Am J Cancer Res 2023;13(1):216-226
www.ajcr.us /ISSN:2156-6976/ajcr0147370

Original Article

Temozolomide combined with ipilimumab plus nivolumab enhances T cell killing of MGMT-expressing, MSS colorectal cancer cells

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Received October 30, 2022; Accepted December 9, 2022; Epub January 15, 2023; Published January 30, 2023

Abstract: Colorectal cancer (CRC) is the third most frequently diagnosed cancer and third-deadliest cancer globally. Over 95% of patients with metastatic CRC have tumors that are microsatellite stable (MSS) and do not respond to immune checkpoint inhibitors (ICI). Results from the 2022 MAYA clinical trial suggest that the DNA-damaging agent temozolomide (TMZ), which is usually used to treat glioblastoma (GBM), sensitizes patients with MSS, MGMT-silenced CRC to ipilimumab + nivolumab ICI. The benefit of adding ipilimumab + nivolumab to TMZ and the impact of MGMT silencing remain unclear. Here, we aimed to determine in a controlled *in vitro* system if adding ICI to TMZ enhances T cell killing of MSS CRC cells. We also aimed to determine the contribution of MGMT to this response. Western blot analysis indicated that CRC cells (n = 4) had significantly elevated MGMT expression as compared to GBM cells (n = 4) likely due to MGMT promoter methylation in GBM cells. In line with this, CRC cells were slightly more resistant to TMZ compared to GBM cells after five days of treatment. TMZ + ICI sensitized MGMT-expressing, MSS CRC cells to T cell killing. TMZ alone did not enhance T cell killing of MSS or MSI CRC cells but did slightly enhance T cell killing of T98G GBM cells. Our results indicate that TMZ sensitizes MSS, MGMT-expressing CRC cells to ipilimumab + nivolumab ICI. Importantly, this suggests that TMZ-mediated sensitization to ipilimumab + nivolumab appears independent of MGMT status and the patient cohort that may benefit from TMZ + ipilimumab + nivolumab may be expanded to CRC patients with MGMT-expressing, MSS tumors.

Keywords: Colorectal cancer, temozolomide, ipilimumab, nivolumab, immune checkpoint inhibitor, MGMT

Introduction

Colorectal cancer (CRC) is the world's third most diagnosed cancer and the third most common cause of cancer-related deaths. In 2020, 9.4% of cancer-related deaths globally could be attributed to CRC and there may be over 3.2 million cases by the year 2040 [1]. The five-year survival rate of metastatic CRC (mCRC) is around 14%, thus better treatment options are needed for patients with this stage of disease [2]. Current treatment options for CRC include surgery, chemotherapy, radiation therapy, targeted therapies, and immune checkpoint inhibitors (ICI) [3]. A majority of patients with mCRC receive chemotherapy regimens that include 5-fluorouracil (5-FU) combined with oxaliplatin, irinotecan, or both. These agents cause lethal DNA damage in cancer cells, triggering the DNA damage response and apoptosis [4]. Though DNA-damaging chemotherapy has been used for decades, their effects on the tumor microenvironment are incompletely understood [5] beyond increasing neoantigen presentation and modulating cytokine secretion [6-8].

CRC is a heterogenous disease that is classified by genetic alterations in the tumor. Approximately 5% of patients with mCRC have

alterations in the mismatch repair (MMR) DNA damage response pathway, which leads to microsatellite instability (MSI) [9]. MSI CRC tumors have a high tumor mutation burden, which leads to high expression of tumor-specific neoantigens and improvement of the anti-cancer immune response. Patients with MSI CRC tumors tend to have a better prognosis as compared to patients with MSS tumors [10] and MSI tumors respond much better to ICI [10, 11]. However, > 95% of patients with mCRC have tumors with proficient MMR systems that are referred to as microsatellite stable (MSS). These patients have relatively low tumor mutation burden, low immune cell infiltrate into the tumor, and are immunologically "cold" [9, 12]. Patients with MSS CRC tumors do not respond to ICI [12, 13]. Investigation of strategies to sensitize MSS CRC patients to ICI and other immunotherapies is an area of great interest and research focus [14, 15].

Glioblastoma (GBM) is the most common primary malignant brain tumor found in adults. Current treatment options include surgical resection, radiation, and chemotherapy. The gold standard chemotherapy treatment for GBM is temozolomide (TMZ) (Temodar®) [16, 17]. TMZ is an oral alkylating agent that damages DNA to trigger cell death in cancer cells [18]. It is especially effective for the 35-55% of GBM patients with tumors that have a methylated O-6-methylguanine-DNA methyltransferase (MGMT) promoter [19-22]. This leads to silencing of MGMT, which is normally responsible for removing DNA-damaging alkyl groups on the DNA [22]. A common resistance mechanism to TMZ is hypermutation of cancer cells. As hypermutation may increase the ability of T cells to recognize cancer cells, there is interest in using TMZ to sensitize cancer cells to immunotherapy such as ICI [13].

Around 40% of patients with CRC have tumors with promoter methylation of MGMT [13]. In 2022, the MAYA clinical trial tested the safety and efficacy of TMZ combined with ipilimumab and nivolumab ICI to treat patients with MGMT-silenced, MSS mCRC. Ipilimumab and nivolumab are monoclonal antibodies that target immune inhibitory receptors CTLA-4 and PD-1, respectively, on T cells. The combination of TMZ + ipilimumab + nivolumab in the MAYA trial resulted in an overall response rate of 45% in patients with MGMT-silenced, MSS mCRC tumors. This finding is significant because the overall response rate of MGMT-methylated MSS mCRC tumors to TMZ is < 10% [23-28] and MSS mCRC tumors do not respond to ICI [12, 13]. Therefore, the results from the MAYA trial suggest that TMZ may sensitize MGMT-silenced, MSS tumors to ICI [13]. However, only patients who initially responded to TMZ received ICI, making it difficult to determine if ICI provided any added benefit. Additionally, because only patients with MGMT-silenced MSS mCRC tumors were enrolled in the study, the role of MGMT in TMZ-mediated sensitization to ICI remains unclear.

The goals of the present study were to determine in a controlled *in vitro* system if the addition of TMZ to ICI enhances T cell killing of MSS CRC cells, and to determine whether MGMT status impacts the ability of TMZ to sensitize cells to ICI. We hypothesized that TMZ would enhance TALL-104-mediated killing of CRC cells -/+ ICI, and that MGMT expression would impact the magnitude of this effect. In a panel of MGMT-silenced GBM cells and MGMT-expressing CRC cells, the 50% inhibitory concentration (IC50) was determined. MGMT-expressing MSS CRC cells were co-cultured with the TALL-104 T cell line -/+ TMZ and -/+ ICI and MGMT-silenced GBM cells were co-cultured with the TALL-104 T cell line -/+ TMZ. Our results suggest that TMZ can sensitize MGMT-expressing, MSS CRC cells to ICI-mediated T cell killing. This suggests that in addition to patients with MGMT-silenced MSS CRC tumors, such as those who were enrolled in the MAYA trial, MGMT-expressing MSS CRC tumors may also benefit from TMZ + ipilimumab + nivolumab combination treatment.

Materials and methods

Cell lines and culture conditions

GBM cells (obtained from ATCC) included U251, SNB19, T98G, and U87. U87 cells were grown in EMEM media supplemented with 10% FBS, 1% Non-Essential Amino Acids (NEAA), 1% sodium pyruvate, 1% Glutamax, and 1% penicillin/streptomycin at 37°C, 5% CO_2. SNB-19, T98G, and U251 cells were grown in DMEM media supplemented with 10% FBS, 1% NEAA, 1% sodium pyruvate, 1% Glutamax, and 1% penicillin/streptomycin at 37°C, 5% CO_2.

CRC cells (obtained from ATCC) included HCT116, HT29, RKO, and SW480. HT29 and HCT116 cells were grown in McCoy's 5A Medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO_2. RKO cells were grown in EMEM media supplemented with 10% FBS, 1% sodium pyruvate, and 1% penicillin/streptomycin at 37°C, 5% CO2. SW480 cells were grown in DMEM media supplemented with 10% FBS, 1% sodium pyruvate, and 1% penicillin/streptomycin at 37°C, 5% CO_2.

Western blot analysis

MGMT expression in CRC and GBM cells was examined using a western blot. A total of 5 × 10^5 cells were plated and incubated for 12 hours to allow the cells to adhere. Cells were harvested and lysed using RIPA buffer containing protease inhibitor. Denaturing sample buffer was added, samples were boiled at 95°C for 10 minutes, and an equal amount of protein lysate was electrophoresed through 4-12% SDS-PAGE gels (Invitrogen) then transferred to PVDF membranes. The membrane was blocked with 5% milk in 1 × TTBS, incubated overnight in the appropriate primary antibody (either MGMT, Cell Signaling Technologies # 58121S or β-actin, Sigma # A5441), and incubated in the appropriate HRP-conjugated secondary antibody (either mouse, Thermo Scientific # 31430 or rabbit, Thermo Scientific # 31460), for two hours. The levels of antibody binding were detected using ECL western blotting detection reagent and the Syngene imaging system.

Establishing IC50 doses

GBM and CRC cells were plated at a density of 5,000 cells per well of a 96-well plate. Cells were treated with TMZ using doses ranging from 0-2000 μM. Cell viability was measured using a CellTiterGlo assay. Bioluminescence was measured using the Xenogen IVIS imager. IC50 doses were determined based on the dose response curve.

Cancer cell + T cell -/+ ICI co-culture

GBM cells and CRC cells were stained with the blue CMAC live cell dye (Thermo Fisher Scientific # C2110). Blue-fluorescent cancer cells were plated at a density of 15,000 cells per well of a 48-well plate. TALL-104 cells were stained with green CMFDA (Cayman Chemical Company # 19583). Green-fluorescent TALL-104 cells were added to the blue-fluorescent cancer cells at a 1:1 ratio. TMZ (90 μM), ipilimumab (25 μg/mL), and nivolumab (1.5 ng/mL), and red fluorescent ethidium homodimer (EthD-1) (1 μM) were added at the same time as TALL-104 cells. After 4 hours of co-culture, images of live cancer cells, live TALL-104 cells, and dead cells were taken using a fluorescent microscope.

Statistical analysis

Live cancer cells, live TALL-104 cells, and dead cells were quantified using FIJI (Fiji Is Just ImageJ) software. The percent dead tumor cells in each well was quantified, and this percentage was normalized by subtracting out baseline death from cancer cell only wells, TALL-104 cell only wells, or both. A two-way ANOVA was used to calculate the interaction effect between drug treatment and TALL-104 cells.

In groups with a significant interaction effect, data was further normalized in the cancer cell + TALL-104 cell + drug treatment well by subtracting out death in the cancer cell + drug well. An unpaired t-test was used to calculate the statistical significance of the difference between this group and the cancer cell + TALL-104 cell well.

Results

HCT116, HT29, RKO, and SW480 CRC cells have elevated protein expression of MGMT compared to U87, T98G, SNB19, and U251 GBM cells

To evaluate MGMT expression of CRC and GBM cells, we conducted a western blot (**Figure 1**). All four CRC cell lines that were evaluated (HCT116, HT29, RKO, and SW480) expressed varying levels of MGMT, while all four GBM cell lines (U87, T98G, SNB19, and U251) did not express MGMT. It is likely that including a greater number of cell lines in the panel would result in an MGMT-silenced proportion that is closer to the frequency seen in patient populations (35-55% for GBM and 40% for CRC) [19-21, 29].

CRC cells are slightly more resistant to TMZ as compared to GBM cells

MGMT expression is a major predictive biomarker for response to TMZ [30]. A Cell-Titer-

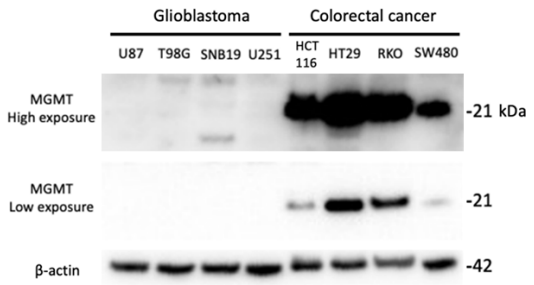

Figure 1. CRC cells have elevated protein expression of MGMT compared to GBM cells. GBM or CRC cells were harvested and the level of MGMT protein in each cell line was detected using a western blot. The level of MGMT expression varied across each CRC cell line, whereas no MGMT was detected in GBM cells even using high exposure times up to 7 minutes. Beta-actin was used as a protein loading control. CRC, colorectal cancer; GBM, glioblastoma.

Glo assay was used to evaluate differential sensitivity of GBM and CRC cells to TMZ and to establish equitoxic IC50 doses for use in cancer cell + T cell co-culture experiments. The IC50 is defined as the concentration of drug in which half of the cells lose viability as compared to an untreated control. In brief, 5,000 cells were added to each well of a 96-well plate. TMZ was added at various concentrations and viability was measured after five days, at which point we observed the cells became sensitive to TMZ. The IC50 values of each cell line were as follows: U87 (100 μM), T98G (90 μM), U251 (100 μM), SNB19 (80 μM), SW480 (150 μM), HT29 (150 μM), and HCT116 (100 μM) (**Figure 2**). These results suggest that CRC cells are more resistant to TMZ than GBM cell lines. This is expected as the CRC cell lines we evaluated all expressed varying levels of MGMT, whereas GBM cell lines did not express MGMT likely due to promoter methylation.

TMZ + ICI enhances T cell-mediated killing of MGMT-expressing, MSS CRC cells

Results from the MAYA clinical trial suggest that TMZ can sensitize patients with MGMT-silenced, MSS CRC tumors to ICI [13]. To test if the addition of TMZ to ICI enhances T cell killing of MGMT-expressing MSS CRC cells in a controlled *in vitro* system, fluorescently labeled HT29 or SW480 cells were co-cultured with fluorescently labeled TALL-104 cells in the presence or absence of TMZ and ICI. In brief, fluorescent SW480 or HT29 cells were stained, plated, and incubated for 24 hours. Fluorescent TALL-104 cells and TMZ -/+ ICI were added and images were taken after 4 hours of co-culture (**Figure 3A**). Images were quantified using FIJI and statistical analysis was performed in GraphPad Prism. TMZ alone and the combination of ipilimumab + nivolumab did not enhance TALL-104-mediated killing of HT29 cells. The combination of TMZ + ipilimumab + nivolumab resulted in a significant interaction effect calculated by two-way ANOVA. A t-test comparing the TALL-104-mediated cell death in the control well versus the TMZ + ipilimumab + nivolumab treated well indicated a significant enhancement of TALL-104-mediated killing in the treated well (**Figure 3B**, **3C**). Together, these results suggest TMZ-mediated sensitization to ICI in HT29 cells. A similar experiment with SW480 cells, which also express MGMT and are MSS, showed a similar result as far as TMZ-mediated sensitization to ICI (**Figure 4A-C**). A similar experiment was conducted with the MGMT-expressing, MSI CRC cell line HCT116 treated with TMZ, and a similar result was observed as far as a lack of T cell killing enhancement with TMZ alone (**Figure 5**). Together, these results suggest that TMZ + ICI significantly enhances TALL-104-mediated killing of MSS, MGMT-expressing CRC cell lines, but each treatment alone had no effect.

TMZ alone enhances T cell-mediated cell killing of T98G cells

To test if TMZ alone enhances T cell killing of MGMT-silenced GBM cells in a controlled *in vitro* system, fluorescently labeled T98G cells were co-cultured with TALL-104 cells in the presence or absence of TMZ. In brief, T98G cells were stained, plated, and incubated for 24 hours. Fluorescent TALL-104 cells and TMZ were added and images were taken after 4 hours of co-culture (**Figure 6A**). Images were quantified using FIJI and statistical analysis was performed in GraphPad Prism. TMZ alone enhanced TALL-104-mediated killing of T98G cells, however the magnitude of this effect was relatively small (**Figure 6B**, **6C**).

Low MGMT mRNA expression occurs at similar frequencies in CRC and GBM patient populations

The frequency of MGMT promoter methylation in GBM is 35-55% and is 40% in CRC [13, 19, 20]. As there is evidence of discordance between MGMT methylation and MGMT expression level [31, 32], we sought to examine

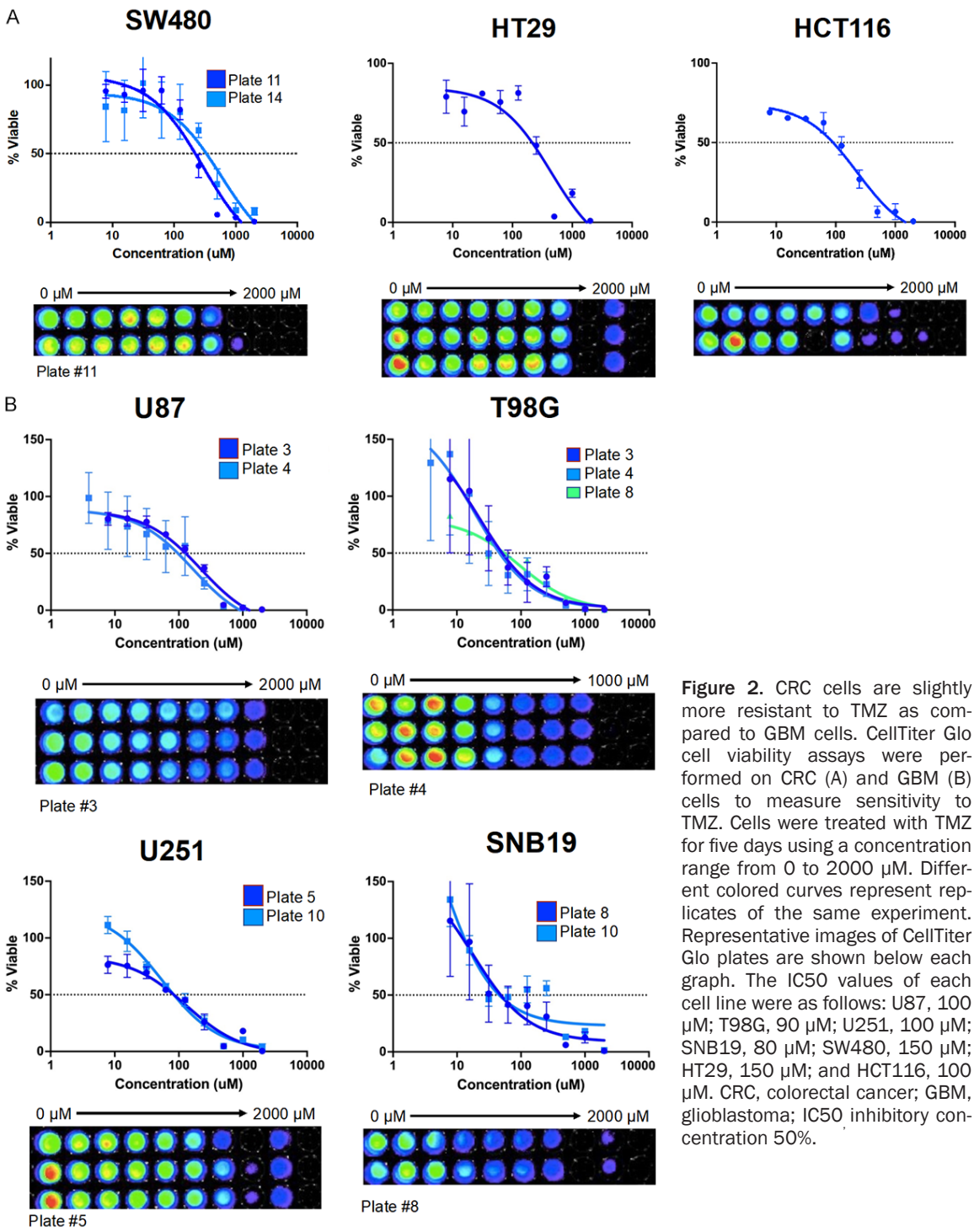

Figure 2. CRC cells are slightly more resistant to TMZ as compared to GBM cells. CellTiter Glo cell viability assays were performed on CRC (A) and GBM (B) cells to measure sensitivity to TMZ. Cells were treated with TMZ for five days using a concentration range from 0 to 2000 μM. Different colored curves represent replicates of the same experiment. Representative images of CellTiter Glo plates are shown below each graph. The IC50 values of each cell line were as follows: U87, 100 μM; T98G, 90 μM; U251, 100 μM; SNB19, 80 μM; SW480, 150 μM; HT29, 150 μM; and HCT116, 100 μM. CRC, colorectal cancer; GBM, glioblastoma; IC50 inhibitory concentration 50%.

MGMT mRNA expression in CRC and GBM tumors using The Cancer Genome Atlas (TCGA) computational tool cBioPortal. The Colorectal Adenocarcinoma PanCancer Atlas and the Glioblastoma Multiforme PanCancer Atlas databases were analyzed. We found that 19% (29/155) of GBM tumors and 14% (84/592) of CRC tumors had low expression of MGMT (**Figure 7**). These findings suggest that the frequency of low MGMT RNA expression is similar in GBM and CRC, providing rationale for the use of TMZ to treat CRC.

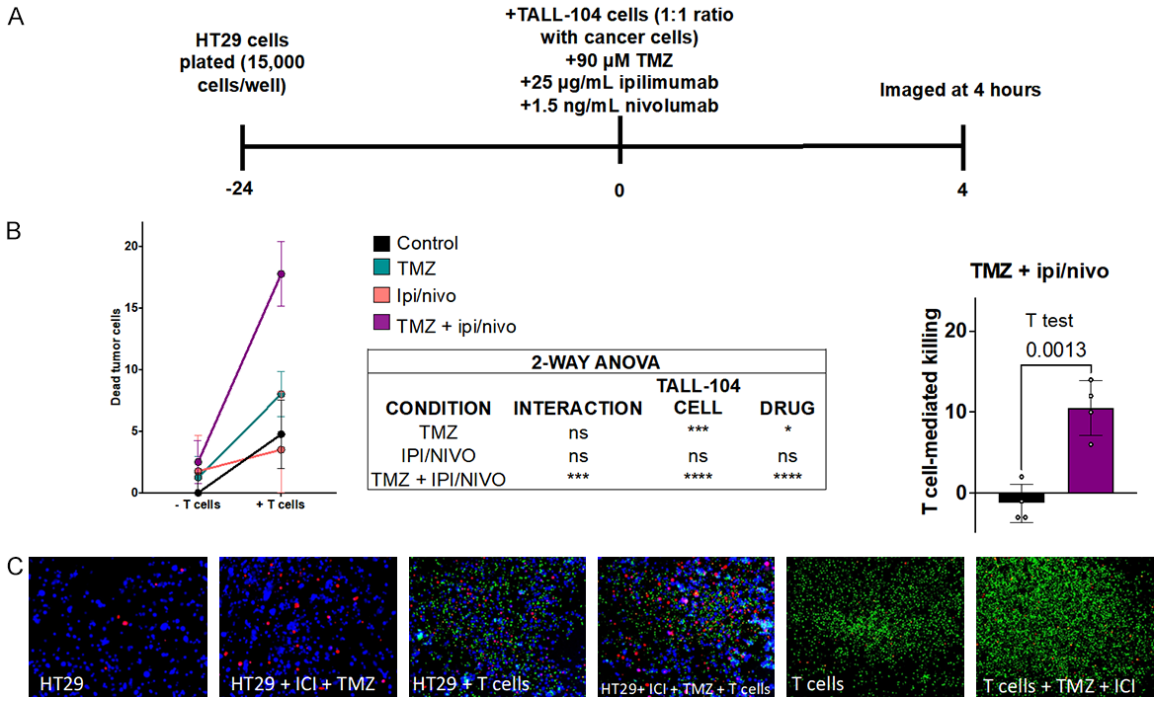

Figure 3. TMZ + ICI enhances T cell-mediated killing of HT29 cells. A. Experimental timeline: Cells were plated and incubated for 24 hours then treated with TMZ -/+ ICI, -/+ T cells. Images were taken after 4 hours of co-culture. B. A significant interaction effect and significant enhancement of T cell killing was observed for HT29 cells that were treated with TMZ + ipilimumab + nivolumab, but not TMZ alone or ipilimumab + nivolumab alone. *p ≤ 0.05, ***p ≤ 0.0005, ****p < 0.0001. C. Representative images. ICI, immune checkpoint inhibitor; Ipi/nivo, ipilimumab + nivolumab; TMZ, temozolomide.

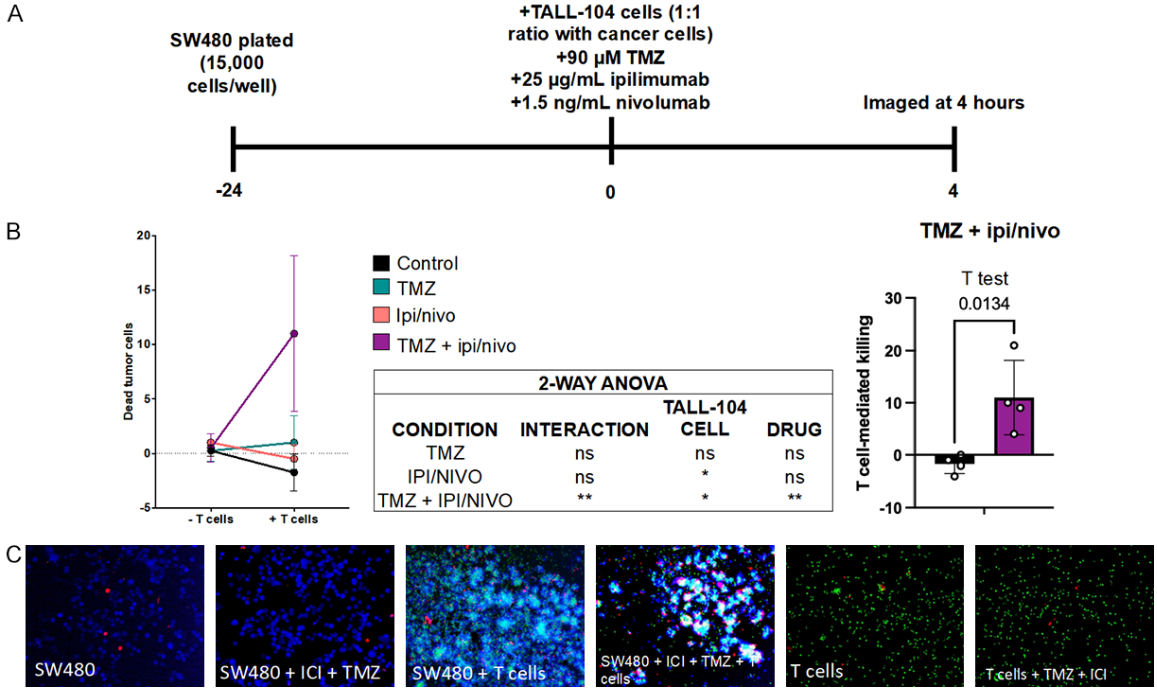

Figure 4. TMZ + ICI enhances T cell-mediated killing of SW480 cells. A. Experimental timeline: Cells were plated and incubated for 24 hours then treated with TMZ -/+ ICI, -/+ T cells. Images were taken after 4 hours of co-culture. B. A significant interaction effect and significant enhancement of T cell killing was observed for SW480 cells that

were treated with TMZ + ipilimumab + nivolumab, but not TMZ alone or ipilimumab + nivolumab alone. *p ≤ 0.05, **p ≤ 0.01. C. Representative images. ICI, immune checkpoint inhibitor; Ipi/nivo, ipilimumab + nivolumab; TMZ, temozolomide.

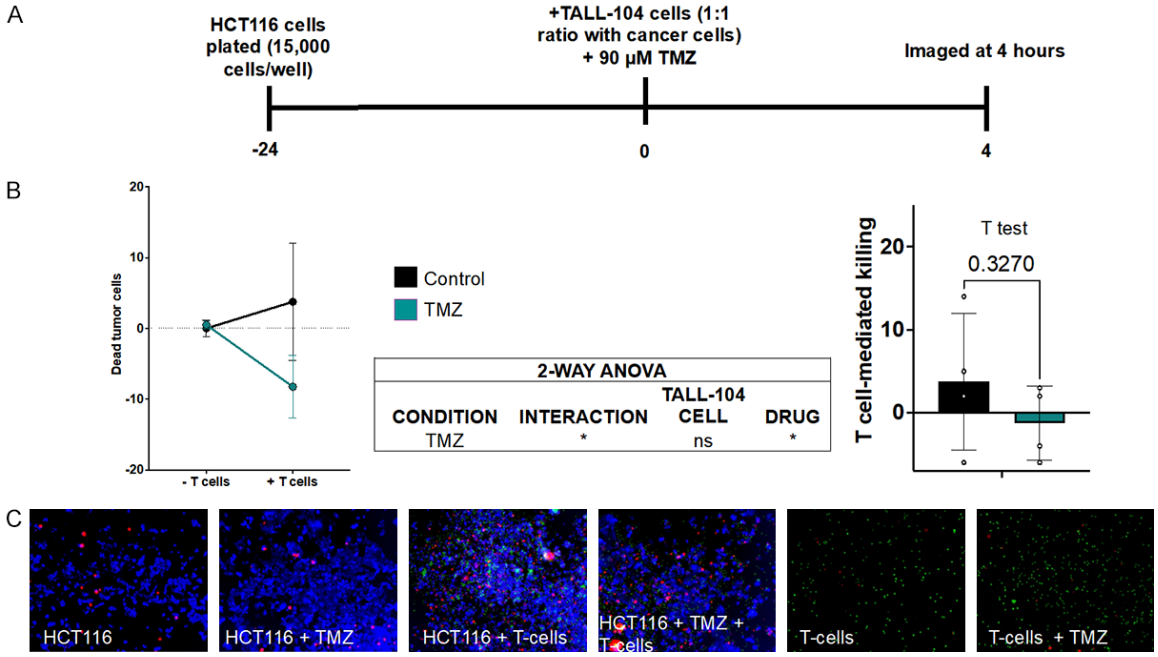

Figure 5. TMZ has no effect on T cell-mediated killing of HCT116 CRC cells. A. Experimental timeline: Cells were plated and incubated for 24 hours then treated with TMZ -/+ T cells. Images were taken after 4 hours of co-culture. B. A significant interaction effect and was observed for HCT116 cells treated with TMZ, but the enhancement of T cell killing in this group was insignificant (P = 0.3270). *p ≤ 0.05. C. Representative images. TMZ, temozolomide.

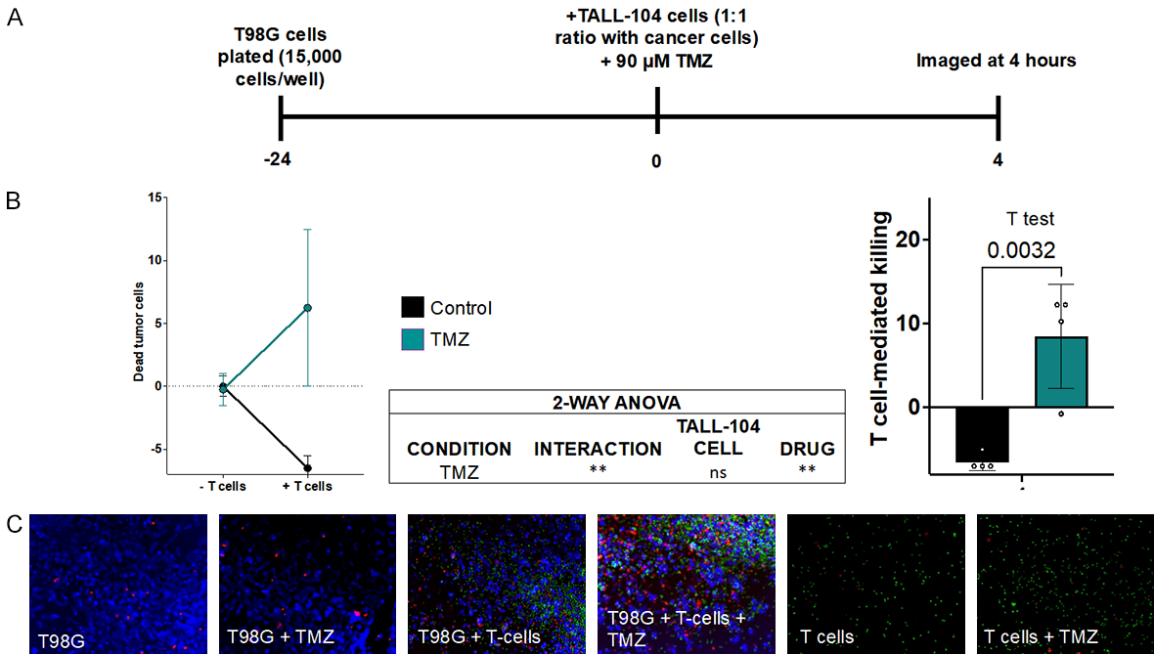

Figure 6. TMZ alone enhances T cell-mediated killing of T98G cells. A. Experimental timeline: Cells were plated and incubated for 24 hours then treated with TMZ -/+ T cells. Images were taken after 4 hours of co-culture. B. A significant interaction effect and significant enhancement of T cell killing was observed for T98G cells that were treated with TMZ. **p ≤ 0.005. C. Representative images. TMZ, temozolomide.

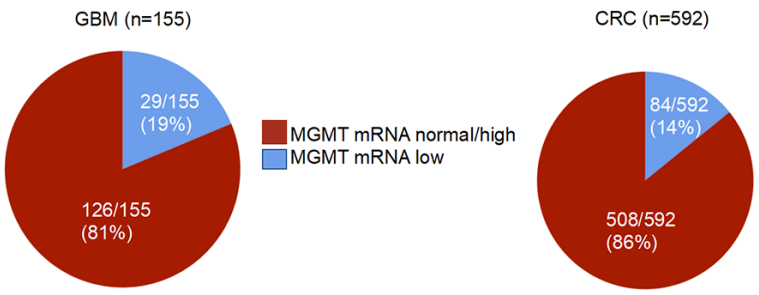

Figure 7. Low MGMT mRNA expression occurs at similar frequencies in CRC and GBM patient populations. The Colorectal Adenocarcinoma PanCancer Atlas and the Glioblastoma Multiforme PanCancer Atlas databases, which contain TCGA mRNA-seq data from CRC and GBM tumors, were analyzed using the computational tool cBioPortal. The frequency of tumors with low (< -1 standard deviation from the mean of all samples) MGMT expression and tumors with normal to high MGMT expression was similar in each database. CRC, colorectal cancer; GBM, glioblastoma; TCGA, The Cancer Genome Atlas.

Low MGMT mRNA expression predicts better survival in GBM but not CRC

TCGA was examined to evaluate if MGMT expression impacted survival in GBM and CRC patients. Data from the Colorectal Adenocarcinoma PanCancer Atlas and the Glioblastoma Multiforme PanCancer Atlas databases were analyzed. Patients with MGMT-low (< -0.5 standard deviation from the mean of all samples) GBM had better survival compared to patients with MGMT-high (> 0.2 standard deviations from the mean of all samples) GBM (log rank *P*-value = 0.0247). Patients with MGMT-low CRC had similar outcomes as patients with MGMT-high CRC (log rank *P*-value = 0.897) (**Figure 8**). This is likely because TMZ is commonly used to treat patients with GBM and is not used to treat patients with CRC.

Discussion

Our findings suggest that in an *in vitro* co-culture system, TMZ sensitizes MGMT-expressing MSS CRC cells to ipilimumab + nivolumab ICI-induced T cell killing. Importantly, it appears that TMZ-mediated sensitization to ICI is independent of MGMT status and the patient cohort that may benefit from TMZ + ICI may be expanded to include CRC patients with MGMT-expressing, MSS tumors. Future investigation could determine if TMZ-mediated sensitization to ICI is independent of MGMT status in GBM.

TMZ alone did not have any effect on TALL-104-mediated killing of MSS CRC cells or the MSI CRC cell line HCT116. TMZ alone did slightly enhance the TALL-104-mediated killing of T98G GBM cells. This variation across CRC and GBM cells may be due to differences in MGMT expression or may indicate variation in TMZ-induced changes in cytokine profiles across cancer type, as we are currently investigating.

It is thought that TMZ sensitizes cancer cells to ICI through hypermutation. However, it is unlikely that the short treatment time used here (4 hours) induced hypermutation in the cancer cells. The mechanism mediating short term TMZ-mediated sensitization to ICI needs to be investigated further. Possible explanations include TMZ-induced secretion of immunostimulatory cytokines by cancer cells and/or immune cells, or TMZ-induced regulation of immunomodulatory receptors or ligands such as PD-1/PD-L1.

It is notable that TMZ-mediated sensitization to ICI was observed in two CRC cell lines (HT29 and SW480) that expressed MGMT. These cells were more resistant to TMZ as compared to GBM cells, as expected, but still experienced TMZ-mediated sensitization to ICI. This is notable because the MAYA clinical trial only evaluated TMZ-mediated sensitization to ICI in MGMT-silenced, MSS CRC tumors which make up < 5% of the CRC patient population. Our study suggests that patients with MGMT-expressing MSS CRC tumors may also benefit, expanding the proportion of patients with CRC that could be treated with TMZ + ipilimumab + nivolumab. Future experiments involving knockdown of MGMT expression could more concretely define the role of MGMT in TMZ-mediated sensitization to ICI.

Other open questions exist, including examination of genes that are co-expressed with MGMT methylation/silencing to evaluate if they affect patient survival. Additionally, *in vivo* evaluation of TMZ-mediated sensitization to ICI in both MGMT-silenced and MGMT-expressing MSS CRC syngeneic and humanized mouse models is needed to establish this mechanism

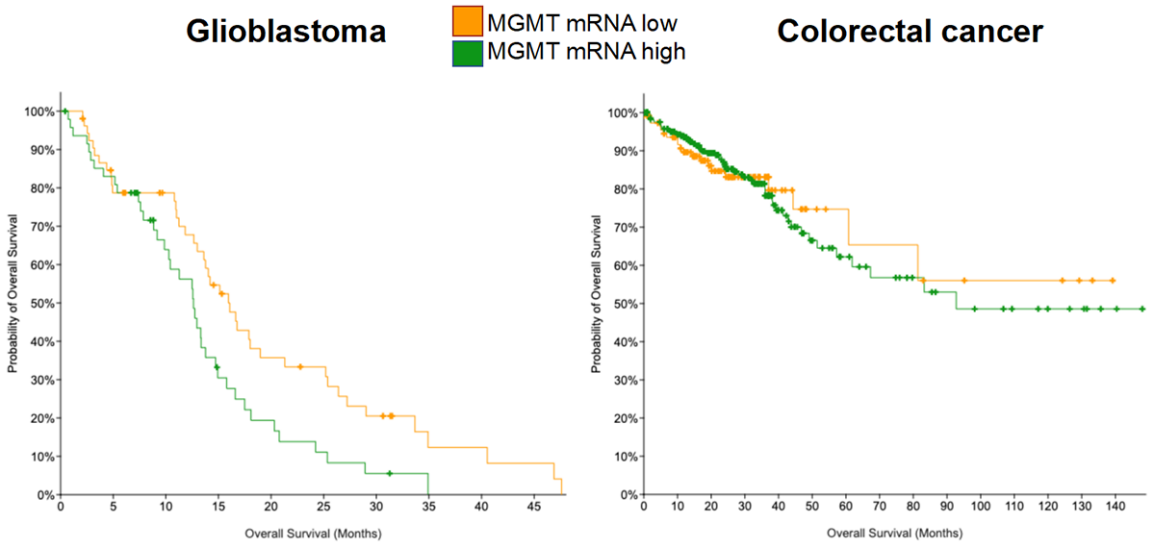

Figure 8. Low MGMT mRNA expression predicts better survival in GBM but not CRC. The Colorectal Adenocarcinoma PanCancer Atlas and the Glioblastoma Multiforme PanCancer Atlas databases, which contain TCGA mRNA-seq data from CRC and GBM tumors, were analyzed using the computational tool cBioPortal. Patients with MGMT-low (< -0.5 standard deviation from the mean of all samples) GBM had better survival compared to patients with MGMT-high (> 0.2 standard deviation from the mean of all samples) GBM (log rank *P*-value = 0.0247). Patients with MGMT-low (< -0.5 standard deviation from the mean of all samples) CRC had similar outcomes as patients with MGMT-high (> 0.2 standard deviation from the mean of all samples) CRC (log rank *P*-value = 0.897).

in a physiologically relevant system that may further help with clinical translation. Lastly, evaluation of immune cell infiltrate in TMZ-treated GBM and CRC clinical samples could further validate this mechanism in human cancer and would provide rationale for logical combination with ICI.

Disclosure of conflict of interest

None.

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