SIRT1 activation confers a therapeutic effect in inflammation, obesity, and neurodegenerative diseases, whereas inhibition of HIF-1α provides protection against inflammation (40, 45), autoimmune diseases (20, 21, 34, 46), and tumors (47). In our studies, we found that the absence of SIRT1 in MDSC-directed M1-MDSC differentiation is dependent on HIF-1α. SIRT1–HIF-1α double knockout mice could nearly completely rescue the M1 phenotype and glycolytic activity induced by SIRT1 deficiency in MDSCs. Thus, SIRT1 targets the HIF-1α pathway to control glycolytic activities and instruct M2-MDSC differentiation.

Recent studies have shown that HIF-1α is responsible for the glycolytic response downstream of mTOR (40, 48). SIRT1 has shown negative regulatory effects on mTOR (49, 50). In our report, SIRT1 deficiency in MDSCs after LPS stimulation could significantly upregulate p-S6 expression, a downstream target molecule of mTOR. Moreover, the mTOR inhibitor, rapamycin, treatments could significantly rescue the SIRT1 deficiency–induced M1-MDSC polarization and upregulate glycolytic activities. However, rapamycin treatment merely partly blocked the upregulation of HIF-1α induced by SIRT1 deficiency, revealing that HIF-1α induced by SIRT1 deficiency is partly determined by mTOR. Therefore, rapamycin-sensitive mTOR signaling, HIF-1α expression, and glycolytic activity, in the downstream of SIRT1, are critical in directing the reciprocal differentiation of M1- and M2-MDSCs. Taken together, mTOR- and HIF-1α–induced glycolytic activity is required for lineage differentiation from MDSCs to M1 cells determined by SIRT1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: G. Liu, Y. Bi
Development of methodology: G. Liu, Y. Bi, B. Shen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Liu, Y. Bi, B. Shen, H. Yang, Y. Zhang, X. Wang, Y. Chu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Liu, Y. Bi, B. Shen, H. Yang, Y. Zhang, X. Wang, H. Liu, Y. Lu, J. Liao, X. Chen, Y. Chu
Writing, review, and/or revision of the manuscript: G. Liu, Y. Bi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Liu
Study supervision: G. Liu

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