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ORIGINAL ARTICLE – CANCER RESEARCH

Decitabine shows potent anti-myeloma activity by depleting monocytic myeloid-derived suppressor cells in the myeloma microenvironment

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Abstract

Purpose Multiple myeloma (MM) remains incurable. The MM microenvironment supports MM cells' survival and immune escape. Because myeloid-derived suppressor cells (MDSCs) is important in the MM microenvironment, and demethylating agent decitabine (DAC) can deplete MDSCs in vitro and in vivo, we hypothesized that DAC treatment could inhibit MM by depleting MDSCs in the MM microenvironment.

Methods In this study, we used the mouse IL6 secreting, myeloma cell line MPC11 as a model. MDSCs were sorted using magnetic beads and cultured. A transwell coculture assay was used to mimic the microenvironment in vitro. And MPC11-bearing mice model was used to observe the efficacy of DAC treatment in vivo.

Results In vitro coculture assay indicated that MPC11 cells showed significantly lower proliferation rate, less IL6 production and more apoptosis when they were cocultured with bone marrow cells without MDSCs (nonMDSCs) or DAC-treated bone marrow cells (DAC BMs) than with MDSCs or PBS-treated bone marrow cells (CTR BM). Supplementation with M-MDSCs rescued the inhibitory effect of DAC BMs, while additional NOHA supplementation further antagonized the rescue effect of M-MDSCs. In MPC11-bearing mice, the combined treatment of DAC with anti-Gr1 antibody showed synergistic effect on inhibiting tumor growth and promoting T cell infiltration in the tumor tissue. M-MDSC reinfusion also antagonized the efficacy of DAC treatment.

Conclusions DAC treatment can inhibit myeloma cell proliferation and induce enhanced autologous T cell immune response by depleting M-MDSCs in the MM microenvironment. We believe that DAC treatment could improve the prognosis of MM in future.

Keywords Decitabine · Myeloid-derived suppressor cells · Myeloma · Microenvironment

Abbreviations

MM	Multiple myeloma
MDSC	Myeloid-derived suppressor cells
M-MDSC	Monocytic myeloid-derived suppressor cells
G-MDSC	Granulocytic myeloid-derived suppressor cells
IL6	Interleukin 6
DAC	Decitabine
CTR	Control
BM	Bone marrow
nonMDSC	Bone marrow cells without MDSC
Tc cells	Cytotoxic T cells
Th cells	Helper T cells

Introduction

Multiple myeloma (MM) is one of the most frequent hematologic malignancies. The malignant cells develop from clonal plasma cells and result in end-organ damage. Conventional anti-myeloma therapy includes cytotoxic chemotherapy and autologous hematopoietic stem cell transplantation. However, the response rate is relatively low, and the long-term prognosis is unsatisfactory. Although many novel agents such as proteasome inhibitors, immunomodulatory

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agents and monoclonal antibodies have all significantly improved the prognosis, MM remains an incurable hematological malignancy (Chim et al. 2018). Most patients ultimately relapse and become refractory to current treatments. Basic research on the pathogenesis of MM concentrates on two main aspects: the myeloma clone itself, and the microenvironment that enables myeloma clone survival. Although accumulated evidence suggests that genetic and epigenetic aberrations occur and evolve in clonal plasma cells, the genetic aberrations are considered insufficient for the development of systematic MM (Amin et al. 2014; Bianchi and Munshi 2015). Therefore, the MM microenvironment has begun attracting increasing attention in MM research.

The MM microenvironment is a very complex network with various cellular and noncellular factors. The bidirectional interaction between malignant myeloma cells and its microenvironment provides a protective niche against cytotoxic agents and immune attacks. Among the various factors that constitute the MM microenvironment, the essential role of myeloid-derived suppressor cells (MDSCs) has already been established, which involves facilitating immune escape, promoting angiogenesis, and supporting MM cell proliferation (Botta et al. 2014; Gorgun et al. 2013; Ramachandran et al. 2013). There are two main populations of MDSCs: monocytic MDSCs (M-MDSCs) and granulocytic MDSCs (G-MDSCs). In tumor-bearing mice, the immunophenotype of M-MDSCs was defined as $Gr1^{dim}/Ly6G^{low}$, while that of G-MDSCs was defined as $Gr1^{high}/Ly6G^{high}$. However, the effects of these two populations on the MM microenvironment remain controversial. In some studies, G-MDSCs were increased in MM patients and induced angiogenesis in vitro and in vivo (Binsfeld et al. 2016; Giallongo et al. 2016), while in other studies, M-MDSCs were regarded as more important than G-MDSCs, because accumulating M-MDSCs in the peripheral blood of MM patients were associated with lower treatment response rates and worse long-term prognosis (Lee et al. 2016; Wang et al. 2015b).

Decitabine, also known as 5-aza-2′-deoxycytidine (DAC), is a hypomethylating agent. Its anticancer activity relies on reactivating methylation-silenced genes. Through its demethylation mechanism, DAC treatment not only upregulates some tumor suppressor genes to inhibit tumor cell proliferation and induces tumor cells to undergo apoptosis but also upregulates many immune-associated genes, leading to the so-called immune modulation effect (Saleh et al. 2016). In our previous studies, we demonstrated that DAC treatment could upregulate cancer-testis antigen expression on leukemia cells and induce autologous cancer-testis antigen-specific cytotoxic T cells in vivo (Zhou et al. 2013). In 2017, we reported the immunoregulatory effect of DAC on immune tolerance. We found that DAC treatment could specifically induce MDSCs to undergo apoptosis in vitro and in vivo and deplete MDSCs in the tumor microenvironment. MDSC depletion by DAC treatment disrupted the tumor-induced immune tolerance and enhanced the autologous anti-leukemia immune response in vivo (Zhou et al. 2017). Such an MDSC-depleting effect of hypomethylating agent, including both decitabine and 5-azacytidine, had also been confirmed by other researchers previously (Mikyskova et al. 2014; Stone et al. 2017; Terracina et al. 2016).

Considering that MDSCs are essential in the MM microenvironment and that DAC treatment depletes MDSC in vitro and in vivo, we hypothesized that DAC may exert anti-myeloma activity by targeting MDSCs in the MM microenvironment. In this study, we attempted to prove our hypothesis in vitro and in vivo using a mice model and to explain the different roles of M-MDSCs and G-MDSCs in the myeloma microenvironment.

Methods

Cell lines

Mouse multiple myeloma cell line MPC11 was purchased from the cell culture center of Peking Union Medical College, Beijing, China. MPC11 cells were maintained in Dulbecco's Modified Eagle Medium's supplemented with 10% fetal calf serum, 100 mg/mL penicillin/streptomycin and L-glutamine. The cells were incubated at 37 °C and 5% CO_2. MPC11 cells could secrete interleukin-6 (IL6). So, the activity of MPC11 cells could also be measured by its ability of IL6 production.

Animals

BALB/C mice ($H\text{-}2^{Kd}$) (male, 6–8 weeks old) were purchased from the laboratory animal center of Southern Medical University (Guangzhou, China). All mice were housed in autoclaved microisolator environments at Forevergen Biosciences Co., Ltd. (Guangzhou, China). To generate the myeloma mice model, 6×10^5 MPC11 cells were injected subcutaneously into the left inguinal skin. All manipulations were performed under a laminar flow hood. For the in vivo treatment, DAC was administered intraperitoneally at a dose of 1 mg/kg for 5 consecutive days. For MDSC depletion, the anti-mouse Gr1 antibody or its isotype (Bio X Cell, NH, USA) was injected intraperitoneally at a dose of 200 μg/mouse/day every 3 days for a total of 4 times. All animal experiments were performed in accordance with national and institutional guidelines for animal care and were approved by the Animal Use and Care Committee of The Second Clinical Medical College (Shenzhen People's Hospital), Jinan University.

Flow cytometry staining and analysis

PE-conjugated anti-mouse Gr-1 and CD8, FITC-conjugated anti-mouse CD11b, and APC-conjugated anti-mouse CD4 were purchased from eBiosciences (San Diego, USA). Propidium iodide (PI) and Annexin V were purchased from Biolegend (San Diego, USA). Fluorescence-labeled cells were analyzed on a BD FACSCalibur cytometer, and data were analyzed using Flowjo software version 7.6.1 (TreeStar, Ashland, USA).

Cell sorting

Mouse MDSC isolation kits, MACS columns and a MACS separator were purchased from Milteny Co. (San Diego, USA). MDSC sorting was performed according to the manufacturer's instructions. Briefly, bone marrow cells from MPC11-bearing mice were first magnetically labeled with anti-Gr1 microbeads and anti-Ly6G microbeads. Then, the cell suspension was loaded onto a MACS column. The $Gr1^{high}/Ly6G^{high}$ cells, which were G-MDSCs, were retained and positively selected in the first step. In the second step, the flow-through fraction was labeled with anti-Gr1 microbeads again and then loaded onto a second MACS column. The retained and positively selected cells in the second column were $Gr1^{dim}/Ly6G^{low}$ cells, which were M-MDSCs. For experiments using total MDSCs, these two MDSC subsets were mixed together after sorting.

Coculture of MDSCs and MPC11 cells in vitro

Primary MDSCs and MPC11 cells were cocultured in a transwell plate (pore size: 0.4 μm) containing Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 mg/mL penicillin/streptomycin and L-glutamine. Human granulocyte colony-stimulating factor (G-CSF) was added at 100 ng/mL, and mouse granulocyte–macrophage colony-stimulating factor (GM-CSF) was added at 250 U/mL. Cultures were incubated at 37 °C and 5% CO2. Recombinant murine IL-13 (R&D Systems, Minneapolis, USA) was added at 80 ng/mL every other day. MPC11 cells were seeded in the upper chamber at a density of 2×10^5/mL, while MDSCs were seeded in the lower chamber at a density of 1×10^6/mL. M-MDSCs secrete arginase-1 (ARG-1); thus, for ARG-1 antagonism, N^G-hydroxy-L-arginase (NOHA) (Cayman Chemical, Ann Arbor, USA) was added to the coculture system at a concentration of 50 μmol/L. G-MDSCs secretes inducible Nitric oxide synthase (iNOS); thus, for iNOS antagonism, *N*-ω-nitro-L-arginine methyl ester (L-NAME) (Sigma, St. Louis, USA) was added to the coculture system at a concentration of 10 mmol/L.

ELISA

After coculture for 120 h, supernatants were collected from the top chamber of the transwell plates and preserved at -20 °C. Since MPC11 cells could secrete IL6, concentrations of IL-6 in culture supernatants were determined using mouse IL-6 ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, USA).

Cell viability assay

The MTT assay was used to detect cell viability. Briefly, the MPC11 cells after coculture were harvested and re-seeded into 96-well plates at a density of 2×10^4 cells/well. The medium for MPC11 cells culture in the 96-well plates was also Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 mg/mL penicillin/streptomycin and L-glutamine. After being cultured under standard conditions for 24, 72, or 120 h, 10 μL of MTT solution (Sigma, 5 mg/mL in PBS, St. Louis, USA) was added to each well and incubated for another 4 h. Then, the supernatant was discarded, and 100 μL of DMSO was added to each well. After shaking the plates for 10 min, the absorbance was recorded at 570 nm using a spectrophotometer.

Statistics

Statistical analysis was conducted using GraphPad Prism (Version 5.0, GraphPad Software, Inc. La Jolla, USA). Student's *t* test was used to compare continuous variables between groups. Mice survival was calculated using the Kaplan–Meier method and compared by the log-rank test. A *p* value less than 0.05 was considered significant.

Results

In vitro coculture assay revealed the impact of DAC BMs on MPC11 cells

We generated an MPC11-bearing BALB/C mice model, sacrificed the mice when the tumors became palpable (about 5 × 5 mm), and then collected the bone marrow cells. The bone marrow cells were separated into two populations by MDSC isolation kits, as indicated in the methods. One population was MDSCs, primarily Gr1+/CD11b+ cells; the other was bone marrow cells without MDSCs (nonMDSCs). MDSCs or nonMDSCs were then cocultured with MPC11 cells in a transwell system. MPC11 cells were seeded in the upper chamber at a density of 2×10^5/mL, while MDSCs or nonMDSCs were seeded in the lower chamber at a density of 1×10^6/mL. After coculture for 5 days, we found that MPC11 cells cocultured with MDSCs showed higher

proliferation rates, more IL6 production, and less apoptosis than those cocultured with nonMDSCs, as shown in Fig. 1, which indicated that MDSCs support MPC11 cell survival in an in vitro microenvironment.

Furthermore, we generated MPC11-bearing BALB/C mice model, treated them with DAC at a dose of 1 mg/kg/day or with PBS as control for 5 consecutive days and then sacrificed them and collected the bone marrow cells (DAC BMs and CTR BMs). DAC BMs or CTR BMs were further cocultured with MPC11 cells in the transwell system as mentioned above. After coculture for 5 days, we found that MPC11 cells cocultured with CTR BMs showed higher proliferation rates, more IL6 production, and less apoptosis than those cocultured with DAC BMs (Fig. 1).

Considering that MPC11 cocultured with DAC BMs showed a pattern similar to those cocultured with nonMDSCs, and since we have already demonstrated that DAC could deplete MDSC in the bone marrow of mice (Zhou et al. 2017), we believe that DAC BMs inhibit MPC11 cell survival and proliferation in vitro probably via MDSC depletion.

M-MDSCs, but not G-MDSCs, rescued the impact of DAC BMs on MPC11 cells in the in vitro coculture assay

To further confirm our hypothesis that DAC BMs inhibited MPC11 cell survival in vitro by depleting MDSCs, we repeated the in vitro coculture assay by replenishing MDSCs into DAC BMs in the coculture transwell system. Briefly, DAC BMs and MPC11 cells were cocultured in the transwell system. M-MDSCs and G-MDSCs were sorted and supplemented into DAC BMs, respectively. For DAC BMs supplemented with M-MDSCs or G-MDSCs, NOHA or L-NAME were further added, respectively. As shown in Fig. 2, supplementation with M-MDSCs significantly rescued the impact of DAC BMs on MPC11 cells in vitro by promoting proliferation, increasing IL6 production and reducing MPC11 apoptosis. Further treatment with NOHA counteracted the rescue effect of M-MDSCs. However, such a rescue effect was not observed in DAC BM supplemented with G-MDSCs or with G-MDSCs and L-NAME treatment.

Therefore, we confirmed that M-MDSCs, but not G-MDSCs, were essential for MPC11 cells' survival and proliferation in the in vitro coculture system. And the reason why DAC BM inhibited MPC11 cells was mainly due to M-MDSC depletion after DAC treatment.

DAC treatment inhibited MPC11 proliferation in vivo by depleting M-MDSCs and increasing T cell infiltration in the tumor tissue

To further confirm the efficacy of DAC treatment in vivo, we employed the mice model as mentioned above. The MPC11-bearing mice were divided into six groups: group 1 received the anti-Gr1 antibody and DAC treatment; group 2 received

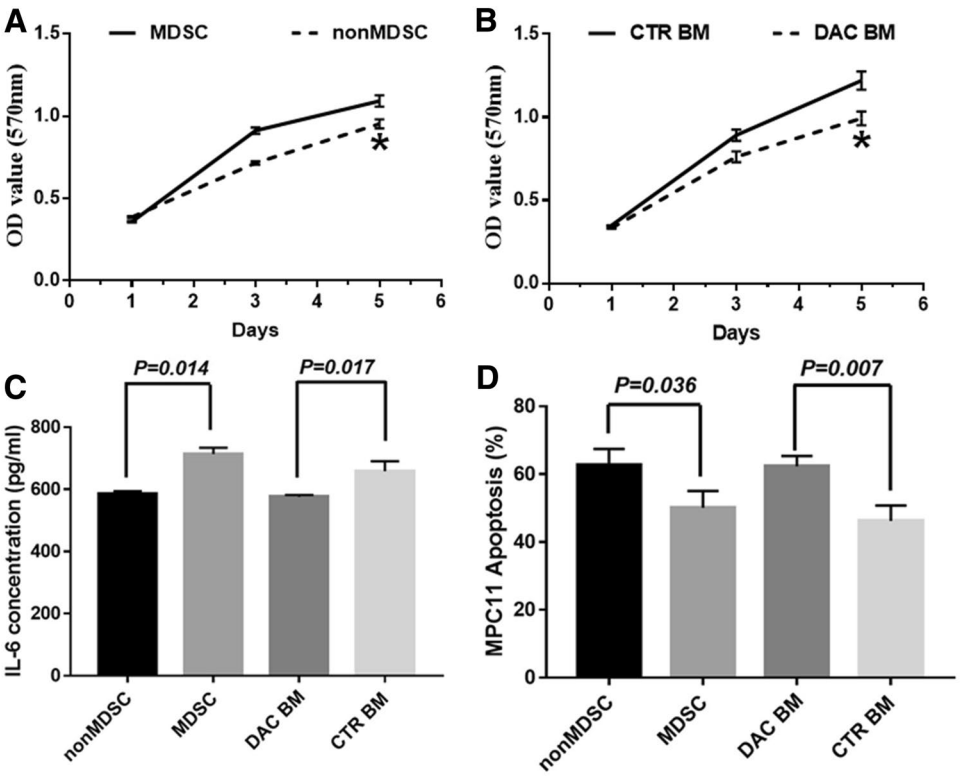

Fig. 1 In vitro coculture assay revealed the impact of DAC BMs on MPC11 cells. **a** MPC11 cells cocultured with MDSCs showed a significantly higher proliferation rate than those cocultured with nonMDSCs; **b** MPC11 cells cocultured with CTR BMs showed a significantly higher proliferation rate than those cocultured with DAC BMs; **c** MPC11 cells cocultured with MDSCs or with CTR BMs secreted more IL6 than those cocultured with nonMDSCs or with DAC BMs; **d** MPC11 cells cocultured with MDSCs and CTR BMs showed less apoptosis than those cocultured with nonMDSCs or with DAC BMs. $^*p < 0.05$. Graphs are shown as the mean ± SEM. Data are representative of three independent experiments with triplicate wells for each group

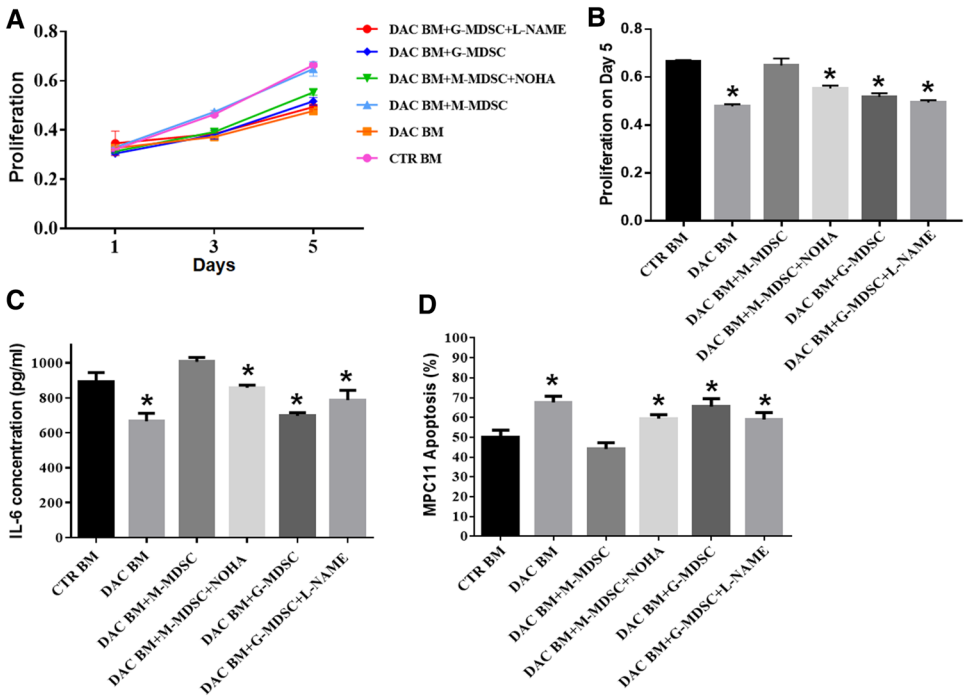

Fig. 2 M-MDSCs, but not G-MDSCs, rescued the impact of DAC BMs on MPC11 cells in the in vitro coculture system. **a** MPC11 proliferation by MTT assay; **b** bar chart of MPC11 proliferation data on day 5 after coculture; **c** IL6 production by MPC11 cells on day 5 after coculture; and **d** MPC11 cell apoptosis on day 5 after coculture. Supplementation with the ARG-1 antagonist NOHA counteracted the rescue effect of M-MDSCs, while supplementation with the iNOS antagonist L-NAME did not affect the effect of G-MDSCs. Graphs are shown as the mean ± SEM. *$p < 0.05$ vs. CTR BM. Data are representative of three independent experiments with triplicate wells for each group

the anti-Gr1 antibody and PBS treatment; group 3 received the isotype and DAC treatment; group 4 received the isotype and PBS treatment; group 5 received the isotype and DAC treatment as well as G-MDSC reinfusion via the tail vein at a dose of 5×10^6/mouse after DAC treatment; and group 6 received the isotype and DAC treatment as well as M-MDSC reinfusion via the tail vein at a dose of 5×10^6/mouse after DAC treatment. For all groups, the day MPC11 cells were inoculated subcutaneously was marked as day 0. Then, anti-Gr1 antibody or its isotype was infused at days 1, 4, 7, and 10 for a total of four doses, while DAC or PBS treatment was administered from day 6 to day 10 for a total of 5 consecutive days. M-MDSCs or G-MDSCs were reinfused via tail vein at day 12.

As shown in Fig. 3a–c, the isotype combined with PBS treatment did not show any therapeutic efficacy, while both the anti-Gr1 antibody and DAC treatment inhibited tumor proliferation in vivo. Combining the anti-Gr1 antibody and DAC treatment elicited a synergistic effect as the most significant inhibition on tumor growth. In addition, for mice treated with the isotype and DAC, reinfusion with M-MDSCs promoted tumor growth, while reinfusion with G-MDSCs did not show any effect on tumor growth. These in vivo results were concordant with what we observed in the in vitro coculture system—M-MDSCs could rescue the effect of DAC treatment.

We then sacrificed the mice on day 25, harvested and digested the tumors, and analyzed the dissociated tumor cells by flow cytometry, as shown in Fig. 3d. The constitution of tumor-infiltrating T cells was shown in Fig. 3e, f. As was shown, in mice treated with isotype and PBS, since no MDSCs were depleted, the percentage of tumor-infiltrating CD4+ and CD8+ T cells were both very low; in mice treated with the isotype and DAC and then reinfused with M-MDSCs, the percentage of tumor-infiltrating T cells was also very low. However, in other groups, tumor-infiltrating CD4+ and CD8+ T cells were all at a similarly high level. According to this result, DAC treatment, by depleting M-MDSCs, could not only disrupt the microenvironment MPC11 cells needed for survival and proliferation, but also enhance the autologous T cell infiltration in the tumor tissue, which indicated stronger immune response.

Discussion

MM remains an incurable hematological malignancy even in the new-drug era. Therapies targeting the MM microenvironment hold promise for a possible cure in future. In our and other researchers' previous studies, hypomethylation treatment, both decitabine and 5-azacytidine, had been reported to deplete MDSCs in the tumor microenvironment, and thus enhance the antitumor immune response in vitro and in vivo (Mikyskova et al. 2014; Stone et al. 2017; Terracina et al. 2016; Zhou et al. 2017). In this study, we not only confirmed that DAC treatment could inhibit myeloma proliferation in vitro and in vivo by depleting MDSCs but also demonstrated that M-MDSCs are an essential subset for myeloma cell survival and immune escape in the MM microenvironment.

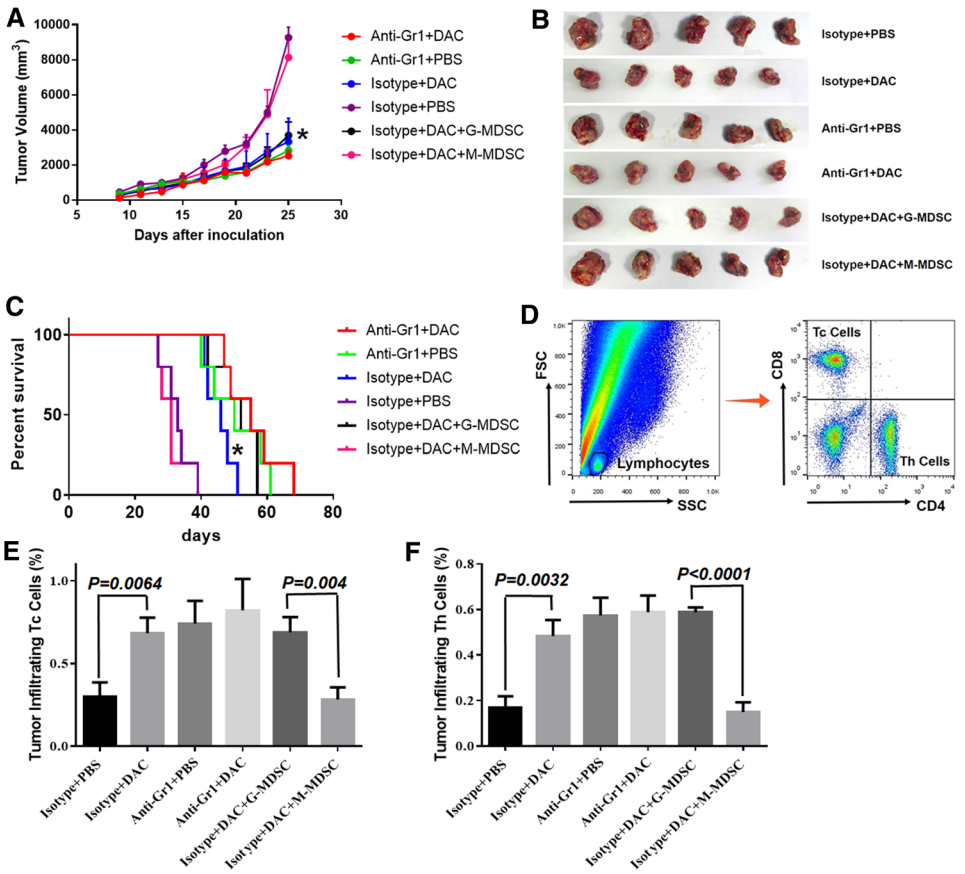

Fig. 3 DAC treatment inhibits MPC11 proliferation in vivo by depleting M-MDSCs and increasing T cell infiltration in the tumor tissue. **a** proliferation shown as tumor volumes between different groups in days after MPC11 inoculation; **b** proliferation data shown as dissected tumors; **c** survival curves of mice in different groups, $^{*}p < 0.05$ vs. isotype + PBS group; **d** gating strategy for analyzing tumor-infiltrating T cells in disassociated tumors; M-MDSC depletion, but not G-MDSC depletion, by either anti-Gr1 antibody, DAC, or both significantly increased tumor-infiltrating Tc (**e**) and Th (**f**) cells in the tumor tissue, which indicated an enhanced autologous T cell immune response. Data are representative of three independent experiments with 5 replicates for each group

MDSCs can be subdivided into M-MDSCs and G-MDSCs. Many studies have reported that M-MDSCs, but not G-MDSCs, were significantly increased in various tumors such as breast cancer (Speigl et al. 2018), diffuse large B cell lymphoma (Wu et al. 2015), peripheral T cell lymphoma (Du et al. 2017), urothelial carcinoma (Ornstein et al. 2018), glioma (Domenis et al. 2017), non-small cell lung cancer (Pogoda et al. 2016), and myelodysplastic syndromes (Kittang et al. 2016). Moreover, an increase in the level of M-MDSCs in the peripheral blood may predict a poor treatment response. For example, Giallongo et al. reported that in CML patients who received dasatinib treatment, the peripheral M-MDSC count was positively correlated with patients' BCR/ABL transcript levels. M-MDSC accumulation in the peripheral blood indicated minimal residual disease and disease progression (Giallongo et al. 2018). In MM patients, this is also the case. Wang et al. reported that the levels of M-MDSCs in newly diagnosed and relapsed MM patients were significantly increased compared with those in MM patients in remission as well as healthy donors. Moreover, the levels of M-MDSCs were shown to correlate with tumor progression (Wang et al. 2015b). Lee et al. also reported that the increase in peripheral M-MDSCs after lenalidomide-dexamethasone treatment was associated with failure to achieve a response of "very good partial remission" (VGPR) or greater in refractory/relapsed MM patients (Lee et al. 2016). Therefore, M-MDSCs and G-MDSCs may play different roles in the MM microenvironment. Van Valckenborgh et al. purified M-MDSCs and G-MDSCs from a MM mice model and tested their ability to suppress antigen-specific T cell proliferation in a dose-dependent manner. They found that M-MDSCs were, on average, more suppressive than G-MDSCs in the MM microenvironment (Van Valckenborgh et al. 2012), while the function of G-MDSCs lied mainly in promoting angiogenesis in the MM microenvironment, as was reported by other researchers (Binsfeld et al. 2016; Giallongo et al. 2016). In our study, we also found that supplement with M-MDSCs significantly antagonized the therapeutic effect after DAC treatment in vitro and in vivo, while additional NOHA counteracted the antagonistic effect of M-MDSCs in vitro. Supplementation with G-MDSCs or with L-NAME did not show any influence on tumor growth. The reason why G-MDSCs did not show any efficacy on MM survival in our study is possibly due to the short observation duration. Since the main function of G-MDSCs is promoting angiogenesis, the duration of supplementation with G-MDSCs in our experiments may have been too short for angiogenesis in MM tissue. Our results are in accordance with the

notion that M-MDSCs play an important role in myeloma cell survival in the MM microenvironment. In addition, since we employed the transwell coculture system in this study, we believe that the crosstalk between M-MDSCs and MM cells occurred independently of cell–cell contact. Some studies have reported that the crosstalk between MDSCs and MM cells is mediated by exosomes (Wang et al. 2015a, 2016). Illustrating the mechanism underlying how M-MDSCs support MM cell survival and proliferation is a goal of our future work.

Considering the important role of MDSCs in the tumor microenvironment, many researchers have tried different methods to disrupt this microenvironment by targeting MDSCs. Some have attempted to promote the differentiation of MDSCs to more mature cells, for example, using valproic acid (Youn et al. 2013) and all-trans retinoic acid (Iclozan et al. 2013). Others tried to block cytokines secreted by MDSCs to antagonize their function, for example, using a COX-2 inhibitor such as celecoxib (Veltman et al. 2010) or the PDE-5 inhibitors sildenafil and tadalafil (Serafini et al. 2006). Researchers have also tried to deplete MDSCs selectively, for example, using daunorubicin (Belyaev et al. 2018), ipilimumab (de Coana et al. 2017) and gemcitabine (Sasso et al. 2016). All these modalities, by influencing MDSCs in different ways, inhibited tumor cell proliferation and enhanced autologous T cell immune response against tumors. In this study and in our previous work, we found that demethylation treatments such as DAC also showed potent anti-myeloma activity in vivo by depleting M-MDSC. To our knowledge, this is the first study demonstrating the possible immune regulation mechanism of DAC in myeloma treatment.

In conclusion, DAC could deplete MDSCs in vitro and in vivo as was formerly reported. In this study, we provide evidence to show that DAC treatment can inhibit myeloma cell proliferation and induce enhanced autologous T cell immune response by depleting M-MDSCs in the MM microenvironment. We believe that DAC treatment could play a synergistic role with currently available anti-myeloma therapies, and thus improve the prognosis of MM in future.

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Compliance with ethical standards

Conflict of interest Author Jihao Zhou declares that he has no conflict of interest. Author Qi Shen declares that he has no conflict of interest. Author Haiqing Lin declares that he has no conflict of interest. Author Lina Hu declares that he has no conflict of interest. Author Guoqiang Li declares that he has no conflict of interest. Author Xinyou Zhang declares that he has no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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