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Full paper

Valproic acid attenuates immunosuppressive function of myeloid-derived suppressor cells

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ABSTRACT

Immune checkpoint blockade (ICB) is a promising novel therapy for multiple cancer types; however, most patients show limited or no clinical response. Accumulating evidence indicates that myeloid-derived suppressor cells (MDSCs) are a major factor responsible for immunosuppression in patients with cancer. Therefore, identifying effective therapies that deplete or modulate MDSCs is essential. In this study, we focus on the anticonvulsant drug valproic acid (VPA), which has additional activities including anticancer and immunoregulation by inhibition of histone deacetylases. We showed that VPA decreased the proportion of polymorphonuclear (PMN)-MDSCs *in vitro* and showed for the first time that VPA greatly attenuated the immunosuppressive function of MDSCs in a dose-dependent manner. Moreover, we demonstrated that *in vitro* differentiated VPA-conditioned MDSCs exhibited impaired ability to stimulate tumor progression *in vivo*. We also showed the possible involvement of several mechanisms in the VPA-induced attenuation of the immunosuppressive function of MDSCs, including the interleukin-4 receptor-α (IL-4Rα)/arginase axis, programmed cell death 1 ligand 1 (PD-L1) and toll-like receptor 4 (TLR4) signaling pathways, and retinoblastoma 1 (Rb1) derepression. This research highlights the potential of combining VPA with ICB in cancer treatment.

1. Introduction

Cancer is one of the leading causes of death globally. In recent decades, an improved understanding of the immune system has prompted the rapid development of cancer immunotherapies. However, most patients have limited clinical response or even fail to respond to immunotherapy because of the immunosuppressive machinery in the tumor microenvironment.[1] Recently, immune checkpoint blockade (ICB) therapies such as anti-programmed cell death 1 (PD-1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) therapies have shown clinical benefits in multiple cancer types.[2,3] The success of these therapies has highlighted the importance of overcoming tumor-induced immunosuppression to achieve effective cancer immunotherapy.

A multivariate analysis has revealed that myeloid-derived suppressor cells (MDSCs) play important roles in the suppression of antitumor immunity, which leads to tumor evasion from immune surveillance.[4,5] MDSCs are immature heterogeneous cells that accumulate in tumor-bearing hosts because of abnormal myelopoiesis induced by proinflammatory cytokines.[6] In mice, MDSCs are defined as $CD11b^{+}Gr\text{-}1^{+}$ cells with immunosuppressive functions against T cell proliferation.[7] They are divided into two subpopulations, polymorphonuclear MDSCs (PMN-MDSCs) with the phenotype $CD11b^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{int}$ and monocytic MDSCs (M-MDSCs) with the phenotype $CD11b^{+}Ly\text{-}6G^{-}Ly\text{-}6C^{hi}$.[8] PMN-MDSCs are the major subset of circulating MDSCs; however, M-MDSCs have a stronger immunosuppressive function than that of PMN-MDSCs.[9] PMN-MDSCs exert their immunosuppressive functions through the production of arginase and reactive oxygen species (ROS), whereas M-MDSCs exert these functions by producing nitric oxide (NO) and arginase.[10] Although the existence of these two MDSC populations has been demonstrated, the mechanism

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Abbreviation

Ab	antibody
Arg1	arginase 1
BM	bone marrow
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
Cybb	cytochrome b-245 beta polypeptide
FBS	fetal bovine serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
ICB	Immune checkpoint blockade
IL-4Rα	interleukin-4 receptor-α
iNOS	inducible nitric oxide synthase
MDSCs	myeloid-derived suppressor cells
M-MDSCs	monocytic MDSCs
NO	nitric oxide
NOS2	NAPDH oxidase 2
PBS	phosphate-buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed cell death 1 ligand 1
PMN-MDSCs	polymorphonuclear MDSCs
Rb1	retinoblastoma 1
ROS	reactive oxygen species
TLR4	toll-like receptor 4
VPA	valproic acid

responsible for these differences remains unclear. Remarkably, a recent study showed that patients who are refractory to anti-PD-1 therapy have a higher accumulation of MDSCs in the peripheral blood.[11] Therefore, it is important to identify an effective therapy that depletes or modulates MDSCs.

Valproic acid (VPA), a short-chain fatty acid, is an anticonvulsant that has been used for decades for the treatment of seizures. Recently, VPA has been found to have potent anticancer activities and has been tested either alone or in combination with other agents in clinical studies.[12] The anticancer activity of VPA was shown to be attributable to histone deacetylase (HDAC) inhibition.[13,14] HDACs are critically involved in the epigenetic regulation of multiple genes and have been demonstrated to regulate immune cell functions.[15] Furthermore, Youn et al.[16] have shown that VPA inhibition of HDACs can cause differentiation of tumor-induced MDSCs into macrophages and dendritic cells following *in vitro*. However, the exact effect of VPA on MDSCs remains largely unexplored. In this study, we elucidate the effects of VPA on the immunosuppressive activity of MDSCs *in vitro* and explore its potential as a cancer immunotherapy.

2. Materials and methods

2.1. Mice

C57BL/6 J mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan) and were used at 7–8 weeks of age. All animals were bred and maintained under specific-pathogen-free conditions. All animal experimental procedures in this study were performed in accordance with the institutional guidelines for animal experiments of Osaka University.

2.2. In vitro *MDSC differentiation*

Bone marrow (BM) cells were harvested from the tibia and femur of C57BL/6 J mice. The BM cells were suspended in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 2 mM GlutaMAX (Gibco), and 50 nM 2-mercaptoethanol (Sigma–Aldrich), at a concentration of 2.5×10^5 cells/mL. Cytokines were added to the culture medium at the following concentrations: 40 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF, Peprotech, Rocky Hill, NJ, USA), and 0, 0.25, 0.5, or 1 mM VPA (Sigma–Aldrich). The cell suspensions (10 mL) were seeded into 100 mm tissue culture-treated dishes (Corning, New York, NY, USA) and cultured at 37 °C exposed to an atmosphere of 5% CO_2 for 4 days.

2.3. Flow cytometry analysis

Cells were washed and suspended in phosphate-buffered saline (PBS) supplemented with 2% FBS (2% FBS/PBS), blocked with TruStain fcX (anti-mouse CD16/32) antibody (BioLegend, San Diego, CA, USA), and stained with the following antibodies (Abs): APC anti-mouse CD11b (eBiosicence, San Diego, CA, USA), Pacific Blue anti-mouse Gr-1, PE anti-mouse IL-4Rα, Pacific Blue anti-mouse CD4, and fluorescein isothiocyanate (FITC) anti-mouse CD8α (BioLegend). Then, the cells were washed and resuspended in 2% FBS/PBS. Immediately before the flow cytometry analysis, 7-amino actinomycin D (7-AAD) viability staining solution (BioLegend) was added to the suspension to identify dead cells. Flow cytometry analysis was performed using a BD FACSCanto II device (BD Biosciences), and the acquired data were analyzed using FlowJo software (TOMY Digital Biology).

2.4. In vitro *suppression assay*

The spleens were harvested from C57BL/6 J mice, ground to release the splenocytes, and then treated with ammonium-chloride-potassium lysis buffer to eliminate the red blood cells. Next, the splenocytes were subjected to $CD4^+$ T cell and $CD8^+$ T cell isolation using the MojoSort magnetic cell separation system and Mouse CD4 or CD8 Nanobeads (BioLegend). Isolated cells were then labeled with proliferation dye eFluor 670 (eBioscience) and seeded into a 96-well plate at 1×10^5 cells/50 μL per well. The wells were pre-coated with anti-CD3ε Ab (BioLegend) diluted with PBS to a concentration of 1 μg/mL and stored at 4 °C overnight before use. Differentiated MDSCs were added at ratio of 1:1, 0.5:1, or 0.25:1 to T cells. Anti-CD28 Ab (BioLegend) was added to each well to a final concentration of 0.5 μg/mL. After 3 days of incubation at 37 °C exposed to an atmosphere of 5% CO_2, the proliferation of $CD4^+$ and $CD8^+$ T cells were analyzed using flow cytometry.

2.5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the *in vitro* differentiated MDSCs and used to synthesize the cDNA using the QuantiTect reverse transcription (RT) kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quantitative RT-polymerase chain reaction (qRT-PCR) was performed using SYBR Premix Ex Taq (Tli RNaseH Plus; TaKaRa, Kusatsu, Japan) on a CFX96 touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Primer sequences are listed in Supplementary Table 1. Relative gene expression data were normalized to against glyceraldehyde 3-

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