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Original Articles

Myeloid-derived suppressor cells induce multiple myeloma cell survival by activating the AMPK pathway

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ABSTRACT

Multiple Myeloma (MM) is an incurable malignancy of terminally differentiated plasma cells, which are predominantly localized in the bone marrow. Myeloid-derived suppressor cells (MDSC) are described to promote MM progression by immunosuppression and induction of angiogenesis. However, their direct role in drug resistance and tumor survival is still unknown. In this study, we performed co-culture experiments of myeloma cells with 5TMM derived MDSC *in vitro*, leading to increased survival and proliferation of MM cells. Co-culture experiments resulted in MDSC-induced AMPK phosphorylation in MM cells, which was associated with an increase in the anti-apoptotic factors MCL-1 and BCL-2, and the autophagy-marker LC3II. In addition, 5TMM cells inoculated in mice showed a clear upregulation of AMPK phosphorylation *in vivo*. Targeting the AMPK pathway by Compound C resulted in apoptosis of human myeloma cell lines, primary MM cells and 5TMM cells. Importantly, we observed that the tumor-promoting effect of MDSC was partially mediated by AMPK activation. In conclusion, our data clearly demonstrate that MDSC directly increase the survival of MM cells, partially through AMPK activation, identifying this pathway as a new target in the treatment of MM patients.

1. Introduction

Multiple Myeloma (MM) is a hematological cancer with an uncontrolled growth of plasma cells. The tumor cells display a preferential localization in the bone marrow (BM), where the local microenvironment offers the most optimal niche for tumor cell survival and proliferation and also protects the MM cells from drug-induced apoptosis [1]. The MM cells, on the other hand, modulate the BM microenvironment resulting in osteolytic lesions, anemia, and immunosuppression, leading to the typical clinical symptoms of MM disease [2]. Moreover, it has been proven in the clinic that treating the 'abnormal' BM can lead to an improvement in the management of MM. Proteasome inhibitors like bortezomib as well as immunomodulatory agents such as lenalidomide are compounds that target both the BM microenvironment and MM cells. These agents have significantly contributed to the increased survival of MM patients in the last decade, however the majority of patients relapse after a certain period of time [3,4]. This emphasizes the need for further investigation of the MM-BM interactions to develop new targeted therapies.

An important cell type present in the MM-BM is the myeloid-derived suppressor cell (MDSC) population. MDSC are described as a heterogeneous population of immature myeloid cells, that accumulate during cancer development [5]. The most important role of MDSC is to regulate the immune system mainly by their immunosuppressive capacity and thereby promoting tumor development. Besides immune regulation, MDSC also promote tumor angiogenesis and tumor growth by the secretion of cytokines and growth factors [6–8]. We previously described the presence and the immunosuppressive capacity of MDSC in

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MM in the syngeneic, immunocompetent 5TMM mouse models using CD11b, Ly6G and Ly6C as MDSC markers [9]. Furthermore, Mcl-1 was demonstrated to be an important survival factor of MDSC in MM pathology [10]. In the 5TMM models we described that depleting MDSC with anti-Gr-1 antibodies or 5-fluorouracil could indeed reduce tumor burden in diseased mice [10]. Other groups have reported the MDSC immunosuppressive activity in MM patients [7,8]. Since it is suggested that targeting MDSC would be beneficial for the treatment of cancer patients, MDSC-targeting agents are currently under investigation [10–13].

In the current study, we investigated the role of MDSC on MM cell survival and drug resistance focusing on the AMPK pathway as an important mediator. As a cellular energy sensor, AMPK regulates lipid and glucose metabolism and could be essential in cancer cell survival during stress conditions. We further elucidated the role of AMPK in MM.

2. Material and methods

2.1. Mice

C57BL/KaLwRij mice were purchased from Envigo (Horst, the Netherlands). They were housed and maintained following the conditions approved by the Ethical Committee for Animal Experiments, Vrije Universiteit Brussel (license no. LA1230281). The 5T33MM model originated from spontaneously developed MM in elderly C57BL/KalwRij mice and was propagated by intravenous transfer of the diseased marrow into young syngeneic mice [14]. For *in vivo* experiments, mice were intravenously inoculated with 5×10^5 5T33MM cells. The 5TGM1 cell line, kindly provided by Prof. Dr. Oyajobi, resulted spontaneously from cultured 5T33MMvv cells and grow *in vitro* independently from BM stroma. 5TGM1 cells were previously modulated to express eGFP [15].

2.2. Compounds

Compound C (Dorsomorphin) was obtained by Sigma-Aldrich (Bornem, Belgium) and used *in vitro* at micromolar concentration. 5-fluorouracil (5FU) (Sigma-Aldrich), melphalan (Sigma-Aldrich) and bortezomib (Bz) (Selleckchem, Munich, Germany) were used for *in vitro* and *in vivo* studies. Bz and 5FU were dissolved in dimethylsulfoxide according to manufacturer's instructions. For *in vivo* use, both were further diluted in PBS to their appropriate concentration.

2.3. Cell culture and purification

BM was isolated from diseased 5T33MM mice (~18–20 days after inoculation) [16] and red blood cells were depleted by red blood cell lysis. 5T33MMvv cells were further purified by a negative selection ($CD11b^-$) by using CD11b MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions, while $CD11b^+$ cells were collected for the MDSC fraction. After coculture of MDSC with 5T33MMvv cells (1/1 ratio), a second CD11b MACS selection was performed to obtain pure 5T33MMvv cells (CD11b- fraction) for western blot analysis (> 90% purity, defined by May-Grünwald-Giemsa stained cytosmears). Primary human MM cells were purified from BM samples of MM patients after written informed consent in accordance with the Declaration of Helsinki. The research is approved by the Ethical Board of the UZ Brussel (B.U.N. 143201316382). Mononuclear cells were obtained after Ficoll density gradient centrifugation (Nycomed, Lucron Bioproducts, De Pinte, Belgium) and purified by CD138 positive selection by MACS beads (Miltenyi Biotec). The human myeloma cell lines (HMCL) LP-1, U266 and RPMI8226 were obtained from the American Type Culture Collection. RPMI8226, LP-1, U266, 5T33MMvv cells and 5TGM1 cells were cultured in RPMI 1640 medium (Lonza), supplemented with 10% fetal calf serum (HyClone), 2mM L-glutamine, and antibiotics (Lonza).

2.4. Viability and apoptosis assay

Cell viability was determined by the use of a CellTiter-Glo® luminescent assay (Promega, Madison, WI, USA). Apoptosis was quantified on the $CD11b^-$ population (CD11b-PECy7 staining, Biolegend, San Diego, CA, USA) by an AnnexinV-APC staining (Becton Dickinson, Franklin Lakes, NJ, USA) (gating strategy in supplemental Figure 1) and active Caspase-3-FITC staining (gating strategy in supplemental Figure 2B) (Becton Dickinson) followed by flow cytometric analysis with the FACS Canto and FACSDiva software (BD Pharmingen, Erembodegem, Belgium). AnnexinV/7-AAD (Becton Dickinson) staining was used to analyse apoptosis in HMCL.

2.5. Western blot/PathScan® Intracellular Signaling Array

Cells were harvested, lysed, and protein extracts were blotted as previously described [17]. The following western blot antibodies were used: pAMPK (#2535), total AMPK (#5831), MCL-1 (#5453), BCL-2 (#2870), LC3I/II (#4108) and B-ACTIN (#4967) (Cell Signaling Technology, Boston, MA). Pathways were analyzed by a PathScan® Intracellular Signaling Array Kit (#7323) (Cell Signaling) according to the manufacturer's instructions. The pixel densities of proteins were quantified by ImageJ.

2.6. BrdU staining

Purified 5T33MMvv cells (CD11b-) and MDSC (CD11b+) were cocultured and BrdU (Sigma-Aldrich) was added for 24 h. The mixture of cells were fixed, permeabilized and afterwards stained with a FITC-anti-BrdU antibody (Roche Diagnostics, Manheim, Germany) and 3H2 (idiotype) antibody [14]. Flow cytometry was performed using a FACS Canto and the FACSDiva software. BrdU incorporation was analyzed on the 3H2+ 5T33MMvv cell fraction (supplemental Figure 2A).

2.7. Cell cycle analysis

Cells were stained with propidium iodide (PI) solution containing 0.1% Triton-X100 (Merck, Barmstadt, Germany), 1 mg/ml sodium nitrate (Merck), 100μg/ml RNaseA (Boehringer Ingelheim, Germany) and 50μg/ml PI (Sigma). Cells were analyzed by flow cytometry (FACSCanto) using FACSDiva software.

2.8. In vivo experiment 5T33MM model

To investigate the effect of 5FU and Bz, 5T33MM mice (n = 7/group) were injected with vehicle (DMSO diluted in PBS), with one single injection of 5FU intraperitoneal (i.p) (50 mg/kg) on day 4, with Bz (0.7 mg/kg, on day 10, day 13 and day 16, subcutaneous) or with the combination of both. MDSC subpopulations were analyzed by flow cytometry using following antibodies: CD11b-FITC, Ly6G-PECy7 and Ly6C-APC (Biolegend). MDSC subpopulations were identified by gating strategies as previously described [10]. Tumor burden in the BM was assessed at day 17 by measuring the percentage of idiotype positive cells ($3H2^+$) by flow cytometry in the BM [14]. Additionally, serum M-spike was measured by means of serum electrophoresis.

2.9. Microarray data of primary Multiple Myeloma cells

To analyse PRKAA1 and PRKAA2 expression, we used publically available expression data (Affymetrix Human Genome U133 Plus 2.0 array platform) as published by Zhan et al. [18]. These datasets contain gene expression data of CD138 + selected plasma cells obtained from 22 healthy donors, 44 MGUS patients, 12 SMM patients (Gene Expression Omnibus) and 345 MM patients enrolled in total therapy 2 from the University of Arkansas for Medical Science (UAMS, Little Rock, AR) (Gene Expression Omnibus GSE5900 and GSE2658) [18,19].

The datasets were normalized using MAS5 (scaling factor 500) and are available through the Genomicscape webtool (http://www.genomicscape.com/; dataset GS-DT-48). For PRKAA1 and PRKAA2, we used the 225984_at and the 227892_at probes respectively. This section has now been adapted in the material and methods.

2.10. Statistics

Statistical analysis was done using GraphPad Prism 5 software. All data represent the mean ± standard deviation (SD), and results were analyzed using the Mann-Whitney *U* test and One-way ANOVA.

3. Results

3.1. MDSC induce a survival benefit for MM cells both by cytokine secretion and direct cell-cell contact

5T33MMvv cells isolated and purified from 5T33MM-diseased mice were cultured in the presence or absence of syngeneic CD11b$^+$ MDSC. After 16 h co-culture, cells were treated with bortezomib (Bz) or melphalan (Mel) for 24 h and apoptosis was analyzed in 5T33MMvv cells by AnnexinV/7-AAD flow cytometry staining. In Fig. 1A, we observed a significant reduction in apoptotic cell number when 5T33MMvv (CD11b$^-$) cells were co-cultured with MDSC (CD11b$^+$). Even in the presence of Bz (2.5nM-5nM) and Mel (15–30μM), we could observe a protection against induced apoptosis in the co-cultures of 5T33MMvv cells with CD11b$^+$ MDSC. In Fig. 1B, we performed similar co-culture experiments of MM cells and MDSC in the presence or absence of a transwell. We could observe a survival benefit in both conditions, however the strongest pro-survival effect could be observed by direct cell-cell contact. Next, we further examined apoptosis by active caspase-3 staining and proliferation by BrdU staining of 5T33MMvv cells in the presence of MDSC. We observed a significant reduction in caspase-3$^+$ cells (Fig. 1C) and a significant increase in proliferating cells (Fig. 1D). These results indicate that proliferation induction as well as apoptosis reduction are both involved in the 5T33MMvv survival benefit induced by MDSC.

3.2. Combination therapy of MDSC depleting agent 5-fluorouracil and bortezomib significantly reduces tumor load compared to single agent therapy

As our *in vitro* data indicate that MDSC stimulate survival of MM cells even in the presence of Bz we investigated whether targeting MDSC in combination with Bz would lead to a better reduction in tumor load *in vivo*. Therefore, we combined the MDSC depleting agent 5FU together with Bz in the 5T33MM mouse model (Fig. 2A). Previous work demonstrated that 5FU is able to reduce the MDSC population *in vivo* and this was associated with a decrease in tumor load [10]. Bz alone significantly reduced the number of monocytic MDSC (CD11b$^+$, Ly6-G^{low}) (Fig. 2B). The combination of 5FU and Bortezomib more specifically decreased the immature myeloid cell population (IMC, Ly6G^{low}, Ly6C^{low}) (Fig. 2C). Importantly, 5FU in combination with Bz showed a strong reduction in tumor load compared to single agent therapy, as evidenced by both a reduced serum M protein and reduced tumor burden in the BM (Fig. 2D and E).

3.3. MDSC induce AMPK activation, MCL-1 and BCL-2 expression in myeloma cells

To investigate the underlying pathways of the *in vitro* induced survival of MDSC in 5T33MMvv cells we performed a Pathscan® Intracellular Signaling Array demonstrating an increase in pERK1/2, pSTAT3 and pAMPK (phospho Adenosine Monophosphate-activated Protein Kinase) in 5T33MMvv cells after co-culture with MDSC (Fig. 3A). AMPK plays a key role in energy homeostasis and has been described to drive either cell death or cell survival [20]. AMPK activation was confirmed by western blot and measurement of pixel densities (Fig. 3B and C). On the other hand, tumor associated macrophages were not able to increase AMPK phosphorylation (supplemental Figure 3). In literature, it has been demonstrated that under metabolic stress conditions, AMPK could maintain cancer cell survival by induction of autophagy and increased expression of anti-apoptotic proteins MCL-1 and BCL-2 [21–24]. By western blot, we could observe an increase in the anti-apoptotic factors MCL-1 and BCL-2 and the autophagy marker LC3II upon co-culture of 5T33MMvv cells and MDSC (Fig. 3D). To

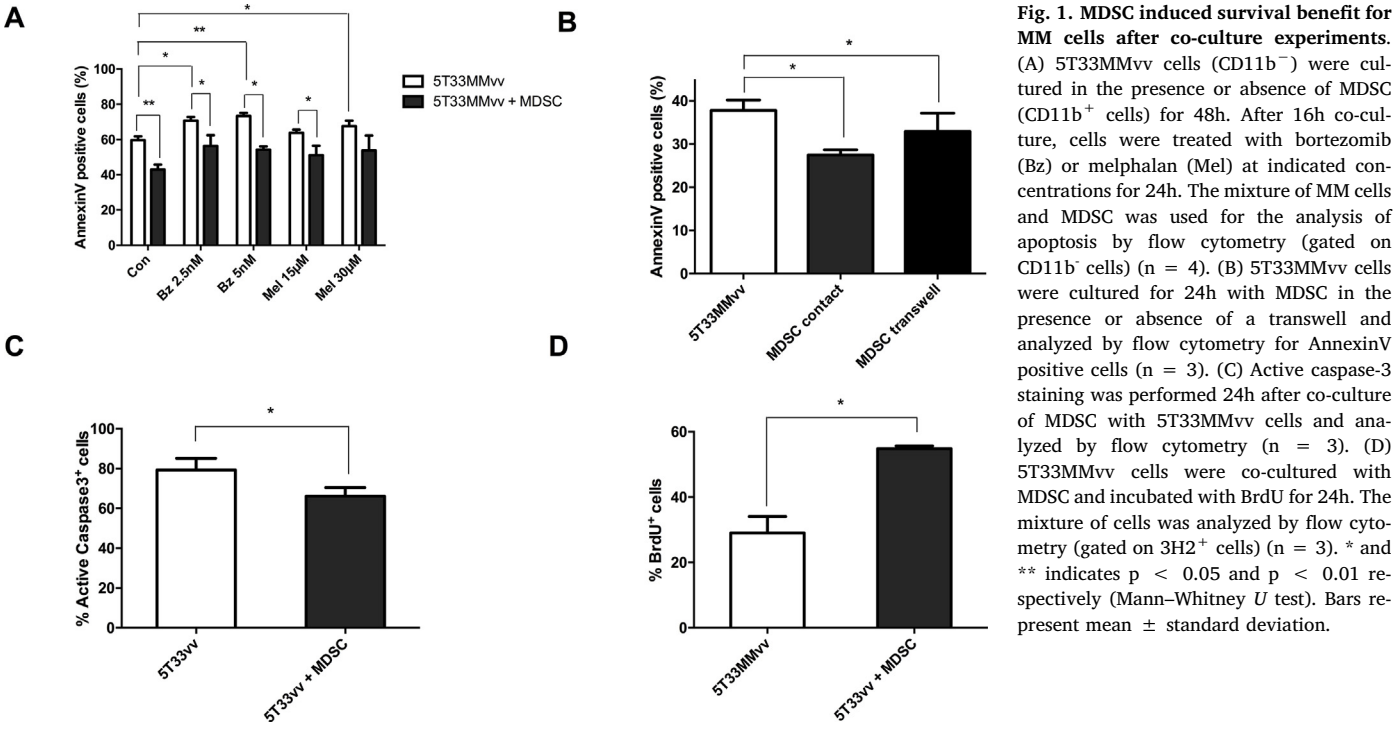

Fig. 1. MDSC induced survival benefit for MM cells after co-culture experiments. (A) 5T33MMvv cells (CD11b$^-$) were cultured in the presence or absence of MDSC (CD11b$^+$ cells) for 48h. After 16h co-culture, cells were treated with bortezomib (Bz) or melphalan (Mel) at indicated concentrations for 24h. The mixture of MM cells and MDSC was used for the analysis of apoptosis by flow cytometry (gated on CD11b$^-$ cells) (n = 4). (B) 5T33MMvv cells were cultured for 24h with MDSC in the presence or absence of a transwell and analyzed by flow cytometry for AnnexinV positive cells (n = 3). (C) Active caspase-3 staining was performed 24h after co-culture of MDSC with 5T33MMvv cells and analyzed by flow cytometry (n = 3). (D) 5T33MMvv cells were co-cultured with MDSC and incubated with BrdU for 24h. The mixture of cells was analyzed by flow cytometry (gated on 3H2$^+$ cells) (n = 3). * and ** indicates $p < 0.05$ and $p < 0.01$ respectively (Mann–Whitney *U* test). Bars represent mean ± standard deviation.

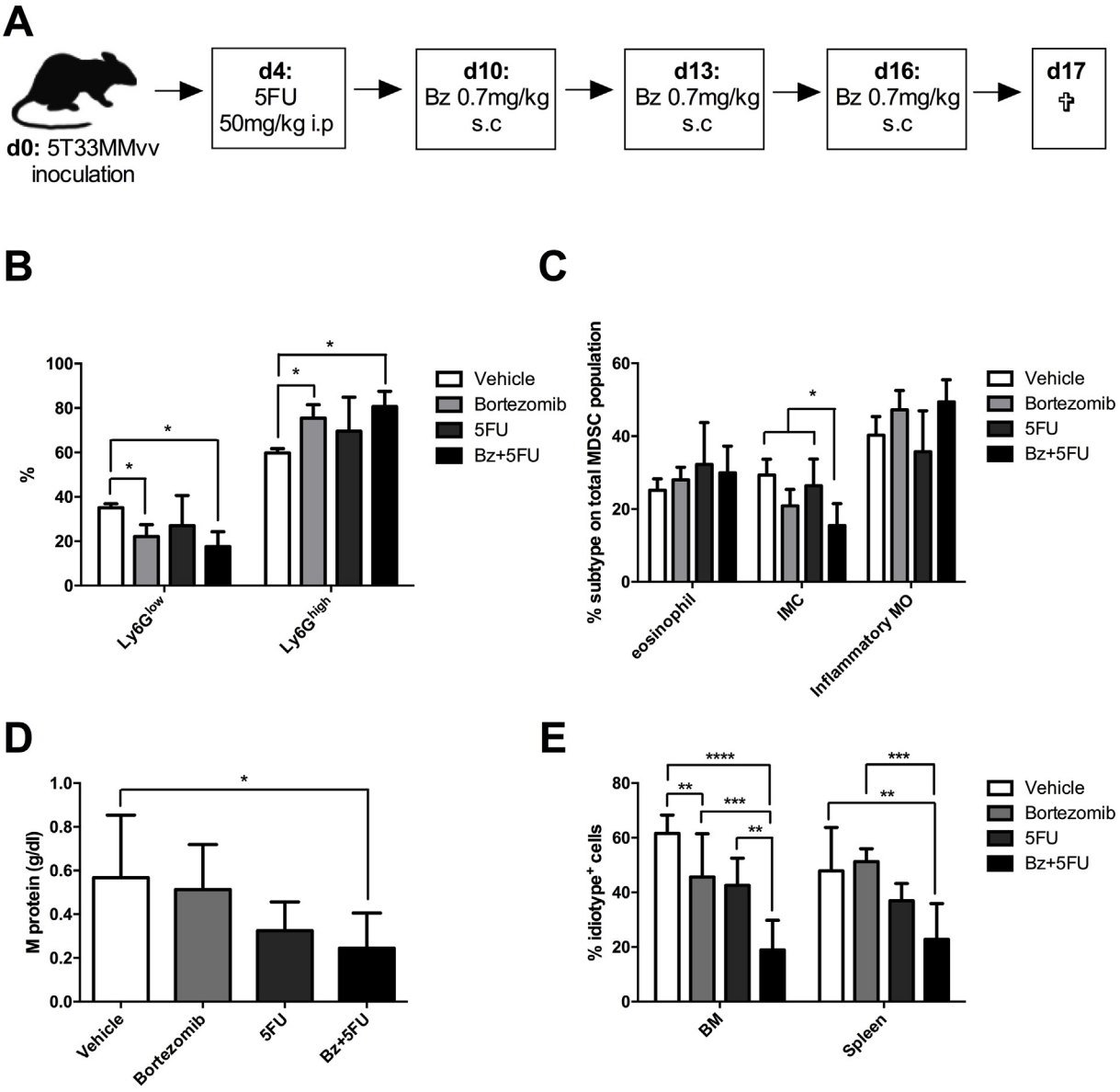

Fig. 2. *In vivo* effect of MDSC-depleting agent 5-fluorouracil in combination with bortezomib on tumor progression. (A) Treatment schedule of 5T33MM mice (n = 7/group). 5T33MM mice were treated with 50 mg/kg 5FU (i.p) on day 4, with Bz (0.7 mg/kg, 3 times, s.c), with the combination of both or with vehicle and MDSC subpopulations and tumor load was assessed at day 17. (B) Percentage $CD11b^+$ $Ly6G^{low/high\ cells}$ of total bone marrow was determined by flow cytometry. (C) In the $CD11b^+$ $Ly6G^{low}$ population, Ly6C expression was analyzed by flow cytometry to distinguish inflammatory monocytes (MO) ($Ly6C^{hi}$), eosinophils ($Ly6C^{intermediate}$), and immature myeloid cells (IMC) ($Ly6C^{low}$). (D) M protein was determined by serum electrophoresis. (E) Tumor load was assessed by specific anti-idiotype FACS staining. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (One-Way ANOVA test). Bars represent mean ± standard deviation.

confirm the observed AMPK activation *in vivo*, we used the 5T33MM derived 5TGM1 model. 5TGM1 cells have the advantage of growing *in vitro* as well as *in vivo* and could be used to investigate the effect of the bone marrow microenvironment on AMPK activation in MM cells by *in vivo* transfer. 5TGM1 cells were injected into mice and when mice showed signs of disease, 5TGM1 cells were isolated, purified by MACS ($CD11b^-$ selection, purity > 90%) and analyzed for AMPK activation. We observed an increase in AMPK phosphorylation of *in vivo* (vv) derived 5TGM1 cells compared to *in vitro* (vt) cultured 5TGM1 cells (Fig. 3E). Furthermore, isolation of *in vivo* derived 5TGM1 cells and subsequent culture *in vitro* (vv/vt) resulted again in reduced AMPK activation, indicating that the BM microenvironment is important in the regulation of AMPK phosphorylation in MM cells.

3.4. AMPK activation partially mediates the MDSC induced MM cell survival

To unravel the role of AMPK in MM cell survival, we targeted AMPK by Compound C, also called BML-275 or dorsomorphin. Compound C has been widely used in cell-based, biochemical and *in vivo* assays as a potent and selective AMPK inhibitor [25–27]. 5T33MMvv cells were co-cultured with MDSC and treated with Compound C. We observed an increase in apoptosis of 5T33MMvv cells (Fig. 4A) and a decrease in AMPK phosphorylation, MCL-1 and BCL-2 expression (Fig. 4B). The effect of Compound C on apoptosis was slightly more pronounced in the presence of MDSC (25% induced apoptosis compared to 20% in single cultured MM cells treated with 20μM Compound C) (Fig. 4A).

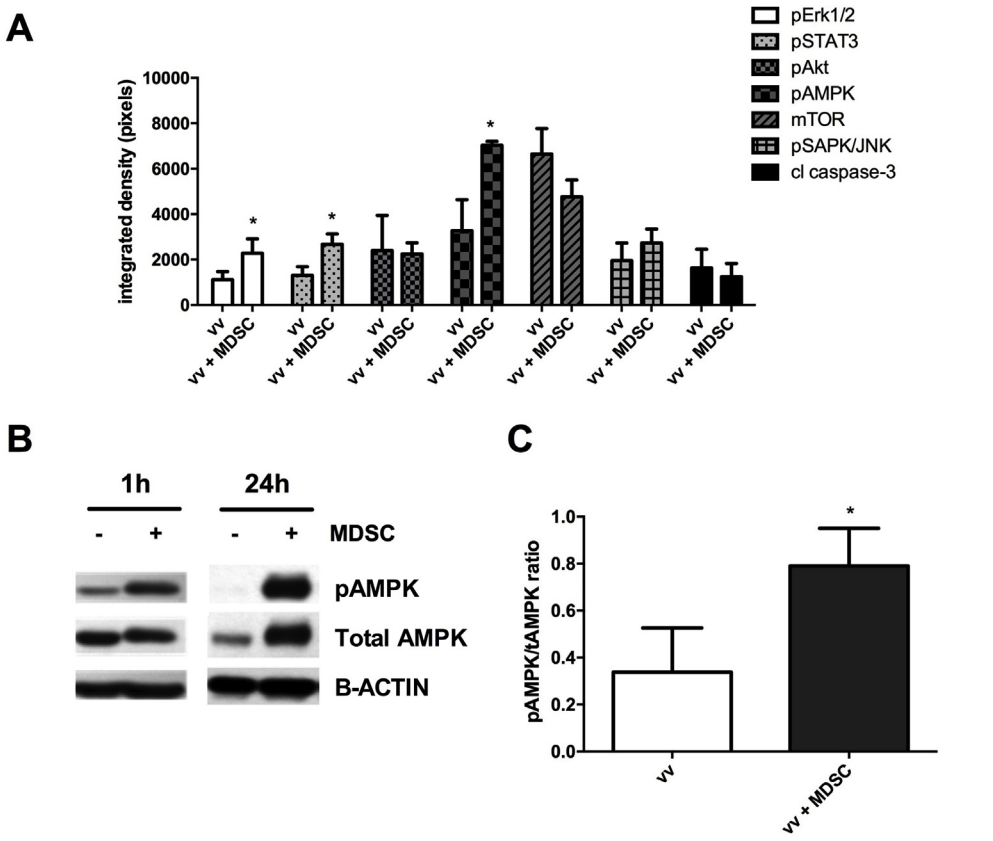

Fig. 3. MDSC induce AMPK activation, MCL-1 and BCL-2 expression in myeloma cells. (A) 5T33MMvv cells were cultured in the presence or absence of MDSC ($CD11b^+$ cells) for 24 h. Afterwards, 5T33MMvv ($CD11b^-$ cells) were purified by MACS isolation and pathways were analyzed by a PathScan® Intracellular Signaling Array Kit and expression was calculated by ImageJ (n = 3). (B) AMPK expression was analyzed by western blot after 1 h and 24 h of co-culture (n = 3). (C) The ratio of pAMPK/total AMPK after co-culture of 5T33MMvv with MDSC for 24 h was calculated by ImageJ (n = 3). (D) 5T33MMvv cells were cultured for 24 h with MDSC, CD11b-cells were purified and analyzed by western blot for MCL-1, BCL-2 and LC3I/II expression (n = 3). (E) 5TGM1 myeloma cells were injected and when mice showed signs of disease, 5TGM1 cells were isolated (vv) and part of the cells were cultured *in vitro* again (vv/vt) and collected after 7 days. AMPK expression and phosporylation in 5TGM1vt cells, vv cells, vv/vt cells and MDSC ($CD11b^+$) was analyzed by western blot. One experiment representing three is shown. *indicate $p < 0.05$ (Mann–Whitney *U* test). Bars represent mean ± standard deviation.

3.5. AMPK targeting by compound C induces apoptosis in human myeloma cell lines and patient samples

We further analyzed the AMPK pathway in primary human MM cells. The heterotrimeric protein AMPK is formed by α, β, and γ subunits. The human genes PRKAA1 and PRKAA2 encode for the catalytic α-subunit containing the kinase domain for AMPK activation by phosphorylation [28]. Gene expression of PRKAA1 and PRKAA2 was

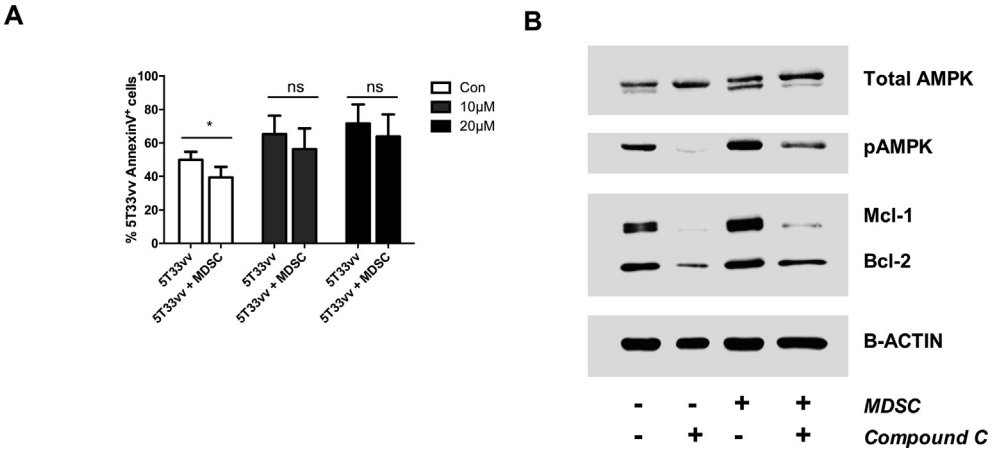

Fig. 4. AMPK activation as underlying mechanism of MDSC induced MM cell survival. (A) 5T33MMvv cells were cultured in the presence or absence of MDSC ($CD11b^+$ cells) and treated with Compound C for 24h. Apoptosis was analyzed by AnnexinV staining (gated on $CD11b^-$ cells) (n = 6). *indicate $p < 0.05$ (Mann–Whitney *U* test). Bars represent mean ± standard deviation. (B) 5T33MMvv cells were co-cultured with MDSC and treated with compound C for 24 h. MCL-1, BCL-2, AMPK and AMPK phosphorylation were analyzed by western blot. B-ACTIN was used as a loading control. One experiment representing three is shown.

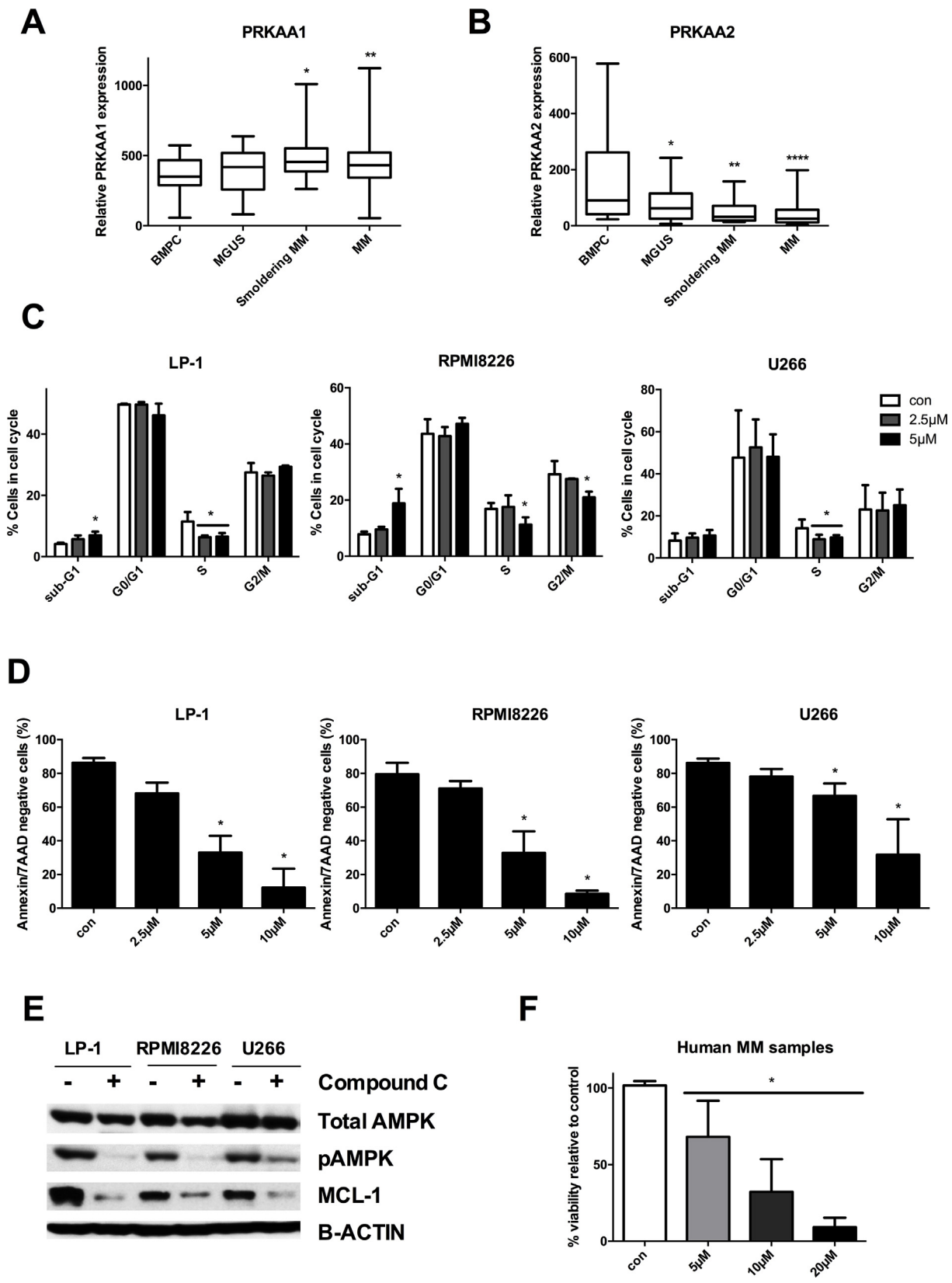

Fig. 5. AMPK targeting by Compound C induces apoptosis in human myeloma cell lines and patient samples. (A–B) PRKAA1 and PRKAA2 expression levels in MGUS, smoldering MM (SMM) and MM patients compared to bone marrow plasma cells (BMPC). (C–D) Different HMCL including LP-1, RPMI8226 and U266 were treated with Compound C at indicated concentrations for 48 h and cell cycle and apoptosis were analyzed by flow cytometry using propidium iodide staining and AnnexinV/7-AAD staining respectively (n = 3). (E) HMCL treated with Compound C for 24 h were analyzed by western blot for MCL-1, B-ACTIN, AMPK and pAMPK expression. One experiment representing three is shown. (F) CD138^{+} cells were MACS sorted from BM derived MM patient's samples and treated with Compound C (5,10,20μM). Cell viability was analyzed by Cell Titer Glo assay after 24 h (n = 3). * indicate $p < 0.05$, ** indicate $p < 0.01$, ****$p < 0.0001$ (Mann–Whitney *U* test). Bars represent mean ± standard deviation.

analyzed by the use of publically available expression data [18,19] and demonstrated a significant upregulation in PRKAA1 expression in smoldering MM and MM patient samples compared to healthy bone marrow plasma cells (BMPC) and MGUS patients (Fig. 5A). PRKAA2 was expressed at low levels in these patient samples and was significantly downregulated compared to healthy BMPC (Fig. 5B). To investigate the role of AMPK in MM cell survival, human myeloma cell lines LP-1, RPMI8226 and U266 were treated with indicated concentrations of Compound C, followed by cell cycle and apoptosis analysis by propidium iodide (PI) staining and AnnexinV/7-AAD staining respectively. In Fig. 5C, we found a significant increase in SubG1 and a decrease in S phase, while no clear G1/G2 arrest could be observed after treatment with Compound C. Furthermore, we demonstrated a clear reduction in viable cells (AnnexinV/7-AAD negative cells) upon treatment with Compound C (Fig. 5D). By western blot analysis, we observed a reduction in AMPK phosphorylation and MCL-1 expression (Fig. 5E). In addition, human primary $CD138^+$ MM cells were treated with Compound C for 24 h and a significant decrease in cell viability was found (Fig. 5F).

4. Discussion

Various cell types in the BM microenvironment contribute to cell survival and tumor progression in MM [29–31]. Our aim was to unravel possible mechanisms of MDSC-mediated myeloma cell survival and identify new targets in the treatment of MM disease. We found that MDSC induce the survival of MM cells *in vitro* and that the addition of Bz and Mel could not abrogate this effect. Secondly, we observed that the MDSC-mediated survival benefit was mediated by both cytokine secretion and direct cell-cell contact. MDSC secrete numerous cytokines that have been described to induce cell survival including IL-6, IL-10 and IL-23 [11,32]. *In vivo,* we combined the MDSC-depleting agent 5FU together with Bz in the 5T33MM mouse model. Previously, we observed MDSC accumulation already one week after MM cell inoculation [10]. Therefore, one single injection of 5FU was administered at day 4, rather as a preventative therapy, to target the MDSC population. Bz-treatment started at day 10 and was continued twice a week. Interestingly, we could demonstrate that combining 5FU together with Bz could significantly decrease the immature myeloid cell population and resulted in reduced tumor burden in 5T33MM-diseased mice compared to single treatments. This result is in agreement with our previous paper, where we demonstrate that 5FU specifically decrease the $CD11b^+$ $Ly6G^{low}$ $Ly6C^{low}$ immature myeloid cells [10]. As 5FU was administered at an early disease stage, the compound could possibly alter tumor engraftment/growth and not just enhance the effects of Bz. From these data we can conclude that MDSC are an important component of the BM microenvironment. We are the first to demonstrate that besides their immunosuppressive activity, they directly contribute to survival of MM cells.

We further investigated the underlying mechanism of MDSC-induced survival of MM cells. Interestingly, a significant AMPK activation was observed in MM cells co-cultured with MDSC. AMP-activated protein kinase (AMPK) is a crucial nutrient and energy sensor that regulates cellular energy homeostasis. Once activated by reduced energy levels, AMPK promotes ATP production by increasing the catabolic processes, while conserving ATP by switching off anabolic pathways [28]. Besides nutrient starvation, other mechanisms can induce AMPK activation including hypoxia and oxidative stress [28,33,34]. Hu et al. previously demonstrated the reduced oxygen tension, the exogenous and endogenous hypoxia markers pimonidazole and upregulation of HIF-1α in the BM of 5T33MM compared to naïve mice [35]. Importantly, we also demonstrated AMPK activation *in vivo*. 5TGM1 cells isolated from the BM of diseased mice have an increased AMPK phosphorylation compared to 5TGM1 cells cultured *in vitro*. In addition, AMPK phosphorylation decreased again after culture of 5TGM1 cells isolated from mice, indicating the importance of the BM microenvironment to regulate AMPK activation.

Although AMPK has generally been considered as a tumor suppressor, there is increasing evidence that under stress conditions AMPK may serve as a contextual oncogene. The pro-survival effect of AMPK may be attributed to the induction of autophagy [23,24] and increases in β-oxidation, ATP production, and NADPH levels [36–38]. Gao M. et al. demonstrated that AMPK-induced upregulation of the anti-apoptotic protein MCL-1 comprises the anti-cancer effects of aspirin [21]. Another anti-apoptotic protein, BCL-2, has also been linked with AMPK-mediated protection against apoptosis of cardiomyocytes [25]. In our study, we observed an increase in MCL-1, BCL-2 and autophagosome formation marker LC3II. Previous research on macrophages demonstrated that anti-inflammatory cytokines IL-10 and TGF-β were able to induce AMPK activation which was important in macrophage polarization [39]. As MDSC produce high quantities of IL-10 and TGF-β [40,41], these cytokines could be responsible for the MDSC induced AMPK phosphorylation in MM cells. Another explanation for AMPK activity is the release of ROS by MDSC. MDSC derived from tumor bearing mice and cancer patients produce high amounts of intracellular ROS [42].

To further elucidate the role of AMPK activation in MM cells, we investigated the expression of PRKAA1 and PRKAA2 expression, genes encoding for the catalytic α-subunit in patient samples. We observed a significant increase of PRKAA1 in smoldering MM and MM patients, while PRKAA2 was low expressed and even downregulated in these patients. This is in accordance with other cancers including prostate cancer, cervical cancer and melanoma where they also observe a high expression of PRKAA1 or AMPKα1 in neoplastic tissue compared to normal tissue [43]. To investigate the effect of AMPK inhibition in MM, we added Compound C (BML-275) to the co-cultures and observed a reduced AMPK phosphorylation, MCL-1 and BCL-2 expression, and induced apoptosis of neoplastic plasma cells both in the presence and absence of MDSC. Since the knowledge on the AMPK pathway is limited in human MM, we further analyzed the effect of Compound C on human myeloma cell lines and patient samples in order to demonstrate its clinical relevance. In contrast to previous studies that observed a G2/M cell cycle arrest by the use of Compound C [44,45], we only observed a decrease in S phase and increase in Sub-G1 phase. In addition, there was a significant increase in apoptotic cells in all HMCL, associated with decreased AMPK phosphorylation and MCL-1 expression. This was confirmed on BM derived $CD138^+$ cells of MM patients, where we also observed a reduction in viability upon treatment with Compound C. This compound has been described as a potent AMPK inhibitor and demonstrated significant anti-tumor effects in other hematological malignancies [25–27].

In conclusion, our data demonstrate that MDSC-mediated AMPK phosphorylation could increase survival of MM cells. In addition, AMPK targeting or MDSC targeting strategies showed promising pre-clinical results that could be applied for future treatment of MM patients. Further research on the underlying mechanism of AMPK activation and autophagy is necessary as it has been demonstrated that both mechanisms could promote cancer cell apoptosis or survival depending on the environmental conditions.

Conflicts of interest

No conflict of interest to disclosure.

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CRediT authorship contribution statement

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Appendix A. Supplementary data

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