Tumor-suppressing effect of metformin through regulation of myeloid-derived suppressor cells and M2 macrophages in the tumor microenvironment of colorectal cancer

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This certifies that the Master's Thesis of

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마지막으로, 늘 제 곁에서 부족한 저를 믿어주고 응원해주는 저의 가족, 아빠, 엄마 그리고 오빠에게 감사하고 또 부끄럽지 않은 제가 되겠습니다.

다시 한번 학위를 도와주신 모든 분들께 감사드립니다.

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강조연 드림

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Tumor-suppressing effect of metformin through regulation of myeloid-derived suppressor cells and M2 macrophages in the tumor microenvironment of colorectal cancer

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(Directed by Professor Tae Il Kim)

Various types of cells are associated with progression and regression of tumor in tumor microenvironment. Myeloid-derived suppressor cells (MDSCs) and M2 macrophages in tumor microenvironment contribute to tumor progression by inducing immune tolerance to tumor antigens and cancer cells, in contrast to role of M1 macrophages for the rejection of the tumor. It has been reported that

metformin, which is one of the most common diabetes drugs, has anti-inflammatory and anti-tumor effects. However, it is not known how metformin affects on inflammatory cells of tumor microenvironment and its mechanism. Thus, I determined the effect of a metformin on M2 macrophage and MDSC using THP-1 cells and the mouse colon cancer model. In flow cytometry analysis using the THP-1 cell, metformin decreased the fractions of MDSCs expressing CD33 and arginase and M2 macrophages expressing CD206 and CD163. Meanwhile, metformin caused the activation of p-AMPK and the inhibition of p-S6 in the western blotting analysis.

Moreover, the fraction of MDSCs and M2 macrophages was decreased by AICAR (AMPK activator) and increased by Compound C (AMPK inhibitor) treatment, and the inhibitory effect of metformin on MDSCs and M2 macrophages was reversed by the treatment of Compound C and mevalonate. In addition, the treatment of rapamycin or simvastatin also decreased the fractions of MDSCs and M2 macrophages in the culture of THP-1 cells, which was reversed by mevalonate. Furthermore, the induction of PGE_2, a mediator for M2 differentiation by PMA and/or mevalonate was inhibited by metformin treatment. Metformin treatment induced the inhibition of protein prenylation in mevalonate pathway and thus reduced the proportion of MDSCs/M2 macrophages. In the mouse colon cancer

model of $Apc^{Min/+}$ mouse treated with DSS, metformin reduced the number and volume of colorectal tumors. In immunohistochemistry stains, MDSCs expressing CD33 and arginase and M2 macrophages expressing CD206 and CD163 were significantly reduced by metformin treatment in the tumor microenvironment. In conclusion, the inhibitory effect of metformin on MDSCs and M2 macrophages in the tumor microenvironment of colon cancers is mediated by AMPK-activation and mTOR inhibition leading to inhibition of the mevalonate pathway, which includes HMG-CoA reductase and protein prenylation.

Key words: M2 macrophages, MDSCs, Metformin, Tumor microenvironment

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I. INTRODUCTION

The third most common cancer worldwide, colorectal cancer (CRC) is a complex tumor condition that is caused by various genetic and environmental factors. Recent advancements in cancer screening and treatment have improved the level of prevention and the outcome of CRC. However, scientific understanding of CRC initiation and progression still remains limited and elusive

in many ways. One flourishing field in cancer treatment is cancer immunotherapy, or the use of immune cells to interact with cancer cells and retard cancer progression. The basic principles of cancer immunotherapy concern the tumor microenvironment, the heterogeneous and intricate microenvironment around the tumor which promotes tumor growth.[1]

The tumor microenvironment consists of blood vessels, fibroblasts, inflammatory cells, and the extracellular matrix.[2] In this heterogeneous microenvironment, macrophages and myeloid progenitors play important roles in cancer regression and survival.[1,2] Macrophages can serve both pro- and anti-tumor functions. Termed M1 and M2 polarized macrophages, the two subsets display different phenotypes with differing functions. The M1 phenotype is activated by Toll-like receptor ligands and interferon gamma (IFN-γ) and produces tumor cytotoxicity through pro-inflammatory cytokines like TNF-α, IL-1β and inhibits tumor progression.[3] In contrast, the alternatively-activated M2 phenotype is activated by cytokines IL-4 and IL-13. M2 macrophages promote and produce high levels of cytokines that stimulate tumor growth and progression with the production of anti-inflammatory stimuli such as TGF-β1 and IL-10.[3] Thus

macrophages can both positively and negatively influence tumor growth.[3] Some of the molecules produced by M2 macrophages attract additional pro-inflammatory mediators to the tumor site, thereby amplifying the inflammatory microenvironment.[4]

On the other hand, myeloid-derived suppressor cells (MDSCs) are associated with greater tumor burdens and worse prognosis,[5] MDSCs are actively recruited to primary and metastatic tumor sites. This process is regulated by chemokines produced by the tumor with little specificity in the types of chemokine. Inflammatory factors that induce MDSC recruitment and expansion in the tumor microenvironment, including IL-6, IL-10, IL-1β can display potent immunosuppressive and tumor-promoting functions in the tumor microenvironment via multiple mechanisms like induction of immunosuppressive cells, blocking of lymphocyte homing and production of reactive oxygen and nitrogen species.[6] Amidst the complex dichotomy of M1 and MDSC/M2 leukocytes, few studies have explored the effects of therapeutic drugs on these populations.

Metformin, a biguanide subclass molecule, is the most commonly prescribed drug used in the treatment of type II diabetes. This drug is known to reduce hepatic gluconeogenesis and insulin resistance by increasing peripheral glucose uptake and consumption in the liver and skeletal muscles via the inhibition of the oxidative phosphorylation pathway. In addition, as direct anti-tumor effect of metformin, AMPK-activation mediated mTOR inhibition, suppression of cancer stem cell, and inhibition of cellular transformation are involved in mechanisms of tumor suppression of metformin.[7] Also for the anti-tumor effect, metformin targets the respiratory chain complex Ⅰ in the mitochondria, which leads to metabolism including the TCA (tricarboxylic acid) cycle and oxidative phosphorylation (OXPHOS).[7] In retrospective clinical data, metformin also reduces the development and recurrence of colorectal polyp and increase CRC survival in DM patients and decrease aberrant crypt foci as a potent source of colorectal cancer in non-diabetic patients.[8,9] Therefore, recently, metformin has been deemed a potential adjunctive drug in cancer treatment or chemopreventive agents due to its anti-tumor capabilities and relative non-toxicity to the human body. In addition, recent studies have also shown that metformin exhibits anti-

inflammatory effects in human vascular endothelial cells and smooth muscle cells via the AMPK pathway.[10] These findings suggest that the impact of metformin on tumor may be related with both direct effect on tumor cells and inflammatory cell-mediated effect on tumor microenvironment.[11] Therefore, we investigated the effect of metformin on tumor-promoting MDSC and M2 macrophage, and its mechanism in tumor microenvironment of CRC.

II. MATERIALS AND METHODS

1. Cell culture and reagents

The human monocytic myeloid cell line, THP-1 (KCLB, Seoul, South Korea) was used, which is derived from an acute monocytic leukemia patient. THP-1 cells were cultured in RPMI-1640 (Gibco-Life Technologies, Grand Island, NY), supplemented 10% fetal bovine serum (Gibco-Life Technologies, Grand Island, NY) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in 5% CO_2 .

THP-1 cells were seeded in the medium and cultured with the presence or absence of metformin 0.25 to 5mM and PMA 100nM (Sigma, St. Louis, MO), mevalonate 200μM (Santacruz, Delaware, CA), AICAR 50 and 125μM, Compound C 20μM, rapamycin 20 and 50nM and simvastatin 2μM (Merck Millipore, Darmstadt, Germany). Also, FTI-277 10 and 20μM (Sigma, St. Louis, MO), GGTI-298 5 and 10μM (Tocris Bioscience, Bristol, UK) and YM-53601 (Cayman Chemical, Ann Arbor, MI) were treated to the THP-1 cells.

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2. Flow-cytometric analysis for M2 macrophages and MDSCs

Before Flow-cytometry analysis, metformin, mevalonate and other reagents were used to treat THP-1 cells plated at a density of 2×10□ in six-well plates in 2ml mediμM and incubated at 37°C in 5% CO_2 for 48 hrs. FACS buffer (1×PBS, 1% bovine serum albumin and 2 mM ethylene diamine tetra-acetic acid) was used for Flow-cytometric analysis of macrophage marker antibodies (PE-Cy3-conjugated anti-CD68), MDSC marker antibodies (FITC-conjugated anti-CD33 and anti-Arginase-1), M2 macrophage marker antibodies (FITC-conjugated anti-CD206 and anti-CD163) and M1 macrophage marker antibodies (FITC-conjugated anti-iNOS). Primary antibodies (Santacruz, Delaware, CA) and secondary antibodies (FITC-rabbit, goat, Cy3-mouse; Abmgood, Vancouver, Canada) were added and incubated for 10 min at 4°C. Cells were washed with FACS buffer and analyzed using a FACSVerse (BD Biosciences, San Diego, CA) coupled to a computer with BD FACSuite software for data analysis.

3. Western blotting analysis for the AMPK pathway

To analyze the AMPK-mTOR pathway, the expression of phosphorylated AMPK (pAMPK) and phosphorylated S6 (pS6) was evaluated. Cells were treated with metformin 1mM, 2.5mM or 5mM in 2ml medium in six-well plates, incubated for 48 hrs and washed twice with PBS, pelleted by centrifugation, and lysed at 4°C for 15 min in protein extraction solution. (iNtRON Biotechnology, Gyeonggi-do, South Korea). Protein concentrations of the samples were determined by a protein assay that used bicinchoninic acid (BCA; Pierce, CA) and were run to sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto the polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA) in a methanol-based tris-glycine buffer.

Membranes were then blocked with 5% Skim milk/TBST (Tris-buffered saline and Tween 20) for 1 hr. Each of the primary antibodies (anti-AMPK, anti-p-AMPK (Thr172), anti-S6, anti-p-S6 (Ser235/236); Cell Signaling Technology, Danvers, MA,) was incubated over the membrane with continuous mixing overnight at 4°C.

Membranes were rinsed at least three times with TBST and then incubated with secondary antibodies for 1 hr at room temperature. After the final TBST rinse,

the ECL(enhanced chemiluminescence) western blotting detection kit (Amersham Biosciences, Freiburg, Germany) was used and exposed by Kodak film to express the emission of proteins.

4. PGE_2 ELISA assay

To measure PGE_2, cells were plated at a density of 1×10□ cells/well in six-well plates, co-treated with PMA for differentiation into macrophage, and added with metformin and/or mevalonate for 48 hrs. The amounts of PGE_2 of supernatants were determined by the PGE_2 ELISA assay kit (ADI-900-001; Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol. Enzymatic reactions were measured by spectrophotometry at 405nm.

5. *In vivo* experiments using a mouse model of colorectal cancer

Six-week old male C57BL/6J $Apc^{Min/+}$ mice (Jackson Laboratory, Bar Harbor, ME) were used and all the mouse experiments were performed according to

institutional guidelines and policies. All mice were subjected to 3% Dextran Sulfate Sodium Salt (DSS) for six days daily via drinking water, and allowed to recover by drinking regular water for three wks. Experimental group were injected with 250 or 350mg/Kg of metformin i.p. (intraperitoneal injection) while the control group were injected with PBS. After three wks, all mice were sacrificed and swiss rolls of large intestines were fixed in 4% PFA (paraformaldehyde) and embedded in paraffin blocks for immunohistochemistry staining.

6. Immunohistochemistry for M2 macrophages and MDSCs

Paraffin-embedded sections were de-paraffinized in xylene and rehydrated in gradually decreasing concentrations of ethanol. Antigen retrieval was performed using a sodium citrate buffer (10mM, pH 6.0) in a heated pressure cooker for 5 min. After incubation with 3% hydrogen peroxide to block endogenous peroxidase activity for 30 min, the sections were incubated in 5% BSA/TBS solution for 30 min at room temperature.

Anti-CD11b (MDSC marker, 1:4000 dilution; Abcam, Massachusetts, US), Anti-CD206 (M2 marker, 1:200 dilution; Abcam, Massachusetts, US) and Anti-

CD86 (M1 marker, 1:200 dilution; Santacruz, Delaware, CA) were incubated with the sections overnight at 4°C, and then secondary antibodies were incubated for 30 min at room temperature. After slides were detected with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA), immunostaining was performed using DAB solution (Dako, Carpinteria, CA). After counterstaining with hematoxylin, IHC staining was evaluated by light microscopy and immuno-activity was assessed based on the proportion of immunostained MDSC, M2 and M1 macrophage cells counted in ten different fields for three samples with x200 magnification.

7. RNA isolation and analysis of gene expression by quantitative RT-PCR

Total RNA of THP-1 cells were isolated using TRIzol reagent (Gibco-Life Technologies, Grand Island, NY). cDNA synthesis from 2μg total RNA was performed using Reverse Transcription Master Premix (ELPISBIOTECH, Daejeon, South Korea). Real-time qPCR was performed using SYBR Green Master mix (Enzynomics, Daejeon, South Korea) and the following primers:

HMGCR (forward, 5'-CCCAGCCTACAAGTTGGAAA-3', and reverse, 5'-AACAAGCTCCCATCACCAAG-3') and GAPDH as a housekeeping gene.

8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 20.0 (IBM Co.,Armonk, NY, USA). A student's one-tailed t-tests were performed for continuous and categorical variables, as appropriate. P values lower than 0.05 were considered statistically significant.

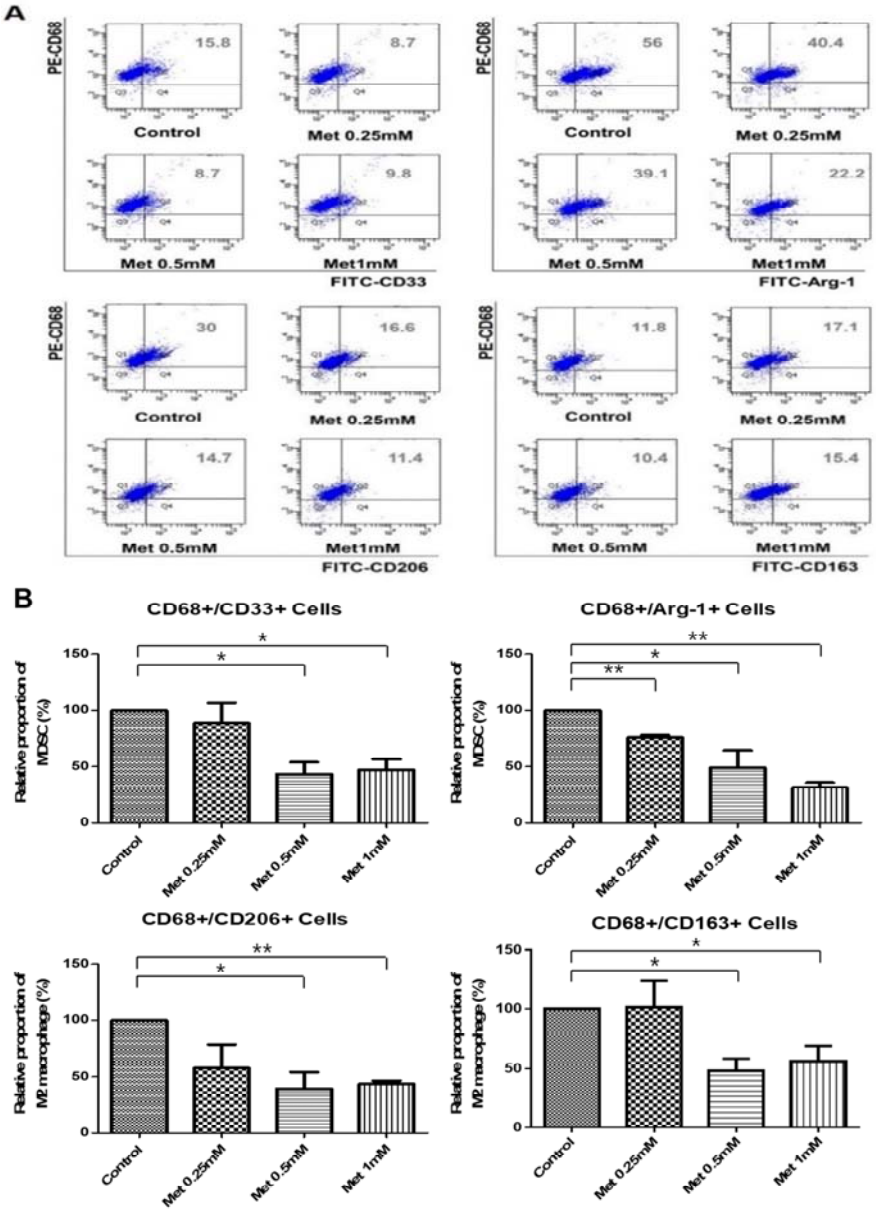
III. RESULT

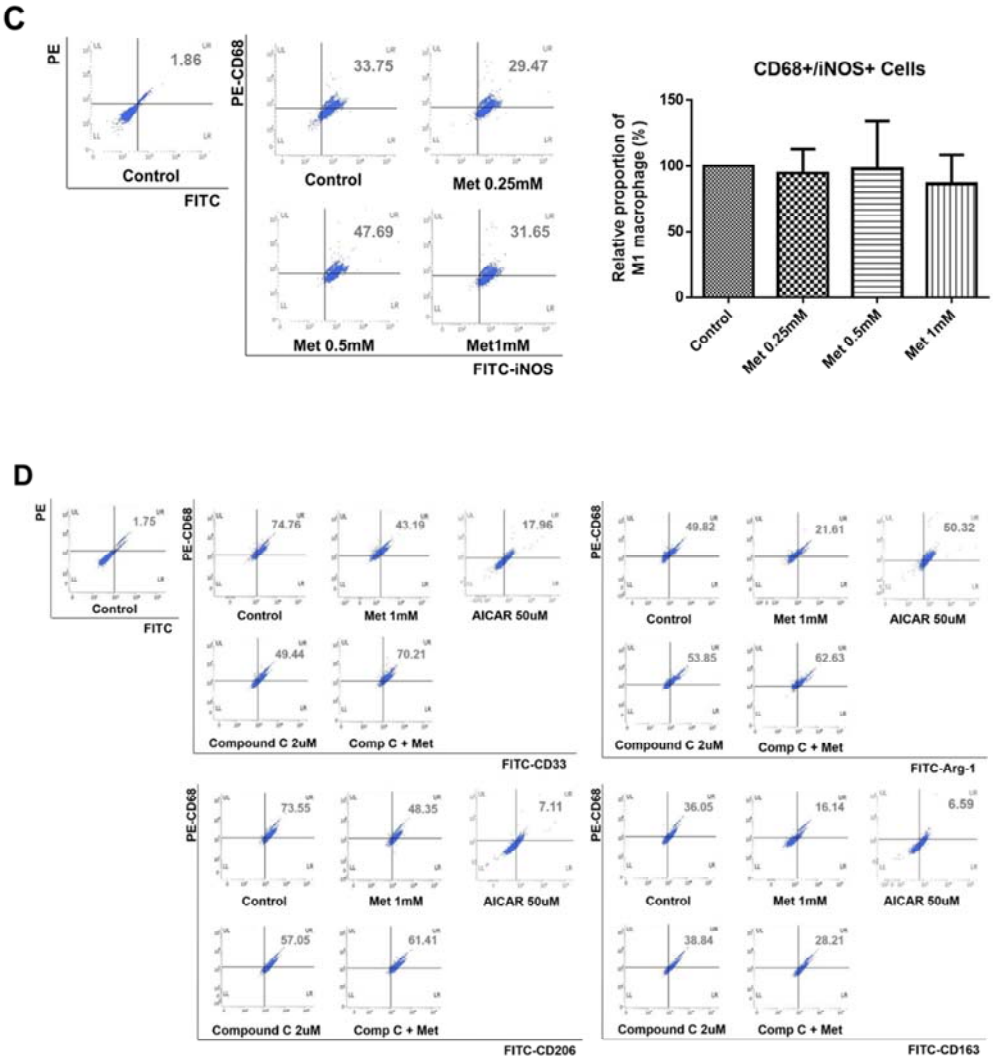
1. Metformin and AMPK pathway modulated the fraction of MDSCs and M2 macrophages in THP-1 cells

I examined whether metformin would reduce MDSC and M2 populations in an AMPK dependent fashion by subjecting THP-1 cells to various AMPK activators and inhibitors. To evaluate the effect of metformin, the AMPK activator (AICAR) and inhibitor (Compound C) on THP-1 cells, I performed Flow-cytometric analysis using macrophage (CD68), MDSC (CD33, Arg-1) and M2 macrophage (CD206, CD163) markers after treatment with metformin (0.25mM, 0.5mM, 1mM), AICAR (50μM), Comp C (5μM) and their combination for 48 hrs. The proportions of MDSC ($CD68^+$ $CD33^+$, $CD68^+$ $Arg\text{-}1^+$) cells and M2 macrophage ($CD68^+$ $CD206^+$, $CD68^+$ $CD163^+$) were determined.

First of all, MDSC and M2 macrophages decreased significantly along with treatment of metformin in dose-dependent manner while M1 macrophages did not changed with metformin (Fig. 1A to 1C).

Both metformin and AICAR (AMPK activator) reduced the population of MDSC and M2 macrophage in a similar fashion, and Compound C (AMPK inhibitor) increased the proportion of MDSC and M2 macrophage. In addition, the simultaneous treatment of Compound C and metformin reversed the inhibitory effects of metformin on MDSC and M2 macrophage, suggesting the inhibitory effect of metformin on MDSC and M2 macrophage through the activation of AMPK (Fig. 1D and 1E).





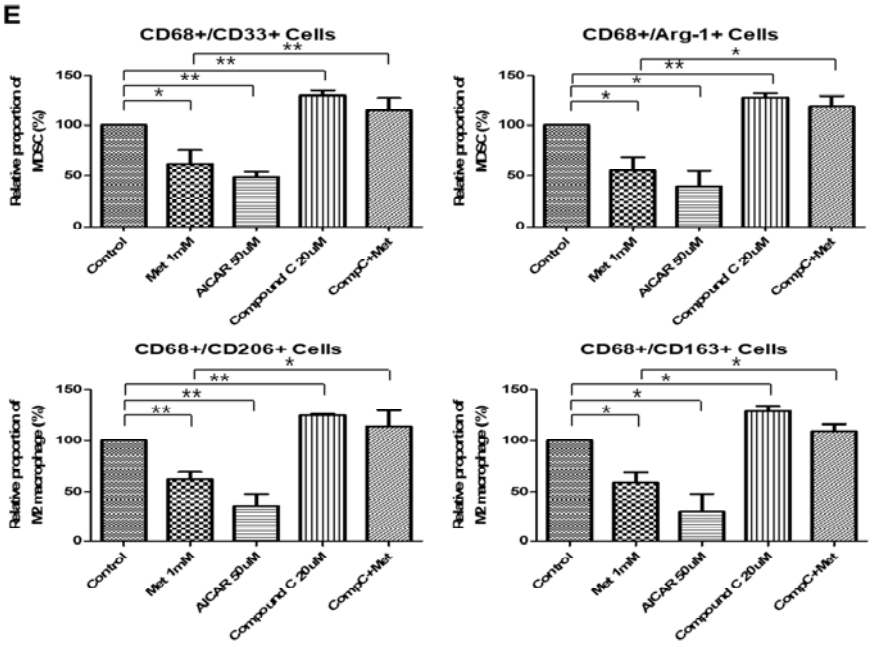


Fig. 1. Inhibitory effect of the metformin and the AMPK activator on MDSC, M2 macrophage and M1 macrophage. (A to C) THP-1 cells were treated with 0.25 to 1mM of metformin in a dose-dependent manner for 48 hrs. (D and E) THP-1 cells were treated with metformin 1mM, AICAR 50μM, Comp C 20μM and a combination of Comp C and metformin for 48 hrs. Then, Flow-cytometric analyses were performed using markers of MDSC ($CD68^+$ $CD33^+$, $CD68^+$ $Arg\text{-}1^+$), M2 macrophage ($CD68^+$ $CD206^+$, $CD68^+$ $CD163^+$) and M1 macrophage ($CD68^+$ $iNOS^+$). Data are expressed as the mean ± standard error of four different experiments; *P < 0.05, **P < 0.005.

2. Metformin induced activation of AMPK and inhibition of mTOR

To confirm the signals through the AMPK-mTOR signaling pathway, I performed western blotting analysis. Treatment of metformin activated p-AMPK and decreased p-S6, a downstream target of mTOR (Fig. 2).

In addition, activated p-AMPK and decreased p-S6 were noted with treatment of AICAR (AMPK activator), and a prominent decrease in p-S6 with treatment of rapamycin (mTOR inhibitor) (Fig. 2).

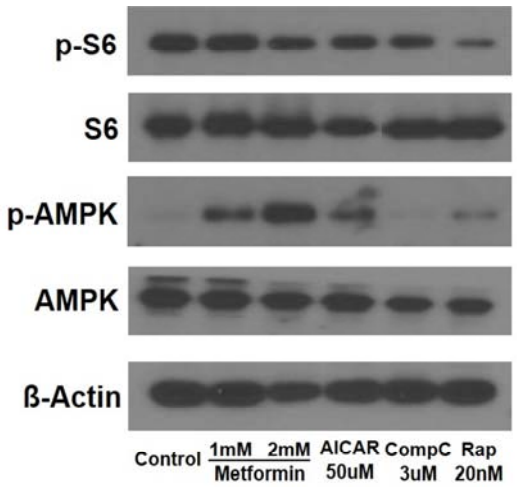
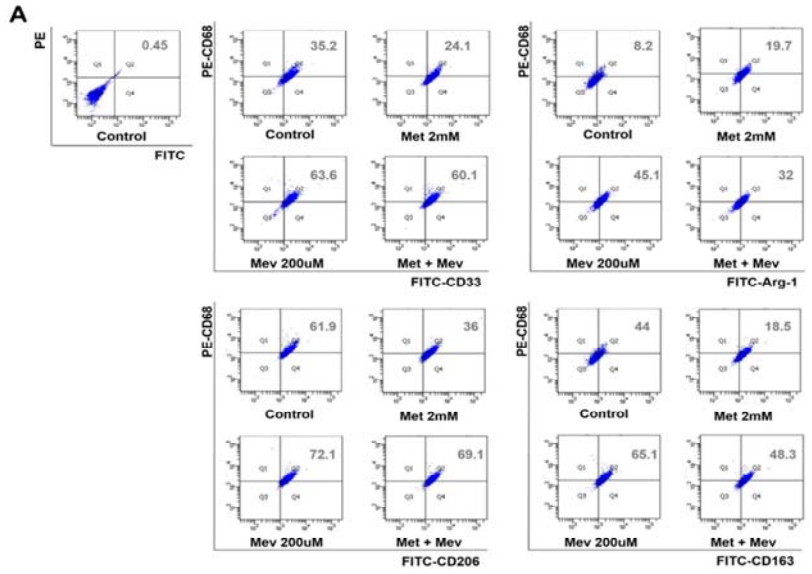
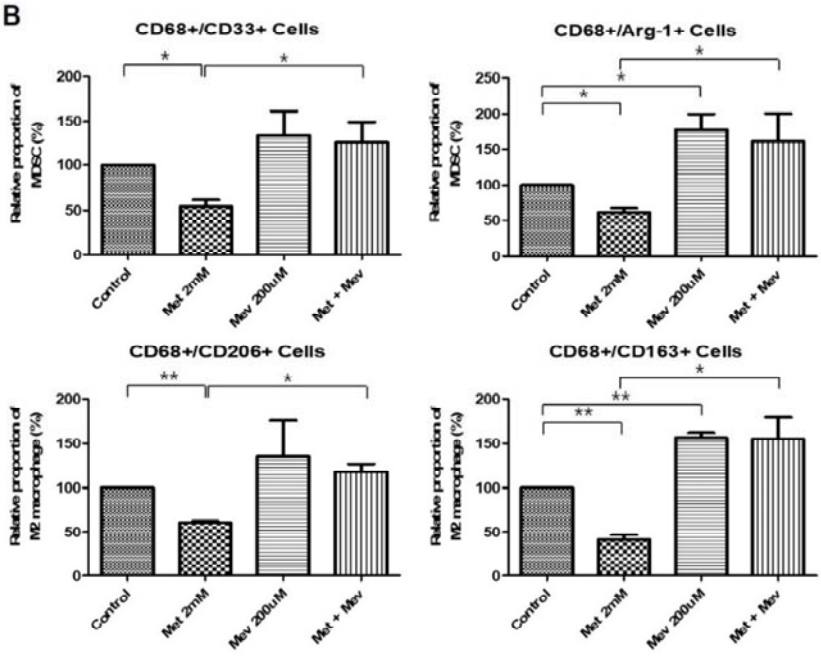


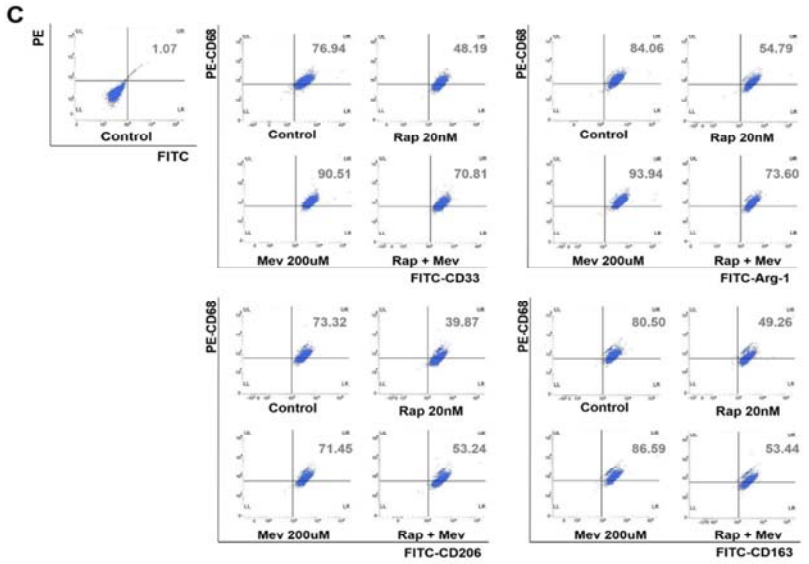
Fig. 2. Expression of AMPK-mTOR signals, after treatment with metformin, AMPK activator and inhibitor and mTOR inhibitor. In Western-blot analysis, the expression of phosphorylated S6 and phosphorylated AMPK was analyzed after 48 hrs of treatment with the control vehicle or metformin 1mM and 2mM, AICAR 50μM, Comp C 3μM and Rapamycin 20nM in THP-1 cells.

3. Mevalonate reversed the inhibitory effect of metformin and rapamycin

Because metformin is also known as a HMG-CoA reductase (HMGCR) inhibitor,[12] to examine the relationship between the mevalonate pathway and the AMPK-mTOR pathway, metformin and rapamycin (mTOR inhibitor) were treated with or without mevalonate to THP-1 cells and flow cytometry was performed (Fig 3A and 3B). Rapamycin treatment also induced the same inhibitory effect on the proportion of MDSC and M2 macrophage, as shown in metformin treatment (Fig. 3C and 3D), and the treatment of mevalonate reversed the inhibitory effects of metformin and rapamycin on MDSC and M2 macrophage (Fig. 3A to 3D), suggesting that AMPK-mTOR pathway and the mevalonate pathway could be involved in the regulation of MDSC and M2 differentiation in THP-1 cell lines.







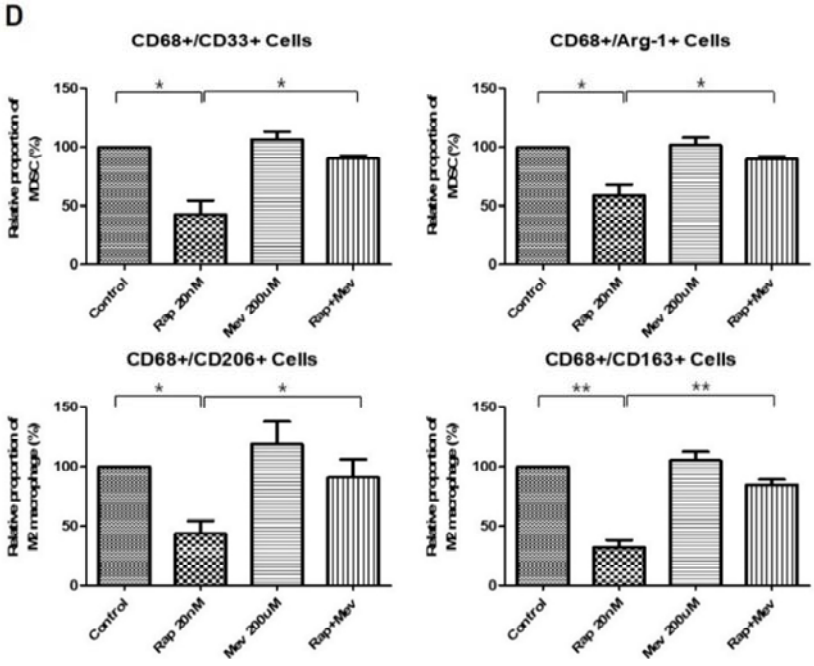
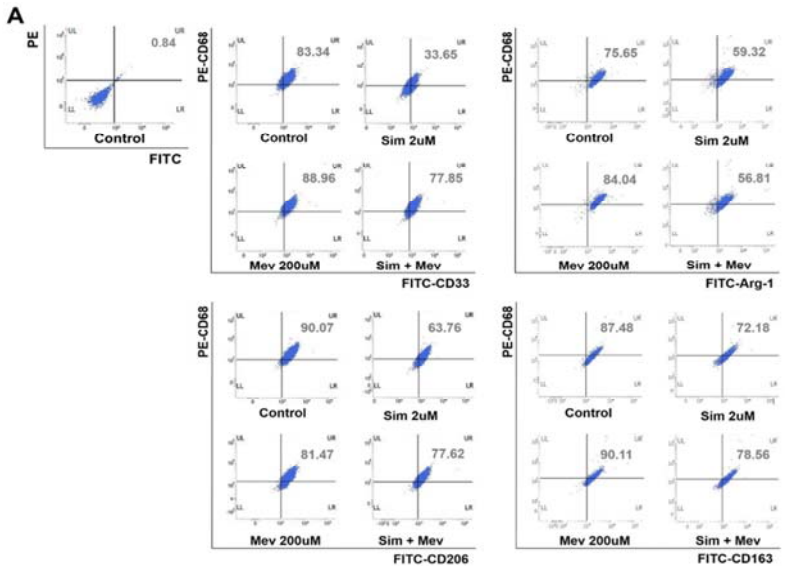


Fig. 3. Addition of mevalonate reversed the effect of metformin and rapamycin on MDSC and M2 macrophage. THP-1 cells were treated with metformin 2mM and rapamycin 20nM with or without mevalonate 200μM. Then, the expression of MDSC ($CD33^+$ /$CD68^+$ and $Arg\text{-}1^+$ /$CD68^+$) and M2 macrophage ($CD206^+$ /$CD68^+$ and $CD163^+$ /$CD68^+$) were analyzed by flow cytometry. Data are expressed as the mean ± standard error of three different experiments; *P < 0.05, , **P < 0.005.

4. Simvastatin reduced MDSC and M2 macrophage, and it was reversed by addition of mevalonate

Because simvastatin is a well-known HMG-CoA reductase inhibitor and cholesterol-lowering agent, to confirm the signals through the mevalonate pathway, THP-1 cells were treated with simvastatin with or without mevalonate and flow cytometry was performed. Inhibition of HMG-CoA reductase by simvastatin reduced the proportion of MDSC and M2 macrophage in THP-1 cells (Fig. 4A and 4B).

In addition, when THP-1 cells were treated with a combination of simvastatin and mevalonate, fractions of the MDSC and M2 macrophage population were recovered from reduced fraction by simvastatin (Fig. 4A and 4B).



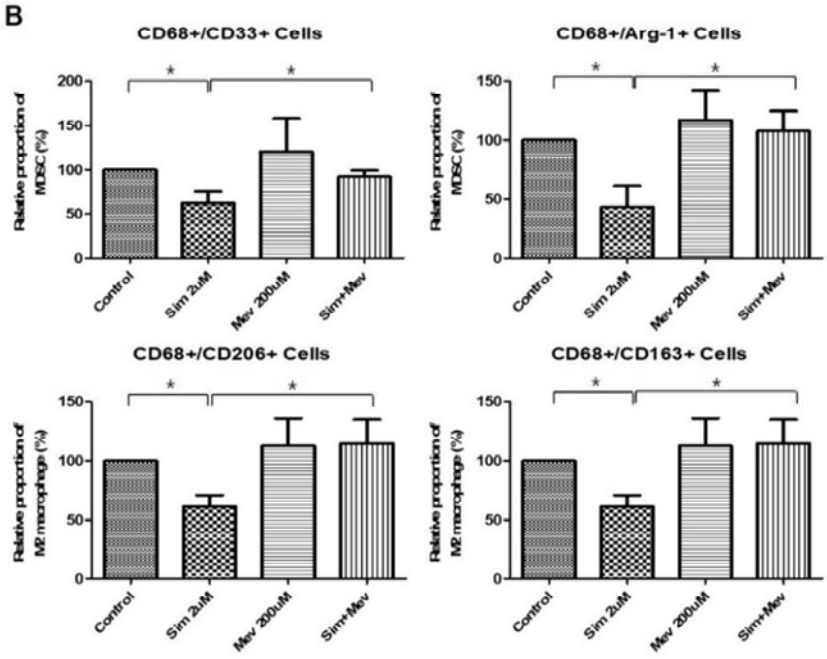
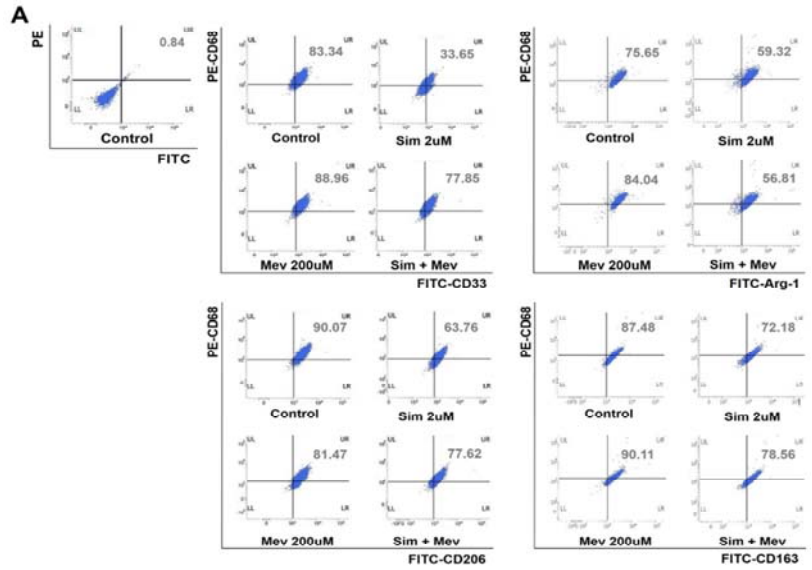
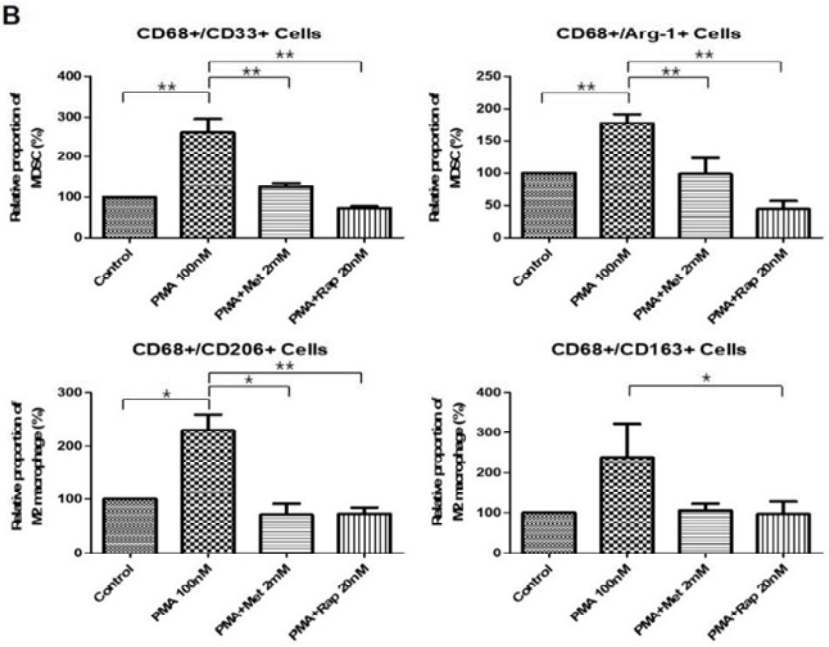


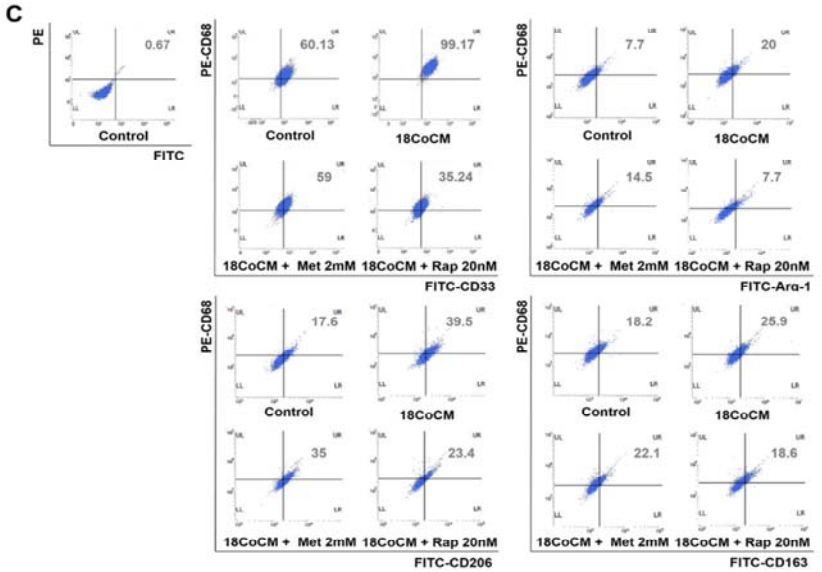
Fig. 4. Simvastatin treatment decreased the fraction of MDSC and M2 macrophage, and it was reversed by mevalonate. Simvastatin 2μM with or without mevalonate 200μM was treated in THP-1 cells for 48 hrs. Then, the expressions of MDSCs ($CD33^+$ /$CD68^+$ and $Arg\text{-}1^+$ /$CD68^+$) and M2 macrophages ($CD206^+$ /$CD68^+$ and $CD163^+$ /$CD68^+$) were analyzed by flow cytometry. Data are expressed as the mean ± standard error of three different experiments; *P < 0.05, **P < 0.005.

5. Metformin and rapamycin reduced PMA- and 18Co CM-induced MDSC and M2 differentiation

To show the same effect of metformin in MDSC/M2 macrophage-activated state, I induced activation of the MDSC/M2 macrophage population by PMA and 18Co cell (myofibroblast cell) CM (conditional media), which is important cell component of the tumor microenvironment. Flow-cytometric analysis results revealed that treatment with PMA and 18Co CM increased MDSC/M2 macrophage population in the THP-1 cells, and this induced population was reversed by treatment with metformin and rapamycin (Fig. 5A to 5D). These results suggest that metformin may be able to reduce pro-tumor immune cell populations in the microenvironment.







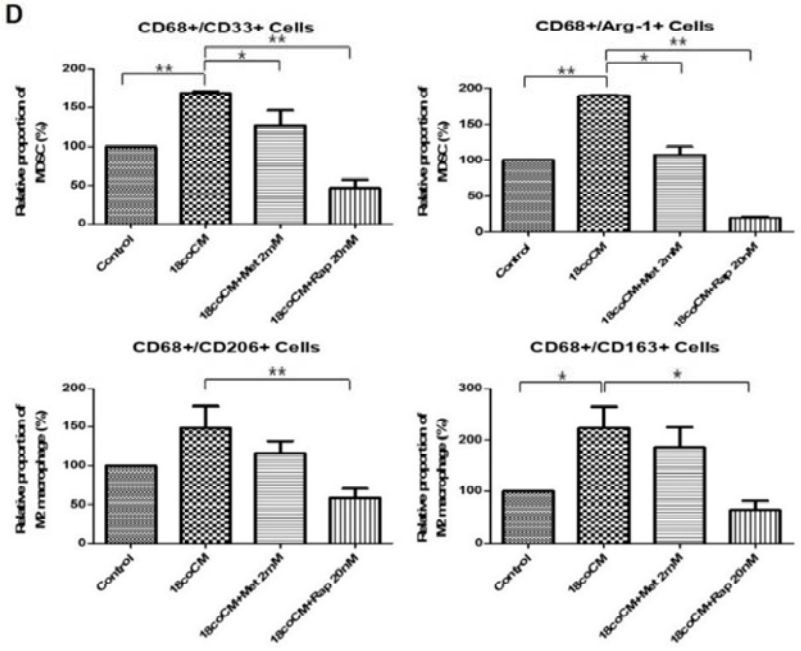
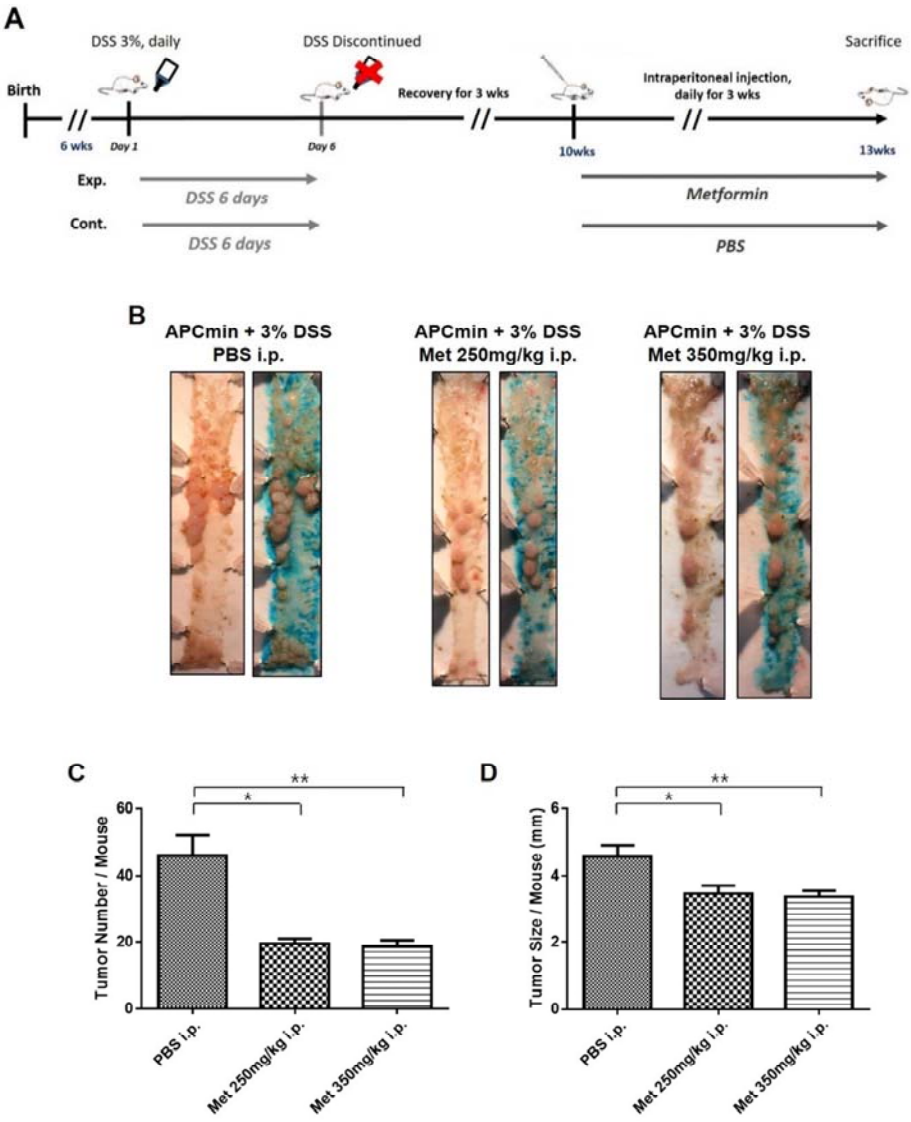


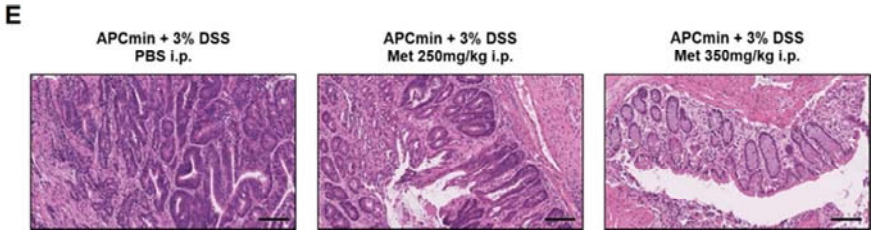
Fig. 5. PMA and 18Co CM-induced MDSC and M2 macrophage population was attenuated by metformin and rapamycin. (A and B) THP-1 cells were pre-treated with PMA 100nM for 6 hrs and then, treated with or without metformin 2mM or rapamycin 20nM for 48 hrs. (C and D) 18Co Cells were grown in serum-supplemented culture media, then washed with PBS and starved overnight in serum-free media. After two days, this medium was harvested and THP-1 cells were grown in 18Co CM and treated with or without metformin 2mM or rapamycin 20nM for 48 hrs. Then, expressions of MDSCs ($CD33^+$ /$CD68^+$ and Arg-1^+ /$CD68^+$) and M2 macrophage ($CD206^+$ /$CD68^+$ and $CD163^+$ /$CD68^+$) were analyzed by Flow-cytometric analysis. Data are expressed as the mean ± standard error of three different experiments; *$P < 0.05$, **$P < 0.005$.

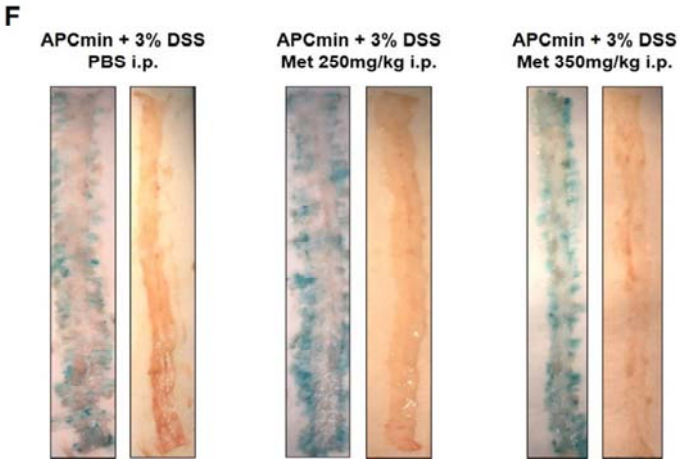
6. Tumor-suppressing effect of metformin with decreased MDSC and M2 macrophage in the mouse colon cancer model

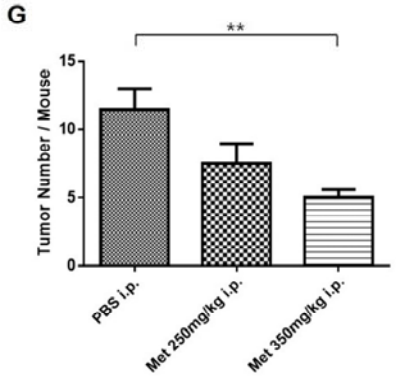
To show the effect of metformin in the *in vivo* mouse tumorigenesis model, colon tumors were developed using $Apc^{Min/+}$ mice with DSS treatment (Fig. 6A). In this mouse colon cancer model treatment with metformin decreased significantly the number and size of colonic tumors, compared to the control group (Fig. 6B to 6E). In addition, in small bowel, the number and size of polyps were noticeably decreased in metformin 350 mg/kg mice compared to control group in this mouse cancer model (Fig. 6F to 6H).

As for immune cells in tumor microenvironment, immunohistochemistry stains of MDSC (CD11b), M2 (CD206) and M1 (CD86) markers in tumor of a mouse colon showed that the number of MDSC-and M2-positive cells decreased remarkably in metformin treated mice when compared to those of the control mice (Fig. 7A and 7B). However, M1-positive cells did not show any significant changes (Fig. 7C and 7D).









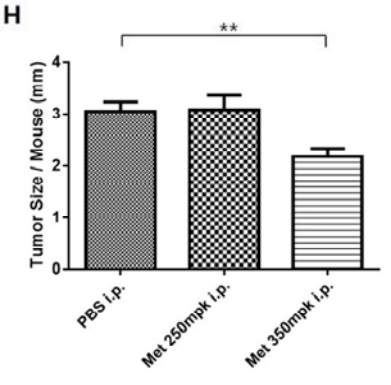


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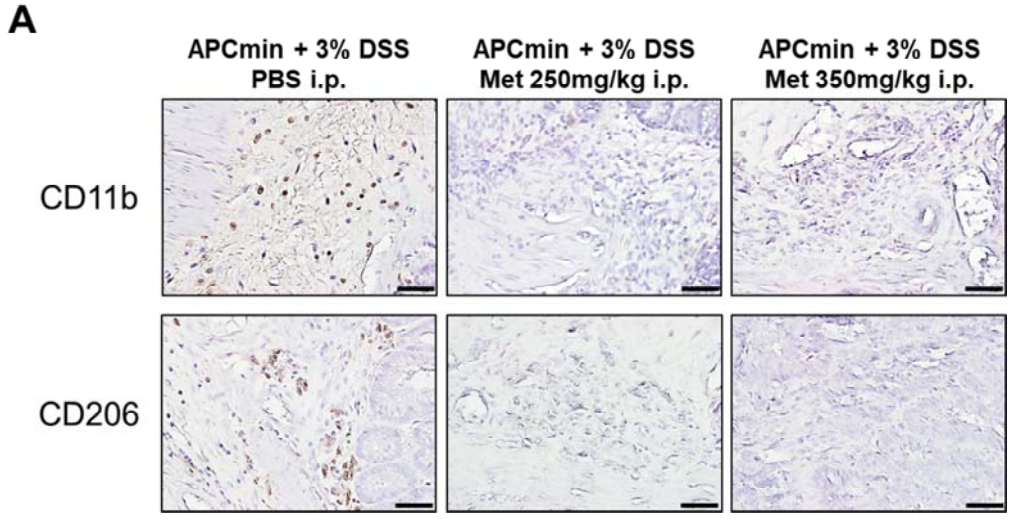
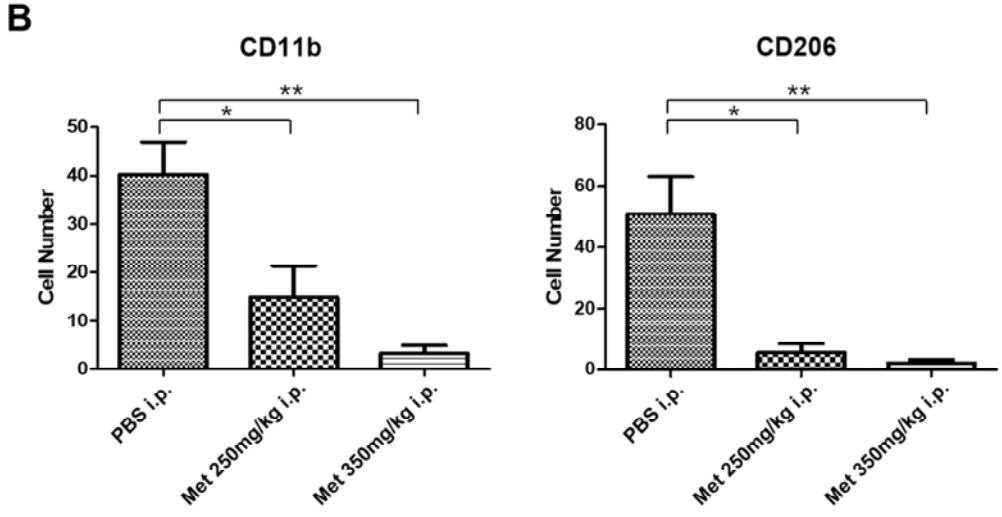
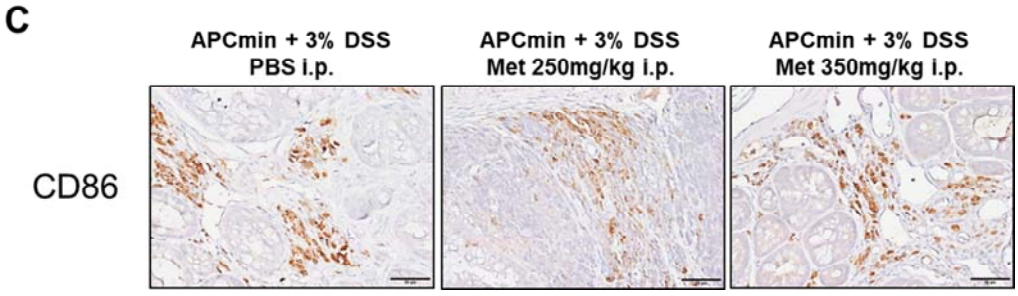
Fig. 6. The effect of metformin on tumors of mouse colon cancer model (A) *In vivo* tumorigenesis model experimental schedule, using $Apc^{Min/+}$ treated with 3% DSS, and metformin treatment schedule. (B to D) After spraying methylene blue, the number and size of polyps in colon were measured. (E) Representative H&E stains of the rectum of mice. (F to H) Mouse small bowel were stained with methylene blue, and polyp size and number were measured. Data are expressed as the mean ± standard error; *P < 0.05, **P < 0.005. n = 3 per group, Scale bar = 50 μm.







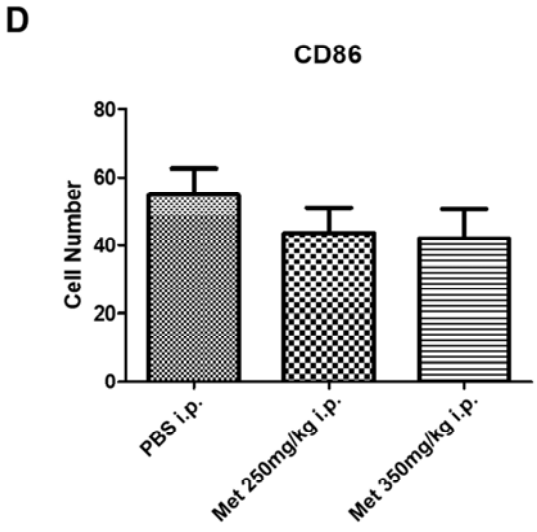


Fig. 7. Immunohistochemistry analysis for MDSC and M1/M2 macrophage. (A to D) The immunohistochemistry (IHC) of the colon sections was performed on paraffin-embedded sections for MDSC marker (CD11b), M2 macrophage marker (CD206), and M1 macrophage marker (CD86). CD11b, CD206 and CD86 stained cells were counted in the ten different fields under x200 magnification. Data are expressed as the mean ± standard error; *P < 0.05, **P < 0.005. n = 3 per group, Scale bar = 50 μm.

7. Metformin and mTOR inhibitor suppressed PMA-induced HMG-CoA reductase

HMG-CoA reductase (HMGCR) is the rate-controlling enzyme of the mevalonate pathway, which converts HMG-CoA to mevalonate. Metformin is recently reported as a potent inhibitor of HMGCR.[15] Also, mTOR which is inhibited by AMPK activation was reported to targeting HMGCR.[16] Thus, I performed real time PCR to examine metformin and mTOR inhibitor can inhibits HMGCR in THP-1 to suppress M2 macrophage activation through the mevalonate pathway (Fig. 8).

The result showed that mRNA level of HMGCR in the THP-1 was increased when treated with PMA only, and PMA-induced HMGCR was decreased by treatment of metformin and rapamycin (Fig. 8).

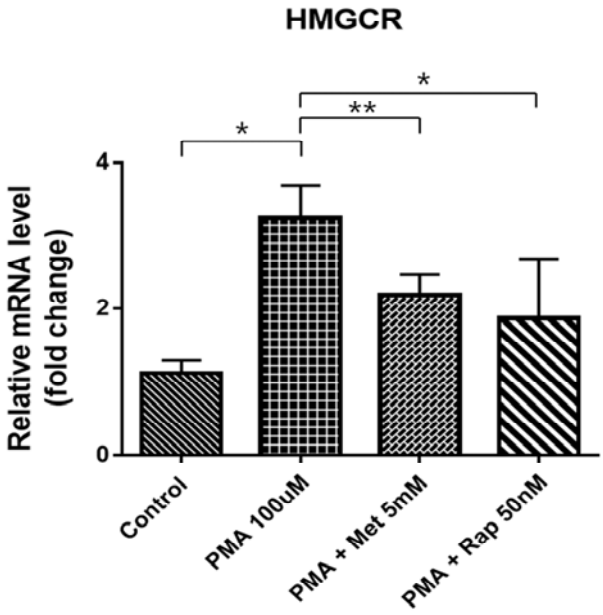
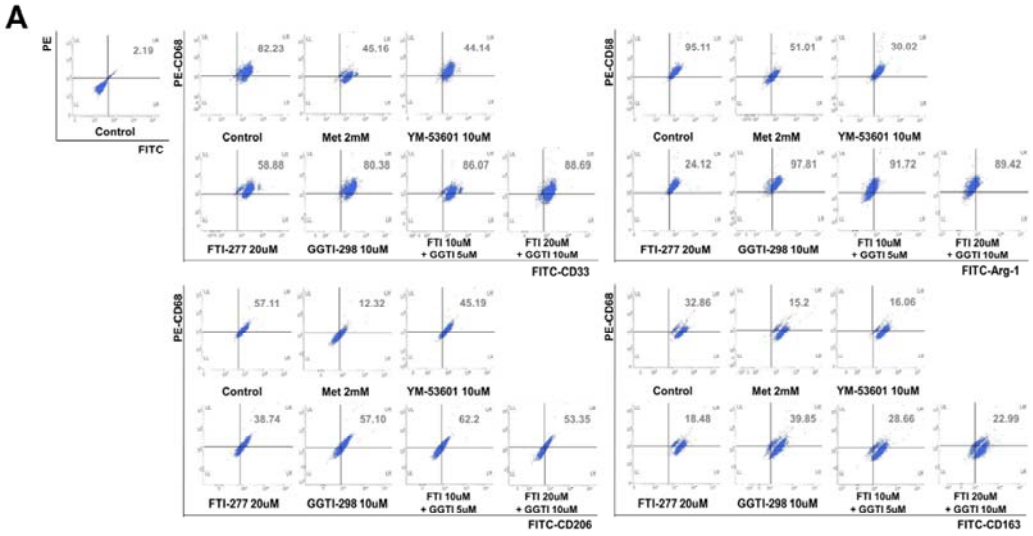


Fig. 8. PMA-induced HMGCR expression in THP-1 was attenuated by metformin and rapamycin treatment. The THP-1 cells were treated with PMA or control vehicle for six hrs and treated with metformin 5mM and rapamycin 50nM for additional four hrs. Cells were harvested and examined by real-time qPCR, normalized to GAPDH expression. Data are expressed as the mean ± standard error of two different experiments; *P < 0.05, **P < 0.005.

8. Protein prenylation inhibitor reduced the proportion of MDSC and M2 macrophage

Mevalonate pathway is divided to cholesterol synthesis and protein prenylation which includes protein farnesylation and geranyl-geranylation.[17] To elucidate the effect of protein prenylation and cholesterol synthesis on MDSC and M2 macrophage, THP-1 cells were treated with YM-53601 (cholesterol synthase inhibitor), FTI-277 (farnesyl transferase inhibitor) and GGTI-298 (geranylgeranyl transferase inhibitor) and analyzed by flow cytometry analysis (Fig. 9A and 9B).

Results showed that YM-53601, FTI-277 and GGTI-298 treatment alone did not reduce MDSC/M2 population, however combination of FTI-277 and GGTI-298 noticeably suppressed MDSC/M2 population in the THP-1 cells (Fig. 9A and 9B), suggesting the important role of protein prenylation on MDSC/M2 macrophage cells.



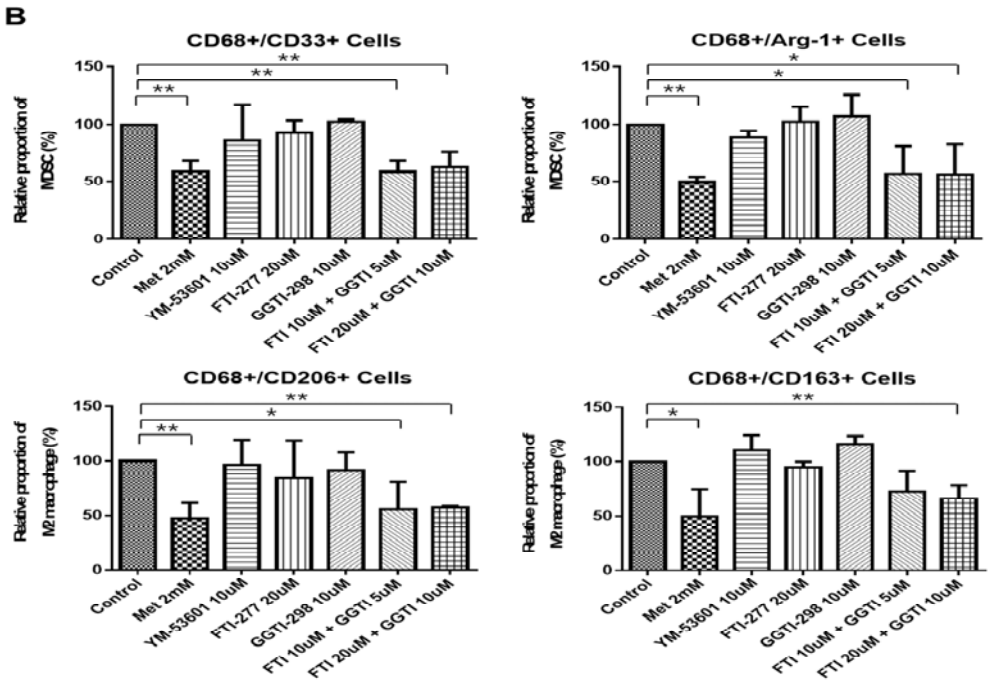


Fig. 9. Combination of FTI-277 and GGTI-298 decreased the proportion of MDSC and M2 macrophage. (A and B) The THP-1 cells were treated with metformin 2mM, YM-53601 10μM, FTI-277 10 to 20μM and GGTI-298 5 to 10μM for 48 hrs. Then, expressions of MDSCs ($CD33^+$ /$CD68^+$ and $Arg\text{-}1^+$ /$CD68^+$) and M2 macrophage ($CD206^+$ /$CD68^+$ and $CD163^+$ /$CD68^+$) were analyzed by Flow-cytometric analysis. Data are expressed as the mean ± standard error of three different experiments; *$P < 0.05$, ** $P < 0.005$.

9. Metformin decreased the PMA-induced increase of PGE_2

PGE_2 can be induced by activation of the mevalonate pathway and ERK,[13] and is related with M2 differentiation of macrophage.[14] Therefore, I performed PGE_2 ELISA assay to measure the PGE_2 concentration of the supernatant of THP-1 after treatment with metformin and/or mevalonate co-treated with PMA for 48hrs. Data showed that the PGE_2 level was increased when treated with PMA alone and PMA-induced PGE_2 was suppressed by metformin co-treatment. In addition, mevalonate induced further increase of PMA-induced PGE_2 production, and this mevalonate-induced effect was also attenuated by metformin (Fig. 10).

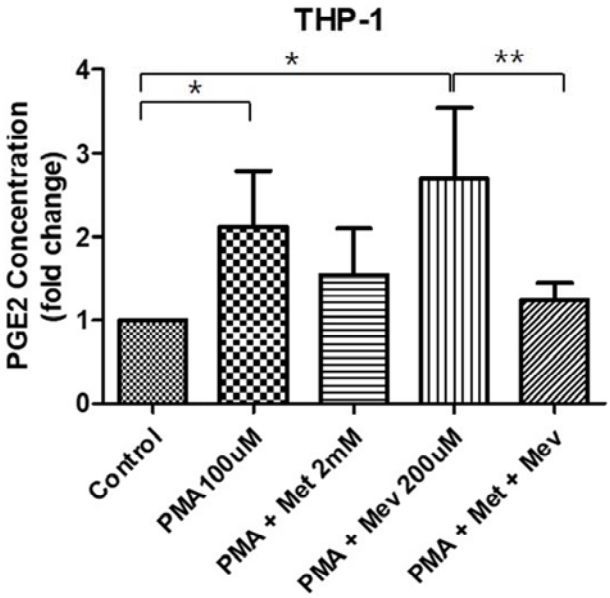


Fig. 10. PMA-induced increase of PGE_2 was attenuated by metformin treatment. The THP-1 cells were treated with PMA for 48 hrs with the control vehicle or co-treated with metformin 2mM and/or mevalonate 200μM. Then, 48 hrs culture supernatant was assayed using ELISA. Data are expressed as the mean ± standard error of four different experiments; *P < 0.05, **P < 0.005.

IV. DISCUSSION

Colorectal cancer (CRC) makes a tumor microenvironment composing cancer in the human body. In addition to harboring carcinoma cells, a tumor microenvironment consists various components that have a major role in influencing the outcome of the malignancy.[2] The tumor microenvironment is composed of various types of components such as the extracellular matrix, stromal cells like fibroblasts, myofibroblasts, immune cells and endothelial cells.[2] When recruiting the tumor microenvironment, especially immune cells in particular are deeply involved with the progression and regression of the tumor.

Metformin has an anti-tumor effect in many aspects of tumorigenesis. Metformin leads to cell cycle arrest and reduces cell growth, proliferations, protein synthesis and cancer stem cells in the tumor and its microenvironment through activation of the AMPK pathway and inhibits the mTOR pathway or the PI3K/AKT pathway.[10,11,18]

This study shows convincing evidence that metformin yields favorable outcomes in the immune cells of the tumor microenvironment. In assembling and

generating this body of work, metformin, mTOR inhibitor, AMPK activator and HMG-CoA reductase inhibitor simvastatin suppressed MDSC/M2 macrophage, and mevalonate and AMPK inhibitor reversed that phenomenon, suggesting that the AMPK/mTOR pathway and mevalonate pathway could be the key to MDSC/M2 macrophage regulation.

Moreover, when the PMA and 18co CM-induced proportion of THP-1 alters monocytes to MDSCs/macrophages, metformin and rapamycin significantly decrease the MDSC/M2 macrophage fraction measured by flow cytometry analysis. Furthermore, in the tumorigenesis mouse model experiment, metformin-treated mice specifically repressed MDSC and M2 macrophage, which is related to tumor progression, without a significant change of M1 macrophage, thus suggesting that the effect of metformin could be more specific to MDSC/M2 macrophage in the CRC microenvironment.

Then, this was followed by an interesting turn of events as it was found that the AMPK activation/mTOR inhibition can also inhibit the synthesis of mevalonate and various intermediates of the mevalonate pathway.[15] Mevalonate, an intermediate of the mevalonate pathway, is necessary for cellular proliferation and growth, and PGE_2 which is end product of mevalonate pathway increases M2 macrophages polarization and enhances development of colorectal cancer.[19] Thus,

I decide to observe the PGE_2 as a principle mediator of macrophage proliferation and differentiation in tumor microenvironment.

In addition, some reports showed that the PGE_2 induces cancer stem cells by directly targeting colorectal neoplasia, and increases mouse oncogenic stem cells via NF-κB-MAPK pathway.[13] As well as being induced in tumor cells, the PGE_2 also induces CD206-positive cells in macrophage, which is representative M2 macrophage marker in the tumor microenvironment.[14] My result also showed that PMA-induced PGE_2 was attenuated by treatment of metformin. These findings suggest that decrease of PGE_2 with metformin could have an effect on a cancer cell itself as well as the tumor microenvironment, including tumor-promoting immune cells like MDSCs and M2 macrophages.

For further investigation on related pathways, I set my research on a demonstration of relationship between protein prenylation led by the mevalonate pathway. Final mainstream of mevalonate pathway has three downstream pathway, including cholesterol synthesis by squalene synthase, protein geranyl-geranylation by geranylgeranyl transferase, and protein farnesylation by farnesyl transferase.[17,20] I focused on protein prenylation, including geranyl-geranylation and farnesylation. Protein prenylation allows the farnesylation of Ras-family

proteins, while most Rho-family proteins are geranyl-geranylated. This leads to activation of COX-2 and PGE_2 through induction of Erk 1/2 or Akt/NF-κB.[21,22]

Previous reports also showed that mevalonate pathway inhibitors may also stimulate immune surveillance, that is, the intrinsic potential of the immune system to control or eliminate cancer.[23] In addition, mevalonate pathway inhibition in dendritic cells may lead to activation of antigen-specific T-cells and NK cells, which can collaborate to produce large amounts of IFN and exhibit potent antitumor cytotoxicity.[20,24] Then, in my results, metformin and rapamycin reduced HMGCR induced by PMA treatment. Moreover, inhibition of protein prenylation by combination of protein geranylgeranyl transferase inhibitor and farnesylation inhibitor significantly reduced MDSCs/M2 macrophages fraction. Therefore, I suggest that metformin-induced suppression of protein prenylation which is induced by HMG-CoA reductase (HMGCR) through the mevalonate pathway would regulate MDSCs and M2 macrophages.

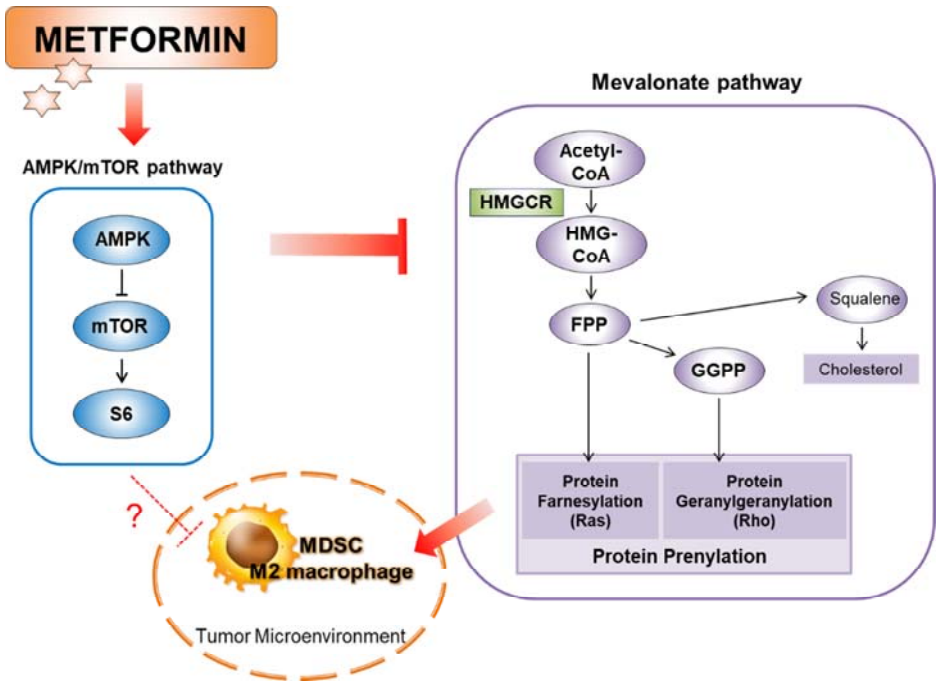


Fig. 11. An overview of inhibitory effect of metformin on MDSCs and M2 macrophages via AMPK pathway and mevalonate pathway in tumor microenvironment. Metformin activates AMPK pathway and inhibites mTOR led to inhibition of mevalonate pathway by suppressing HMG-CoA reductase. Protein prenylation, one of the main stream of mevalonate pathway, is inhibited through this regulation of HMGCR expression on MDSCs and M2 macrophages in tumor microenvironment.

V. CONCLUSION

Metformin can inhibit tumor-promoting immune cells like MDSCs and M2 macrophages via regulation of the AMPK/mTOR pathway and mevalonate pathway related to protein prenylation in tumor microenvironment of CRC.

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ABSTRACT (IN KOREAN)

대장암의 미세종양 환경에서 MDSC 와 M2 대식세포 조절을 통한 metformin 의 종양 억제 효과

< 지도교수 김태일 >

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강조연

암이 진행됨에 따라 형성되는 미세 종양 환경에는 다양한 세포들이 존재하며 각자 고유의 역할에 기여하며 종양 환경을 구성한다. 그 중에, Myeloid-derived suppressor cells (MDSCs)와 M2 대식세포는 항암제에 면역 저항성을 증가시키고 종양의 형성에 기여하며, 그에 반해 M1 대식세포는 종양 억제에 관여하고 있다. 당뇨병 치료제로 널리 알려진 metformin 은 다양한 보고에서 면역 억제와 종양 억제 효과를 보이고 있다고 알려진다. 하지만 metformin 의 면역 세포에 대한 효과와 그 기작에 대해서는 아직 잘 알려져 있지 않은 상태이다.

혈액암 환자로부터 유래한 면역 세포인 THP-1 에 metformin 을 처리하여 대식세포 조절여부를 대해 알아 보았으며, 농도에 따라 MDSC (CD33, arginase)와 M2 macrophage (CD206, CD163)를 감소시킴을 확인하였다. Western blot analysis 을 통하여, metformin 의 처리가 p-AMPK 를 활성화시키고 m-TOR 를 억제시킴을 확인하였다. MDSC 와 M2 는 AMPK 활성제에 의해 감소되며 억제제인 Compound C 에 의해 증가한다는 것을 확인하였다. 대식 세포에 대한 metformin 의 억제 효과는 Compound C 와 mevalonate pathway 의 매개체인 mevalonate 에 의해서 반전되었다. HMG-CoA reductase (HMGCR) 억제제 (simvastatin)와 mTOR 억제제 (rapamycin) 또한 이들 대식 세포를 감소시켰으며, 역시 mevalonate 에 의하여 억제효과가 반전되었다. DSS 를 처리한 APCmin 마우스 대장암 동물 모델을 이용하여, metformin 이 종양의 크기 및 숫자와 MDSC/M2 대식세포를 의미있게 억제함을 확인 하였다. 또한, metformin 과 rapamycin 이 HMGCR 의 mRNA level 을 낮춤을 확인하였고, mevalonate pathway 를 통한 protein farnesylation, geranyl-geranylasion 억제제를 처리 하였을 때, MDSC/M2 대식세포를 효과적으로 억제함을 확인하였다. 결론적으로, 이러한 metformin 의 MDSC/M2 대식세포 억제 효과는 metformin 이 AMPK 활성화를 통해 mTOR 활성을 억제시키고, 이를 통한 mevalonate pathway 의 protein prenylation 억제와 PGE_2 억제를 통해 조절됨을 확인하였다.

핵심되는 말 : M2 macrophages, MDSCs, Metformin, Tumor microenvironment