



# Mitomycin C modulates tumor microenvironment and enhances radiosensitivity in rectal cancer

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**Background:** Neoadjuvant chemoradiation is the standard of care for locally advanced rectal cancer, however, pathological complete response (pCR) rate remains unsatisfactory. Tumor hypoxia in rectal cancer might be one factor of radioresistance. Mitomycin C (MMC), a hypoxia-activated prodrug (HAP), enhances radiosensitivity and is the standard adjunct to radiotherapy (RT) in anal cancer. Our study evaluated the effect of MMC and RT on rectal cancer and its tumor microenvironment (TME).

**Methods:** *In vitro* hypoxia was induced by 1% O<sub>2</sub> culture for CT26 rectal adenocarcinoma cells and HIF-1 $\alpha$  expression was validated with Western blotting. Clonogenicity and MTT assays were used for radiation and drug survival. In syngeneic CT26 model, tumors were treated as follows: control, MMC (2 mg/kg/day), RT (2 Gy  $\times$  2 fractions), and combination with MMC 2 h prior to RT. Tumor volume, body weight, and white blood cell count were monitored for 1 month. Immune cells in TME were analyzed using flow cytometry.

**Results:** MMC inhibited cell viability and enhanced radioresponse in CT 26 cells with delayed repair of DNA double strand break (P<0.05). Expression of *Ifnb1* was augmented by RT and upregulated with addition of MMC. *In vivo* treatment using MMC plus RT suppressed tumor growth with durable effect in comparison to MMC or RT alone (P<0.05). In TME analysis, combination of MMC and RT recruited cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells into tumor and reduced infiltration of regulatory T cells.

**Conclusions:** The combination of MMC and RT may have synergistic therapeutic effect accompanied by modulation of TME towards favorable anti-tumor immunity.

**Keywords:** Mitomycin C (MMC); radiosensitization; colorectal adenocarcinoma; programmed cell death ligand 1 (PD-L1); natural killer (NK) cells

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## Introduction

Colorectal cancer (CRC) is a common malignancy which ranks third in incidence and second in cancer-related deaths globally (1). Neoadjuvant chemoradiation (CCRT) followed by surgery is the standard of care for locally advanced rectal cancer (2). However, only 10% to 25% of patients achieved pathological complete response (pCR) with neoadjuvant CCRT (3). Unsatisfactory pCR rates and potential toxicities remain the major challenge, moreover, the long-term clinical outcome is poor with 5-year overall survival and distant recurrence rates of 76% and 36% (4). These unmet medical needs require exploration of new strategies to increase the efficacy of current standard treatment with reduction of radiation toxicity.

Tumor hypoxia is one of the many molecular pathways and markers related to poor clinical outcome. It is responsible for development of aggressive phenotype and resistance to chemoradiation treatment (5,6). Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) manifests in solid tumors as they outgrow their local blood supply creating a hypoxic tumor microenvironment (TME) (7). Rasheed *et al.* showed that HIF-1 $\alpha$  was expressed in greater than 50% of rectal cancer, which correlated with unfavorable prognosis (8). Besides the effect of hypoxia in upregulating tumor angiogenesis and promoting metastasis, a variety of immune cells in the TME affect the equilibrium of tumor immunity. Cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, and dendritic cells (DCs) are crucial for pro-inflammatory cell killing while tumor infiltrating myeloid cell (TIMC) and regulatory T cells (Tregs) counteract with immunosuppression (9,10). Furthermore, tumor-associated macrophages (TAMs) can be M1 or M2 polarized and the tumor cell expression of ligands for PD-1, programmed cell death ligand 1 (PD-L1) within TME facilitates immune escape (11,12). To potentiate the therapeutic efficacy, use of a radiosensitizer with hypoxia activated feature may provide a novel strategy in targeting radioresistant hypoxic cells and alter the TME balance towards favorable anti-tumor immunity.

Mitomycin C (MMC) is known as a hypoxia-activated prodrug (HAP), which undergo bioreductive activation under hypoxia, translating into enhanced toxicity towards hypoxic tumor cells (5,6). Through altering redox status, MMC generates DNA-damaging species with antitumor profile (13). Chemoradiation with MMC is the standard of care for the treatment of anal cancer, however, MMC as an adjunct to radiotherapeutic management of rectal

cancer is not well established (14). Theoretically, the combination of RT and MMC may present potential benefit of synergistic effect with possible change of intrinsic radiosensitivity in tumors. Currently, the effect of MMC on TME of irradiated rectal tumor is still unknown.

We aimed to investigate if MMC can radiosensitize CT26 adenocarcinoma cells *in vitro* and *in vivo*. In addition, we evaluated the combinatory effect of MMC and RT in modulating TME and enhancing the anti-tumor effect of rectal cancer.

## Methods

### *Cell culture and hypoxic induction*

For *in vitro* and *in vivo* experiments, BALB/c mice bearing CT26 colorectal adenocarcinoma cells were used. The cells were maintained in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, USA). In order to examine the role of MMC-induced radiosensitization under hypoxic condition, the cells were incubated in 1% O<sub>2</sub> incubator for 2 hours prior to any type of treatment.

### *Cell viability assay*

Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method was used for cell viability assay. CT26 cells were cultured in 24-well plate at density  $5 \times 10^4$  cells/mL. Various concentrations of MMC were administered over a period of 24 hours with 2 hours of hypoxic incubation. Tetrazolium salt converted to formazan and tetrazolium dye was added as an indicator. Spectrophotometer at 570 nm was used for measurement of formazan concentrations.

### *MMC treatment and radiation delivery in vitro*

CT26 cells treated with 0, 0.1, 0.25, 0.5  $\mu\text{g/mL}$  MMC and incubated for 2 hours in hypoxic condition for investigation in radiation sensitization. After washing, a linear accelerator (Clinac iX, Varian Associates, Inc., Palo Alto, CA; dose rate 4 Gy/min) was used to deliver 6 MeV of electron beam energy with various doses (0.5, 1, 2, and 4 Gy) in a single fraction. 24 hours after irradiation, the cells were collected and plated for colony formation assay.

### **Colony formation assay**

$10^2$  viable tumor cells were coated in 6-well plate and grown in RPMI-1640 medium supplement with 10% of FCS at 37 °C in a 5% CO<sub>2</sub> incubator. After 10 days of treatment, 0.4% crystal violet was used to stain the dishes and colonies ( $\geq 50$  cells) were counted. Mean colonies/(cells inoculated  $\times$  plating efficiency) represented the surviving fraction. Linear quadratic model was used to plot survival curves. Irradiation dose needed for RT alone divided by the dose needed for MMC plus RT at a surviving fraction of 37% was deemed the sensitizer enhancement ratio (SER).

### **MMC and RT effect on DNA damage repair**

Cells were harvested at various times ranging from 0–24 hours after irradiation with 2 Gy and washed with PBS. Fixation and permeabilization was performed at 4 °C with ethanol (70%) for 1 h, incubated with Triton X-100 (1%), and stained with  $\gamma$ -H2AX antibodies (diluted 1:50; Cell Signaling Technology, Danvers, MA, USA). FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, NJ, USA) collected data from  $10^4$  cells ModFit software (Becton Dickinson, Lincoln Park, NJ, USA) was used for cell cycle analysis and calculation of the proportion of each phase.

### **Western blot**

Phosphate-buffered saline (PBS) was used to wash treated cells and lysis buffer containing 20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% triton, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mg/mL leupeptin (Cell Signaling Technology, Danvers, MA, USA) for collection. Immunoblotting with primary antibodies HIF-1 $\alpha$  (1:1,000; GeneTex, Irvine, CA, USA) was performed after using polyvinylidene fluoride membrane and blocking with 5% de-fatted milk. Horseradish peroxidase-labeled second antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and enhanced chemiluminescence system (GeneTex, Irvine, CA, USA). Internal control was represented by the expression of beta-actin (Millipore, Burlington, MA, USA).

### **Interferon- $\beta$ 1 mRNA expression with reverse transcription and polymerase chain reaction**

RNeasy mini kit (QIAGEN GmbH, Hilden, Germany) isolated total RNA from CT 26 cells. For further use in

PCR, the total RNA was reverse-transcribed to cDNA. UniqueAssay ID qMmuCED0050444 for mouse *Ifnb1* was the PrimePCR SYBR Green assay primers (BioRad) used in the study.

### **Animal model**

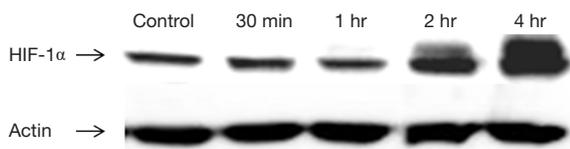
Five-week-old male BALB/c mice were purchased from Animal Resource Center of the National Science Council of Taiwan (Taipei, Taiwan). Experimental Animal Committee of MacKay Memorial Hospital (approval number: MMH-A-S-106-01) approved all animal experimental protocols. Mice were fed with water and laboratory chow (Labdiet 5001; PMI Nutrition International LLC, St. Louis, MO) ad libitum. CT26 cells ( $4 \times 10^6$  cells/100  $\mu$ L PBS) were subcutaneously implanted in the right hind leg of the mice. Tumors were grown for 7 days before treatment.

### **MMC administration and radiation delivery in vivo**

Tumor cells were implanted into four groups of animals, each with 5 mice. The mice were randomly allocated to different treatment arms for two consecutive days: (I) no treatment (control); (II) fractionated radiation therapy (2 Gy  $\times$  2 fractions); (III) MMC 2 mg/kg body weight intraperitoneally (i.p.); (IV) fractionated radiotherapy (RT) (2 Gy  $\times$  2 fractions) in combination with MMC (2 mg/kg i.p., 2 h before irradiation). Zoletil 50 (25 mg/kg) plus Xylazine (10 mg/kg) prior to irradiation was used for anesthesia. The right hind leg was irradiated using the same linear accelerator with 2 Gy (6 MeV electron beam at source-to-surface distance of 100 cm, dose rate 4 Gy/min). MMC 2 mg/kg *in vivo* was chosen based on the conversion of clinically applied human dose (10 mg/m<sup>2</sup>) to mouse equivalent dose (15). 2 Gy per day was given for two consecutive days representing fractionated RT.

### **Evaluation of tumor volume, mouse weight, survival, and white blood cell count**

Implanted tumor size and weight of mouse was measured every other day. Caliper was used to measure the largest (a) and smallest (b) diameters of tumor and volumes were estimated as  $0.5ab^2$ . For evaluation of systemic toxicity, the body weight recorded three times a week. Coulter counter (HEMAVET HV950; Drew Scientific, Inc., Dallas, TX, USA) was used to analyze the white blood cell (WBC) counts of the blood samples.



**Figure 1** Hypoxic condition verified by Western blotting demonstrated HIF-1 $\alpha$  expression after 2 hours of 1% oxygen incubation.

### Measurement of immune cells with flow cytometry

Mice from each treatment groups were sacrificed 21 days after treatment for TME assessment. The mononuclear cells were purified before immunofluorescent staining for T cell markers, DCs, NK cells, myeloid-derived suppressor cells (MDSCs), tolerogenic (M2) TAMs, and T regulatory (Treg) cells. The whole spleen and tumor specimens were removed, cut in 2–4 mm pieces on ice and digested with solution containing Liberase TM (25  $\mu\text{g}/\text{mL}$ ) (Roche Diagnostics GmbH, Mannheim, Germany) and DNase I (10  $\mu\text{g}/\text{mL}$ ) (Sigma-Aldrich, St Louis, MO, USA) at 37  $^{\circ}\text{C}$  for 30 minutes. Single-cell suspensions were obtained with a 70- $\mu\text{m}$  cell strainer. Incubation with ACK solution (Invitrogen, Waltham, MA, USA) was performed to lyse red blood cells. Before staining with cell surface markers, cells were resuspended in IgG from rat serum (1  $\mu\text{g}/1 \times 10^6$  cells) (Sigma-Aldrich, St Louis, MO, USA) and incubated for 15 min at 4  $^{\circ}\text{C}$  to reduce nonspecific binding. Then, cells ( $1 \times 10^6$  cells/mL) were stained with antibodies conjugated with indicated fluorochromes for 30 min on ice, including anti-CD3 (PE), anti-NKG2D (PE), anti-CD11b (PE), anti-CD45 (PerCP), anti-CD206 (FITC), anti-CD8 (FITC), anti-CD83 (FITC), anti-MHCII (FITC), and anti-FoxP3 (APC; BioLegend). After staining, FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, NJ, USA) was used to evaluate infiltrated immune cells DCs (CD83 $^+$ ), M1 macrophages (MHC-II $^+$ ), M2 macrophages (CD206 $^+$ ), NK cells (NKG2D $^+$ ), and total T cells (CD3 $^+$ ). Detection of regulatory T cells (Tregs) staining with FoxP3 was performed according to the manufacture's protocol using the True-Nuclear<sup>TM</sup> Transcription Factor Staining Protocol for 5 mL Tubes (BioLegend, San Diego, CA, USA).

### Immunohistochemical staining

Sections of formalin-fixed and paraffin-embedded mouse lymph nodes and tumors were treated with heat-induced

antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) under 95–100  $^{\circ}\text{C}$  for 15 min, and followed with 3%  $\text{H}_2\text{O}_2$  at room temperature for 10 min. BlockPROT<sup>TM</sup> Blocking Buffer (Visual Protein, Taipei, Taiwan) at room temperature for 1 hour were used for blocking. Primary antibodies against PD-L1 (1:400; GeneTex, Irvine, USA) and HIF-1 $\alpha$  (1:200; GeneTex, Irvine, USA) at room temperature for 1 hour, with secondary antibody of SignalStain<sup>®</sup> Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 hour, and 5–10 minutes with DAB chromogen kit using EnVision<sup>TM</sup> + Dual Link System-HRP (Dako, Santa Clara, CA, USA) were used on the sections before counterstaining with hematoxylin (Merck, Kenilworth, NJ, USA). A pathologist and an oncologist with extensive experience on IHC assessment examined the expression of PD-L1 and HIF-1 $\alpha$ .

### Statistical analysis

All experiments were performed and repeated three times. Mean  $\pm$  standard mean error from three independent experiments were used to express data. Statistical comparison of each experiment was performed with student's *t*-test or one-way analysis of variance (ANOVA). Statistically significant values were indicated by  $P < 0.05$ .

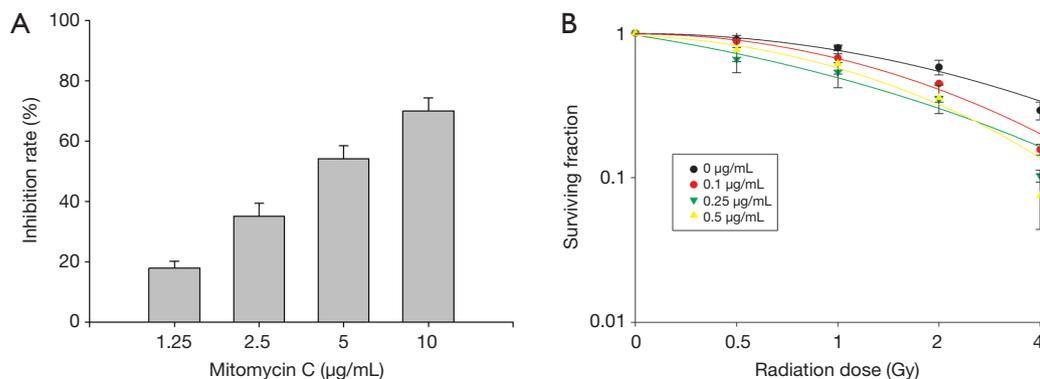
## Results

### Upregulation of HIF-1 $\alpha$ expression in CT26 cells by hypoxia

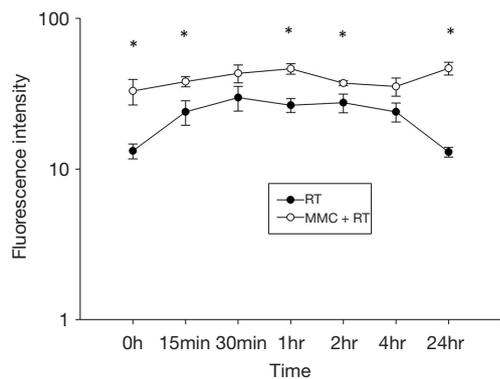
To verify the establishment of hypoxic condition, CT26 cells were incubated in 1% oxygen incubator for 30 min, 1, 2, and 4 hours prior to treatment. Western blotting revealed increasing expression of HIF-1 $\alpha$  after 2 hours of hypoxic incubation (Figure 1). The following *in vitro* experiments were performed with at least 2-h incubation prior to treatment to ensure upregulation of HIF-1 $\alpha$  expression and hypoxic environment.

### Effect of MMC and radiosensitization of CT26 cells *in vitro*

For cell viability, CT26 cells were pretreated with MMC and placed in a hypoxic incubator for 2 h. The highest non-toxic dose with treatment of MMC alone under hypoxia was 1.25  $\mu\text{g}/\text{mL}$  (Figure 2A). To determine the MMC effects on radiation cell killing, radiation was added following MMC



**Figure 2** MMC inhibited CT26 viability and enhanced radiosensitivity. (A) Cell viability by MTT assay; (B) radiation survival curves for CT26 cells. CT26 cells were pretreated with MMC and incubated in 1% oxygen incubator for 2 hours prior to irradiation. Radiation survival was assessed by performing colony formation assay. MMC, mitomycin C.



**Figure 3** Dynamic changes of  $\gamma$ -H2AX after irradiation with or without MMC pretreatment. Significant differences in levels of  $\gamma$ -H2AX in various time intervals after the initial treatment with RT vs. MMC plus RT are indicated by \* $P < 0.05$ . MMC, mitomycin C; RT, radiotherapy.

pretreatment and 2-h hypoxia induction. Cell killing was achieved with a lowered dose of MMC where pretreatment with 0.5  $\mu\text{g/mL}$  MMC was sufficient to sensitize CT26 cells to radiation doses of 2 Gy and above (Figure 2B). The sensitizer enhancement ratio at 37% was 1.4.

#### MMC pretreatment and radiation on DNA damage

Ionizing radiation or cytotoxic drugs induce DNA damage causing double stranded breaks (DSBs) (16). Following DNA damage,  $\gamma$ -H2AX rapidly forms at sites of DSBs to initiate DNA repair and response is maximal at 30 minutes post radiation exposure followed by decrease over time

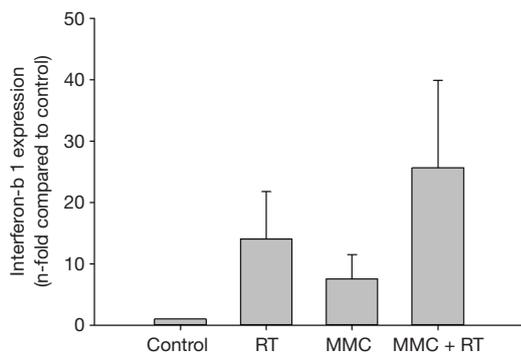
(17,18). We found that pretreatment with 5  $\mu\text{g/mL}$  of MMC prior to 2Gy RT promoted the production and prolonged the reduction of  $\gamma$ -H2AX (Figure 3). Initial levels of  $\gamma$ -H2AX were increased with addition of MMC to RT and maintained over the course of 24 hours compared to RT treatment alone ( $P < 0.05$ ). This indicated that MMC promoted DNA DSBs and delayed DNA damage repair induced by RT.

#### Interferon- $\beta$ mRNA expression with reverse transcription and polymerase chain reaction

To assess the activation of anti-tumor immunity, interferon- $\beta$  (*Ifnb1*) mRNA expression was evaluated via RT-PCR. MMC combined with 2 Gy of radiation showed a 1.8- or 3.4-fold increase in *Ifnb1* mRNA expression compared to treatment with either RT or MMC alone, respectively (Figure 4).

#### MMC enhanced the effect of RT in vivo

CT26 tumor cells were implanted into the right hind legs and randomized into four treatment groups: (I) no treatment (control); (II) fractionated radiation therapy (2 Gy  $\times$  2 fractions); (III) MMC 2 mg/kg body weight intraperitoneally (i.p.); (IV) fractionated RT (2 Gy  $\times$  2 fractions) in combination with MMC (2 mg/kg i.p., 2 h before irradiation). The tumor volume decreased significantly from day 9 and onwards after treatment with MMC and RT (Figure 5A). Inhibition of tumor growth represents durable effect of combined treatment. Significant



**Figure 4** Effect of MMC and RT on *Ifnb1* mRNA expression. MMC, mitomycin C; RT, radiotherapy.

differences in body weight growth kinetics between the control and combined treatment groups were observed on day 21 (Figure 5B). There was no significant adverse effect on WBC counts between the different treatment groups (Figure 5C).

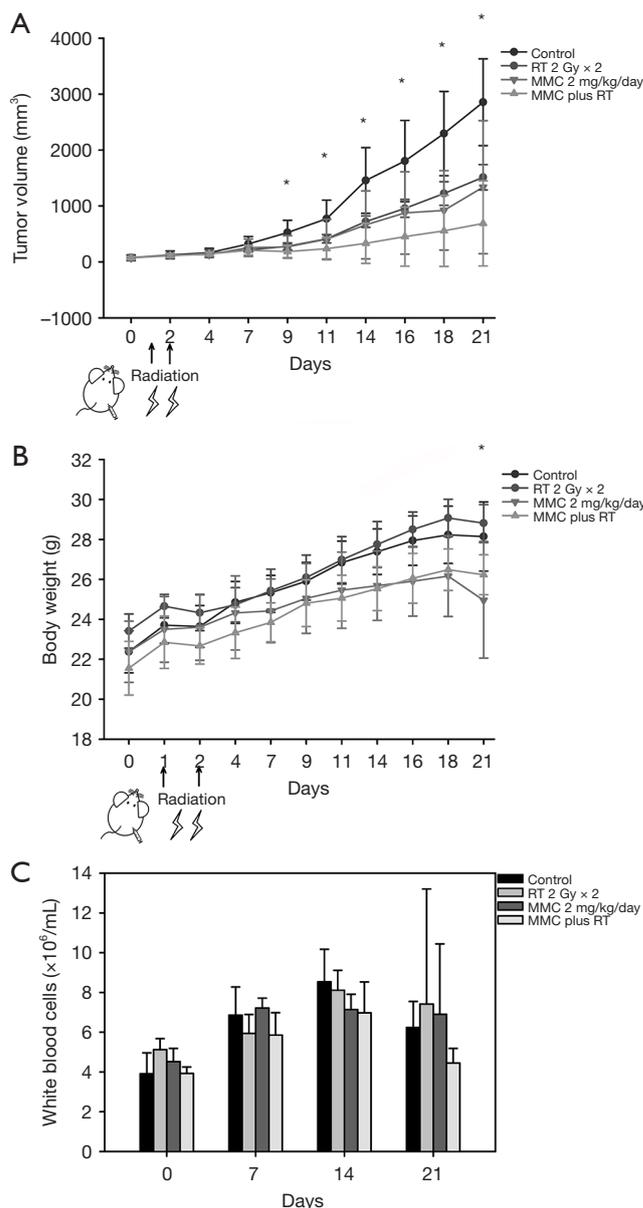
**TME analysis**

We next investigated TME using flow cytometry and analyzed the immune cells with corresponding surface markers: M1 (MHC-II<sup>+</sup>); M2 (CD206<sup>+</sup>); NK (NKG2D<sup>+</sup>); CTL (CD8<sup>+</sup>); DC (CD83<sup>+</sup>); tumor-infiltrating myeloid cell (TIMC) (CD11b<sup>+</sup>); total T cell (CD3<sup>+</sup>); Treg (FoxP3<sup>+</sup>) (Figures 6,7). RT increased intratumoral DCs by 1.2-fold compared to control, while MMC promoted M1 macrophages by 1.5-fold and total T cells by 2.8-fold in comparison to control (Figure 7). TIMC and M2 macrophage expression levels did not vary after treatment. Combined use of MMC with RT showed increased NK cells by 8.5-fold and CTLs by 4.8-fold compared to control (Figure 7). When combined treatment was weighed against RT treatment alone, an increase of NK cells by 9-fold and CTLs by 2-fold was observed (Figure 7). As for Tregs, decrease by half-fold in combination treatment group in comparison to control was noticed (Figure 7).

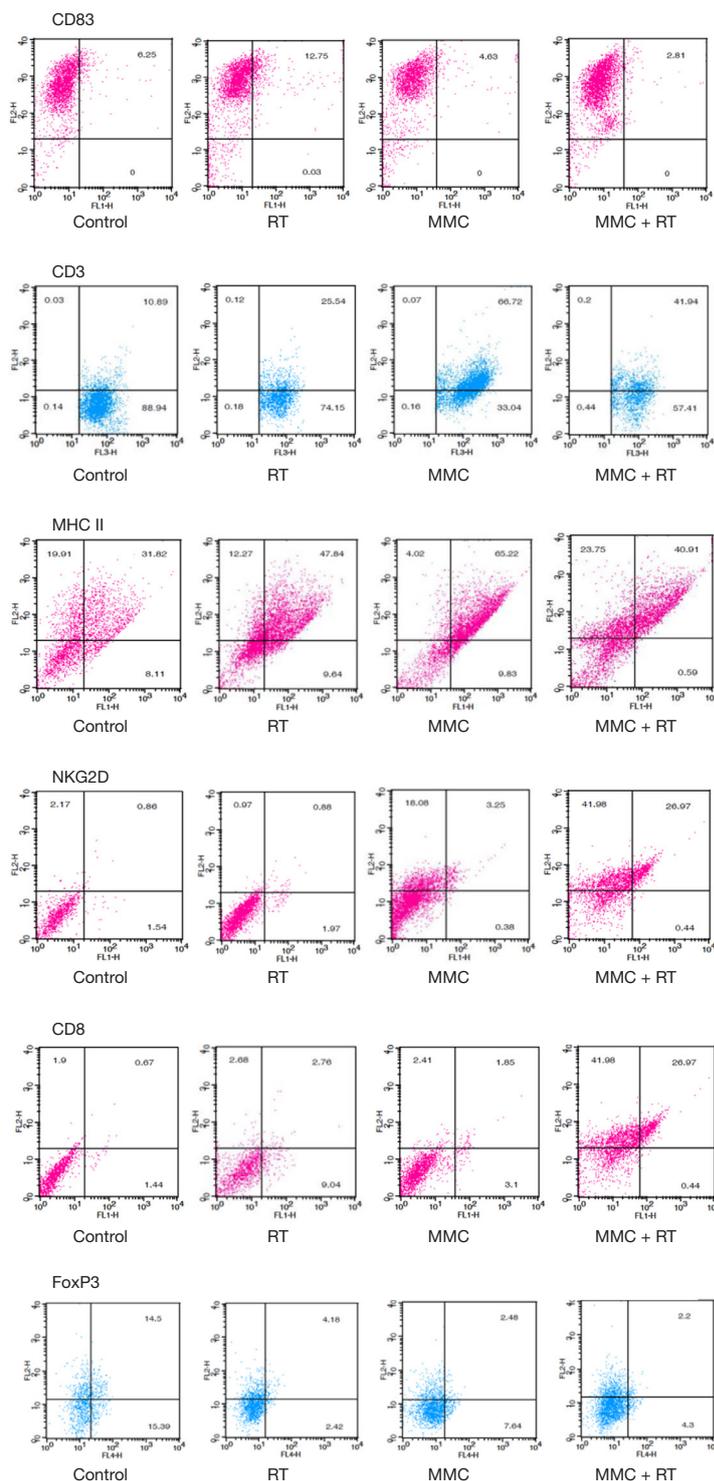
**Immunohistochemistry and histopathological changes**

In our *in vivo* experiment, immunohistochemistry (IHC) staining for HIF-1α was performed for tumor and lymph node sections. This was to verify the presence of hypoxic environment in the *in vivo* setting (Figure 8A,B).

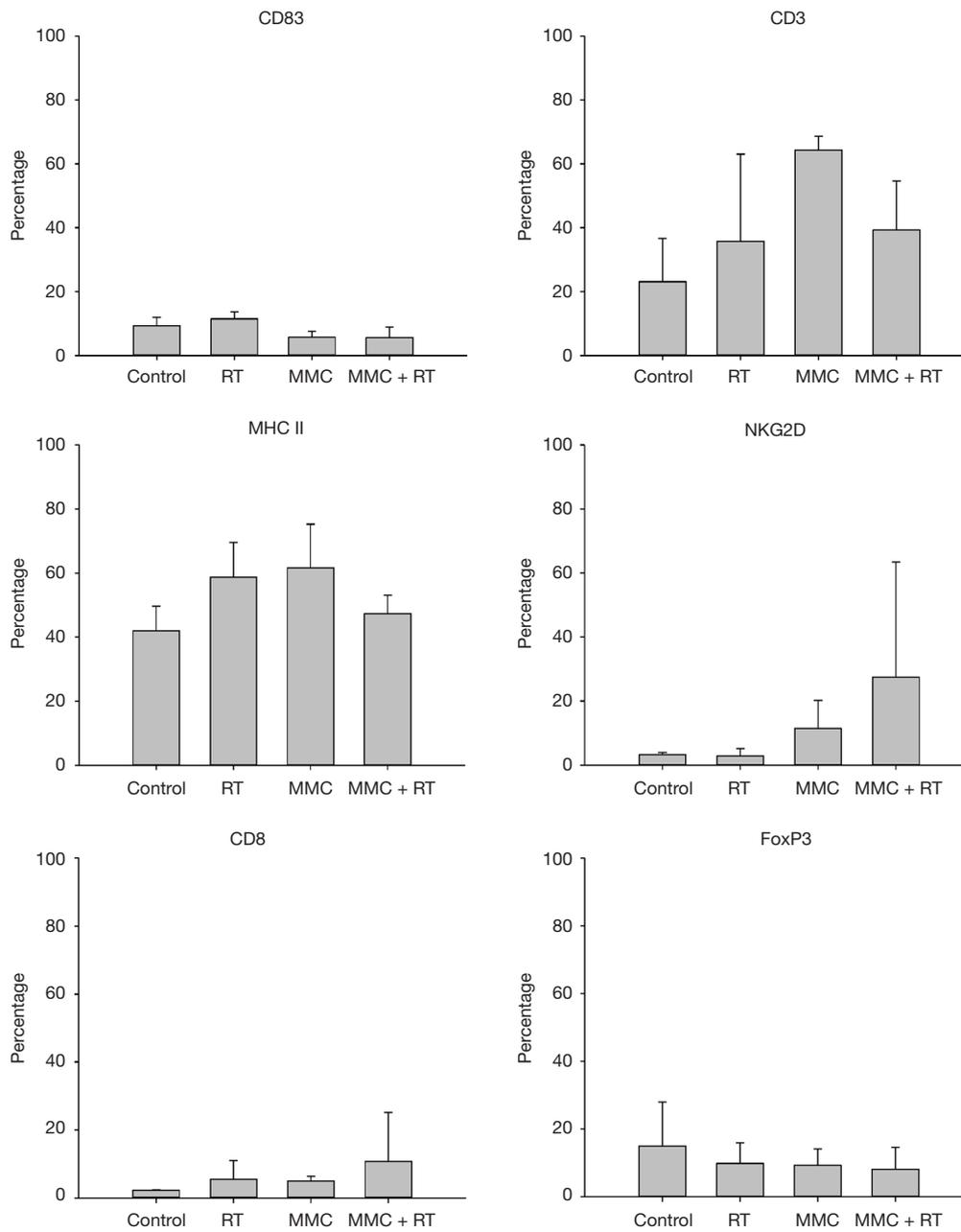
To further investigate possible causes of tumor escape,



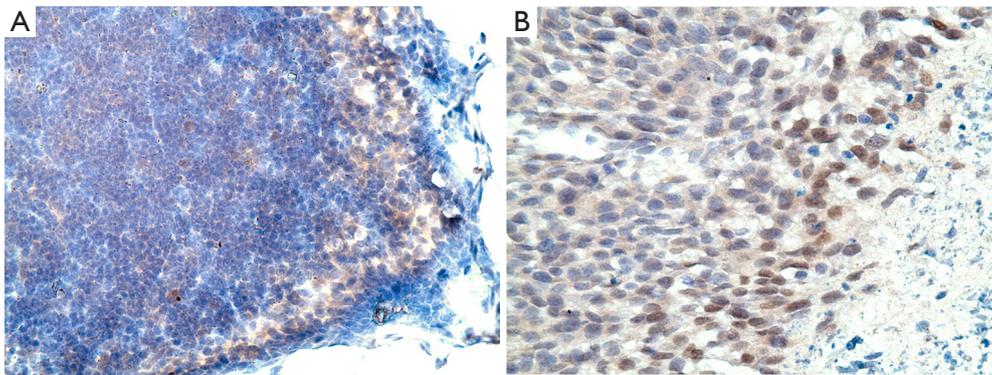
**Figure 5** Effect of MMC and RT on CT26 tumor growth in syngeneic mice. (A) One week after injection of CT26 tumor cells, the Balb/c mice were randomized into the following treatment groups: control; 2 Gy x 2 Fx over 2 days; i.p. implanted MMC (2 mg/kg) over 2 days; MMC + RT in 2 consecutive days with MMC injected 2 hours prior to RT. The tumor volume was significantly smaller in the MMC plus RT treatment group compared to other treatments. (B) Decrease in body weight after treatment. (C) WBC count in various treatment groups. Data from three separate experiments are presented as mean ± standard mean error. N=5 for each group. \*, P<0.05 vs. control. MMC, mitomycin C; RT, radiotherapy; WBC, white blood cell.



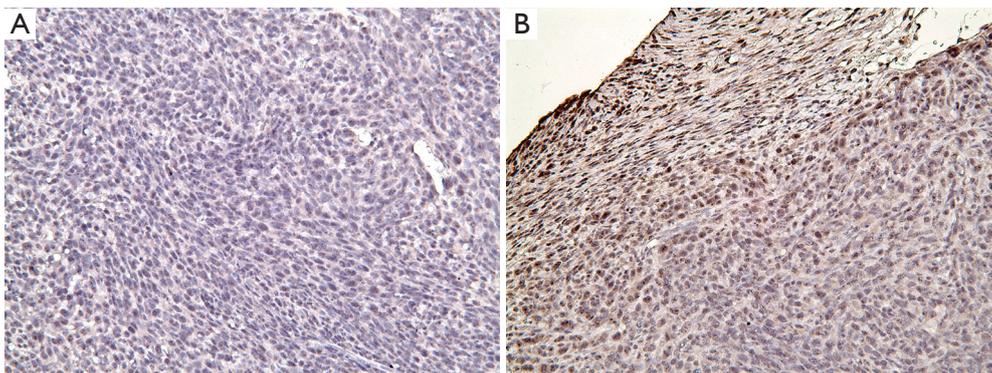
**Figure 6** Tumor microenvironment analysis. Tumor specimens were harvested and analyzed with flow cytometry to detect the surface markers CD83+, CD3+, MHC II+, NKG2D+, CD8+, and FoxP3+ of each immunogenic cell lineages corresponding to DCs, total T cells, M1 macrophages, NK cells, CTLs and Tregs, respectively. Representative dot-plot panels of surface markers in flow cytometry after treatment with control, RT, MMC, and MMC plus RT. DCs, dendritic cells; NK, natural killer; CTLs, cytotoxic T lymphocytes; Tregs, regulatory T cells; MMC, mitomycin C; RT, radiotherapy.



**Figure 7** The average expression levels of CD83+ increased with RT treatment, whereas MMC enhanced CD3+ and MHC II+. In the MMC plus RT treatment group, NKG2D+ and CD8+ were recruited, while FoxP3+ was reduced. MMC, mitomycin C; RT, radiotherapy.



**Figure 8** HIF-1 $\alpha$  nuclear staining in (A) lymph node and (B) tumor. Magnifications 400 $\times$ .



**Figure 9** Tissue expression of PD-L1. Tumor PD-L1 in (A) control and (B) post MMC plus RT treatment. Magnifications 200 $\times$ . MMC, mitomycin C; RT, radiotherapy.

immune checkpoint PD-L1 was stained. IHC results reveal increased expression of PD-L1 in tumor after treatment with combined MMC and RT in comparison to control (Figure 9).

## Discussion

This study revealed that MMC, a hypoxia activated prodrug (HAP), in combination with RT could enhance radioresponse and modulate TME toward inflamed phenotype. MMC demonstrated improve benefit when concurrently used with RT in the management of head and neck squamous cell cancer, but the effect on rectal cancer remains undetermined (19). The need for enhanced tumor radiosensitivity incited the use of MMC on mouse CT26 colorectal adenocarcinoma cells.

Drug repositioning is a concept to apply clinically accepted regents for new indications. The toxicity profile and management of adverse effect of MMC has been well

established, including combined use with RT, which have possible risks of causing febrile neutropenia in 15% and leucopenia in 2% to 13% of patients (20). Our results revealed that MMC enhances the anti-tumor effect of RT against colorectal adenocarcinoma *in vivo* with inhibition of tumor growth and no significant toxicity or body weight loss (Figure 5). This implies safe therapeutic effect of MMC and a promising addition to RT in treatment of rectal cancer. Given that MMC has been widely used in CCRT of anal cancer with manageable toxicity such as moderate myelosuppression which can be prophylactically prevented or treated with colony stimulating factors, the combined administration of MMC and RT may be regarded as relatively safe (20,21). Taken together, this drug repositioning to CCRT of rectal cancer, other than anal cancer, might be reasonable and worthy of verification in the clinical setting.

We began by verifying presence of tumor hypoxia in both *in vitro* and *in vivo* setting as represented by

upregulated HIF-1 $\alpha$  expression (Figures 1 and 8). MMC enhanced the effect of RT as shown in radiation and drug survival assay (Figure 2). Although colon and rectal cancers demonstrate different entities with distinct treatment in clinical setting, the chemoradiosensitivity between colon and rectal cancer cell line does not differ significantly (22). This supports the translational value in our use of CT26 colorectal adenocarcinoma cells, suggesting MMC may be a novel candidate as a radiosensitizer for neoadjuvant CCRT in rectal cancer. In addition, the benefit of MMC in delaying DNA double-strand break repair up to 24 hours after irradiation compared to RT treatment alone, further establishes MMC as a potent radiosensitizer (Figure 3). The activity profile of MMC on targeting hypoxia and prolonging DNA damage repair may be the clue to the putative mechanism of radiosensitization in CT26 colorectal adenocarcinoma cells.

Recent finding revealed DNA damaging agents such as ionizing radiation can induce type I interferon and other cytokine expression (23). The cGAS-cGAMP-STING pathway is the gateway that connects DNA damage and innate immunity, however, STING signaling is found to be suppressed in colorectal carcinoma (24,25). We found that MMC plus RT further upregulated *Ifnb1* mRNA expression compared to RT alone, suggesting possible enhanced activation of cGAS-cGAMP-STING pathway, which may have added effect in anti-tumor immunity (Figure 4). The outcome of anti-tumor response depends on the equilibrium variety of different immune cells, immune factors, and signaling molecules within the TME (10). Therefore, other than being radiosensitizer acting on local hypoxic tumor sites, the immune modulating activity of MMC and RT in the TME of rectal cancer prompted further interest for investigation. Our findings showed that RT promoted intratumoral DCs, which was consistent with previous studies on enhanced anti-tumor immunity of radiation inducing DCs (Figures 6,7) (26,27). The addition of MMC did not augment DC immune response, rather, MMC had more effect on increasing M1 macrophages and total T cells (Figures 6,7). This implicated that although the effector cell ontogeny modulated by MMC might not be DCs, both ionizing radiation and MMC enhanced antigen presenting cells of different phenotypes. In comparison to either RT or MMC treatment as a single modality, the effect in the combination of MMC and fractionated RT is more appealing. Combined treatment markedly recruited CTL and NK cells into tumor and reduced infiltration of Treg, indicating a

favorable immunomodulating effect (Figures 6,7). Part of the effect might be due to ionizing radiation inducing stronger proliferation of effector T cells rather than Treg as discovered by Persa *et al.* (28).

The density in infiltration of lymphocytes and presence of both NK and CD8 cells in the CRC microenvironment represents a favorable prognostic impact that is associated with better overall survival in CRC (29,30). This is due to the enhanced expression of NKG2D ligands, which promotes antitumor response as a result of DNA damage (31,32). In addition, Treg depletion is known to increase NK cell functions and proliferation (33). The reduction of Treg by MMC and RT in our study may render the increase in NKG2D<sup>+</sup> NK cells responsible for synergizing activation of anti-tumoral immunity.

Intriguingly, the generation of active alkylating metabolites of MMC in hypoxic niche within tumor to modulate TME is a unique finding (13). MMC in our study reversed the impairment of T cell infiltration in the hostile TME created by hypoxic stress (34). MMC has the advantages of targeting local intratumoral hypoxia and also escalating intercellular immune response signaling from a local to systemic level. Unlike other immunomodulators acting systemically, HAP such as MMC modifies biological response locally and generates *in situ* immunomodulation in the target tumor. This may provide a novel strategy to develop potent immune booster without interference of pharmacokinetics and pharmacodynamics.

Modulation of TME toward favorable immune-cell lineage commitment with combination treatment of MMC and RT indicate beneficial anti-tumor response. We found that combinatory treatment enhanced PD-L1 expression in tumor (Figure 9). Since PD-L1 on tumor cells or immune host cells are involved in immune evasion and preventing T cell-mediated killing, this implicates use of immune checkpoint blockade such as anti-PD-L1 for beneficial outcome (35,36). Noguchi *et al.* demonstrated PD-L1 on tumor cells can be upregulated when stimulated by interferons, yet, the major contributor of PD-L1 expression is from TAMs on immune host cells (37). Furthermore, TAMs with upregulated PD-1 and PD-L1 expresses M2 polarization which facilitates immune escape (11,12). Our results show that MMC has the added benefit of polarizing TAMs towards M1 anti-tumoral immunity (Figures 6,7). One can speculate that with increase in infiltration of immune cells and PD-L1 expression on tumor after treatment via MMC plus RT, the combination of immune checkpoint blockade renders an attractive modality to

enhance tumor recognition and cancer cell elimination in the inflamed niche.

While it is already known that RT can induce immune cell infiltration, our study demonstrated the addition of MMC augmented anti-tumor immunity to an even greater extent, certifying MMC as an effective radiosensitizer. Low and high dose irradiation act on different pathways in DCs, yet the optimal radiation dose is unexplored in the current study (28). Limitation remains in determining target effector cells and further investigation is required into PD-1/PD-L1 blockade. The causal relationship between cGAS-STING-Interferon type I signaling pathway and the stimulated inflammatory cytokine expression needs further clarification. Upregulated interferon plays important role in regulating immune cell infiltration, as evident with the alteration in TME after treatment. Whether this immunomodulatory effect correlates to increase immune cell infiltration and decreased tumor evasion remains to be elucidated. Further investigation is necessary in identifying biomarkers for hypoxia since it is present in greater than 50% but not all CRC (8). Hypoxic biomarkers are important to determining patient subgroups expected to benefit most from treatment with HAP and RT. Frey *et al.* discovered that antitumor immune responses are timely restricted suggesting variable days of high and low immune cell infiltration (27). Thus, the chronological order of drug and RT administration for synergistic effect may be further studied during the design of multimodal radioimmunotherapies. Our preclinical data on combined treatment of MMC and RT and its safe use with attraction of immune cells into CRC tumors and enhancement of PD-L1 expression in the TME paves the path for future clinical protocols. The translational aspect is valuable for the optimization of MMC and RT with further design of multimodality treatment to include immunotherapy for the improvement in clinical treatment outcomes of CRC.

## Conclusions

Our results implicate the combination of MMC and conventional fractionated RT may have synergistic therapeutic effect accompanied by modulation of TME toward favorable immune-cell lineage commitment. The correlation between impaired DNA repair, upregulated *Ifnb1* mRNA expression, and TME alteration requires additional clarification. Further optimization for combination of MMC and conventional fractionated RT is warranted before translating to clinical research.

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## Footnote

*Conflicts of Interest:* YJC serves as an Editors-in-Chief of *Therapeutic Radiology and Oncology*. The other authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental protocols involving animals were reviewed and approved by the Experimental Animal Committee of MacKay Memorial Hospital (approval number: MMH-A-S-106-01).

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