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Vaccination with mitoxantrone-treated primary colon cancer cells enhances tumor-infiltrating lymphocytes and clinical responses in colorectal liver metastases

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ABSTRACT

Background: Colorectal cancer remains a leading cause of cancer-related mortality worldwide. Metastases to the liver are often present at initial presentation and will form in most patients during their course of disease. We have previously demonstrated that enhanced trafficking and activation of tumor-infiltrating lymphocytes in colorectal liver metastases (CRLM) may improve antitumor immune responses. Thus, development of novel mechanisms to increase lymphocyte infiltration and activation are needed to improve patient outcomes.

Methods: CT26 murine colorectal cancer cells were treated with physiologic levels of the potent inducer of immunogenic cell death mitoxantrone (MTX). An *in situ* vaccine was created with treated cells in an established model of CRLM. Cells were evaluated by flow cytometry for cell cycle evaluation and calreticulin expression. Splenic and tumor-infiltrating lymphocytes were isolated for phenotypic studies.

Results: MTX-treatment of colon cancer cells resulted in a sub-G1 peak, inhibition of G1 cell cycle progression, and increased G2/M cell fractions while simultaneously increasing dynamic exposure of calreticulin on the cell surface ($P < 0.05$). Vaccination with MTX-treated cells resulted in significant decreases in CRLM formation associated with increased tumor-infiltrating leukocytes that displayed increased expression of the T cell surface activation marker CD69.

Conclusions: Vaccination with MTX-treated primary colon cancer cells enhances tumor-infiltrating lymphocytes and clinical responses in CRLM.

Introduction

Colorectal cancer remains one of the leading causes of cancer-related mortality worldwide. Colorectal liver metastases (CRLM) are often present at the time of diagnosis and remain the most common site of metastatic disease.[1] In selected patients, surgical resection of CRLM has improved long-term survival, but unfortunately only 10%-20% of patients are

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surgical candidates. Most stage IV patients with CRLM will be managed with palliative intent utilizing multidrug chemotherapy regimens. However, systemic chemotherapy has limited efficacy with many treatment-related toxicities.[2] New therapeutic strategies are focusing on enhancing the antitumor immune response by attempting to drive increased numbers of tumor-infiltrating lymphocytes into the tumor microenvironment. Although this strategy has increased response rates in microsatellite instable colon cancers, which represent less than 5% of colon cancers, to date current immunotherapeutic strategies have been unsuccessful in achieving this goal for most colon cancers.[3-6]

Previous studies have documented that the presence of activated and proliferating T cells within primary colorectal tumors is associated with improved survival.[7,8] In addition, we have previously demonstrated an association between increased T cell infiltrates and improved outcomes in patients with CRLM.[9,10] Thus, enhancing the antitumor immune response may play a viable role in treating patients with advanced gastrointestinal malignancies, including colon cancer and CRLM.

Although chemotherapy is traditionally linked to its immunosuppressive effects in treating cancer patients, emerging studies have demonstrated that selected chemotherapeutics may enhance tumor immunogenicity.[11] Chemotherapy-induced immunogenic cell death (ICD) is characterized by the release and/or increased expression of defined damage-associated molecular patterns, including calreticulin (CRT),[12-14] and we have previously demonstrated that induction of ICD in colon cancer cells can generate specific antitumor immunity in colorectal cancer.[15] Therefore, chemotherapy agents not traditionally used in colon cancer but that may induce ICD could have potential for enhancing antitumor immunity in colon cancer patients.

Mitoxantrone (MTX) is a well-established anthracenedione antineoplastic agent used in the treatment of multiple sclerosis, lymphoma, leukemia, hormone-refractory prostate cancer, and advanced stage breast cancer.[16,17] MTX has recently been demonstrated to induce ICD and may elicit a tumor-specific immune response in murine colon cancer models.[14,18-20] Therefore, the aim of the present study is to investigate whether MTX-treated murine colon cancer cells can be used as a tumor vaccine to increase tumor-infiltrating lymphocytes and thus impact the growth of CRLM.

Materials and methods

Cell culture

The murine colorectal carcinoma cell line, CT26, was obtained from the American Type Culture Collection (ATCC, Manassas, VA), tested for mycoplasma, and utilized at low passage numbers. Cells were cultivated with RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 1% penicillin/streptomycin in 37°C incubator containing 5% CO2. All cell culture reagents were purchased from Invitrogen (Grand Island, NY) and cells were passaged at 1 to 10 dilutions on confluence.

Cell cycle analysis and CRT expression

CT26 cells were seeded in a 6-well plate at 3×10^5 cells per well and treated with 1 μM MTX (Cayman Chemical Company, Ann Arbor, MI) or 1:1000 DMSO as vehicle control for 24 h. Cells were harvested with 0.05% trypsin and fixed with 80% ethanol on ice for 30 min and then incubated with 1 mL of DNA staining solution (1 μg/mL DAPI, 0.1% NP-40 in phosphate buffered saline [PBS], all from Sigma Aldrich, St. Louis, MO) at room temperature for 30 min followed by flow cytometry analysis.

To detect cell surface expression of CRT, cells were suspended in 100 μL PBS containing 3% FBS without fixation and incubated with fluorochrome-conjugated rabbit anti-CRT antibody (1:250, from Abcam, Cambridge, MA) at 4°C for 30 min. The cells were washed and incubated subsequently with a 1:500 dilution of Fluro-488 labeled goat anti-rabbit IgG (Thermo Scientific, Waltham, MA) for 30 min. The stained cells were analyzed using a CyAn ADP analyzer (Beckman Coulter, Brea, CA). Events were collected and analyzed using FlowJo software (Tree Star Incorporated, Ashland, OR).

In vivo studies

Female, 6- to 12-wk-old CT26 cell Balb/c mice, weighing 18-20g, were obtained from Charles River Labs (Wilmington, MA). All mouse experiments were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago and performed in the animal facility as per the approved protocol. Five animals were utilized per group based on a biological difference in tumor formation of at least 30% at an alpha of 0.05 and power of 80%.

Vaccination with MTX-treated colon cancer cells and generation of CRLM

CT26 cells were treated with 1 μM of MTX or DMSO (1:1000) as vehicle control for 24 h. The cells were then washed and resuspended in PBS. Mice were randomized to separate cages assigned to receive MTX-treated or control cells subcutaneously into the right flank (1×10^6 in 100 μL PBS) on days 0 and 7. On day 14, each mouse underwent intrasplenic wild-type CT26 cell injection to form reliable CRLM as we have previously reported in this well-established model.[21,22] In this model, 1×10^6 CT26 cells are introduced into an isolated and divided hemispleen that is excised after injection, leaving half of the untreated spleen for immunologic studies.

Preparation of spleen mononuclear cells and tumor single-cell suspension

Mice were euthanized on day 28. Spleen tissue was placed into a cell strainer and gently homogenized using a syringe plunger. Cells were pelleted and red blood cells lysed with ACK lysis buffer.

After being weighed and photographed, liver tumors were manually dissected and minced, and incubated in 10 mL RPMI containing 5% FBS, collagenase IV (1 mg/mL, Sigma), with DNase I (50 μg/mL, Sigma) at 37°C for 30 min, and strained to obtain a single-cell suspension.

Immune cell phenotyping

Isolated cells from murine spleen and CRLM were incubated with anti-CD3 APC, anti-CD4 PE, anti-CD8 PE-cy7, anti-CD69 PE-cy5, anti-DX5 PE-cy7 (Natural Killer [NK] cell marker), and anti-CD45 FITC. Single-cell suspension was analyzed with a CyAn ADP analyzer (Beckman Coulter, Brea, CA). Events were analyzed using FlowJo software (Tree Star Incorporated, Ashland, OR).

Statistical analysis

Data are presented as mean and standard error of the mean. Differences between groups were calculated using the two-tailed unpaired *t*-test. Significance was considered for *P*-values <0.05.

Results

MTX treatment of colon cancer cells inhibits cell cycle progression in vitro

Cell cycle progression of CT26 colon cancer cells treated with 1 μM of MTX for 24 h were compared with vehicle-treated cells. MTX treatment resulted in a sub-G1 peak consistent with induction of apoptosis. Treated cells also were characterized by growth arrest characterized by inhibition of G1 cell cycle progression and increased G2/M cell fractions (Fig. 1A).

MTX induces cells surface expression of CRT

CRT translocation to cell surface has been well documented as a hallmark of ICD.[20] MTX treatment caused a significant increase in dynamic exposure of CRT on the cell surface in treated cells compared with control treated cells (Fig. 1B).

In vivo vaccination with MTX-treated colon cancer cells inhibits liver metastatic tumor growth

MTX-treated or control CT26 cells were injected subcutaneously into the flank of mice followed by establishment of CRLM. Aggressive metastases encompassing more than 50% of the liver formed in 100% of control animals. CRLM tumor burden was markedly decreased in animals vaccinated with MTX-treated as opposed to wtCT26 cells (1.8 g *versus* 4.6 g, $P < 0.05$) (Fig. 2)

Vaccination with MTX-treated CT26 cells increases splenic T cells

By immunofluorescence staining and flow cytometry analysis, a much higher fraction of total CD3 cells in spleens from MTX-treated CT26 tumor cell vaccinated mice were identified when compared with that from control mice (7.9% *versus* 20.3%, $P = 0.01$). Both CD4+ (5.9% *versus* 13.9%, $P = 0.01$) and CD8+ (2.4% *versus* 6.6%, $P = 0.02$) T cell populations were similarly increased. NK cell populations were similar (3.2% *versus* 6.4%,

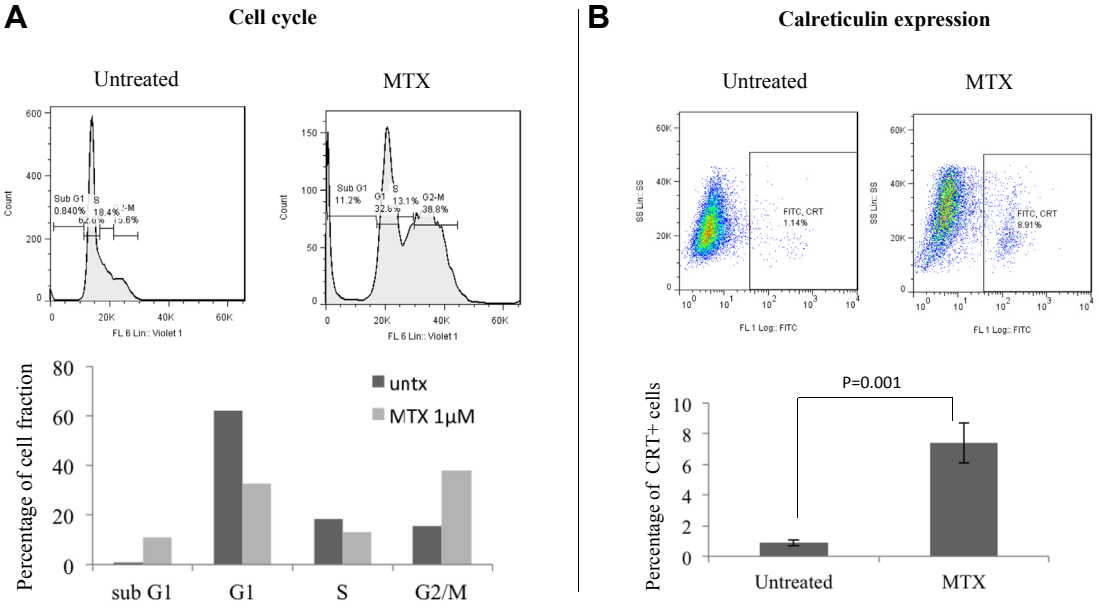

Fig. 1 – MTX induces G2/M growth arrest and CRT expression on colon cancer cells. CT26 cells were treated *in vitro* with physiologic concentrations of MTX (1 μM) for 24 h and stained with either DAPI or anti-CRT followed by flow cytometry analysis. (A) Analysis illustrates the cell cycle profile and the ratio of cells in stages of the cell cycle. With MTX treatment, the fraction of cells in the G2-M phase increased from 15% to 38%, and the fraction of cells in the G1 phase decreased correspondingly from 62% to 33%, consistent with cell cycle growth arrest. The fraction of cells in the sub-G1 phase increased from 1% to 11%, consistent with DNA degradation and apoptosis ($n = 1$). (B) A representative flow cytometry profile (upper panel) reveals enhanced CRT expression with MTX treatment. Summary of the ratio of CRT+ cells based on three independent assays (lower panel). (Color version of figure is available online.)

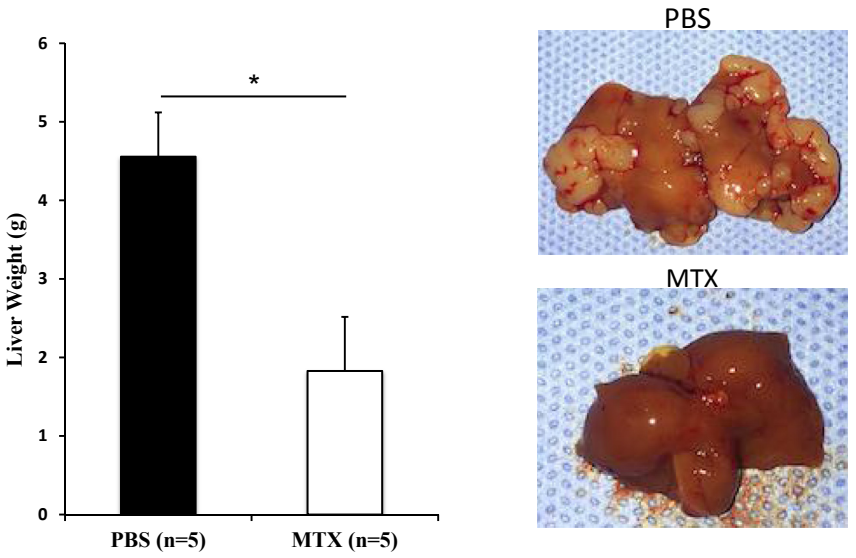

Fig. 2 – Vaccination with MTX-treated CT26 cells inhibits tumor growth of CRLM. CRLM were significantly decreased in animals vaccinated with MTX-treated CT26 cells compared with control treated cells resuspended in PBS. Liver weights reflected large differences in tumor burden (left panel) that were evident on gross examination (representative explanted livers, right panel). *P < 0.05. (Color version of figure is available online.)

$P = 0.11$) in MTX-treated tumor cell vaccinated mice relative to that of control vaccinated mice (Fig. 3).

Vaccination with MTX-treated CT26 cells increases intratumoral lymphocyte infiltration and T cell activation in metastatic tumors

Evaluation of isolated CRLM revealed that the total leukocyte fraction (CD45 + cells) was significantly increased in MTX-treated colon cancer cell vaccinated mice than in control vaccinated mice (11.4% *versus* 38.9%, $P < 0.05$) (Fig. 4A). CD3+ cells in the MTX-treated CT26 cell group were 16.8% compared with 23.6% in the non-MTX–treated vaccination group ($P = 0.09$) and demonstrated an increase in the T cell activation marker CD69 (5.8% *versus* 9.6%, $P = 0.002$). The NK population was significantly increased in the MTX-treated vaccine group (2.5% *versus* 16.4%, $P < 0.05$) (Fig. 4B).

Discussion

It is well established that increased infiltration and activation of lymphocytes into primary and metastatic colon cancer is associated with improved patient outcomes, and we have previously demonstrated that strategies to increase tumor-infiltrating lymphocytes in the tumor microenvironment can result in antitumor immune responses and tumor regression of CRLM.[10,21,23-26] Currently available checkpoint blockade immunotherapies that have generated antitumor immune responses in many tumor histologies have had limited efficacy in most gastrointestinal tumors and specifically on CRLM.[4,6] Therefore, new strategies to enhance lymphocyte proliferation and activation in this disease are needed. One approach to incite an antitumor immune response in colon cancer is through generation of ICD.[15] We show herein that treatment of murine colorectal cancer cells with MTX generates cell cycle arrest accompanied by dynamic increases in cell surface expression of CRT, consistent with prior reports of ICD in this tumor histology. Moreover, vaccination of animals with MTX-treated colon cancer cells, as compared with vaccination with wild-type tumor cells, resulted in marked decrease in CRLM growth and significant increases in tumor-infiltrating lymphocytes.

The cell cycle of CT26 cells was blocked at the G2/M phase, consistent with previous studies and most likely secondary MTX-induced DNA damage.[27,28] Furthermore, previous reports have demonstrated that MTX treatment can induce multiple hallmarks of ICD including CRT translocation, ATP secretion and HMGB1 release,[19,20,29] and we also detected increased expression of CRT at the cell surface after MTX treatment, confirming that the tumor cells treated with MTX have the potential of enhancing antitumor immunity.

The murine colon cancer liver metastasis model is well established in our lab and is considered a reproducible and clinically relevant metastatic tumor model that mimics many aspects of the human course of disease.[21,22,30,31] We found that all control mice harbored a large tumor burden of isolated CRLM, whereas there was significantly less tumor in the experimental group. We also sought to link the tumor inhibition with the host's immune function. Based on the finding of increased numbers of T cells in the spleen of MTX-treated vaccinated mice, we postulate that the vaccination may enhance the systemic immune response specifically against colon cancer tumor cells, although additional coculture and cytotoxicity assays will be necessary to definitively make this determination. In this model, vaccination occurs 2 wk before metastatic tumor initiation. This functioned to elicit an adaptive immune response before implantation of metastases, and also to insure that any identified antitumor immune response was not

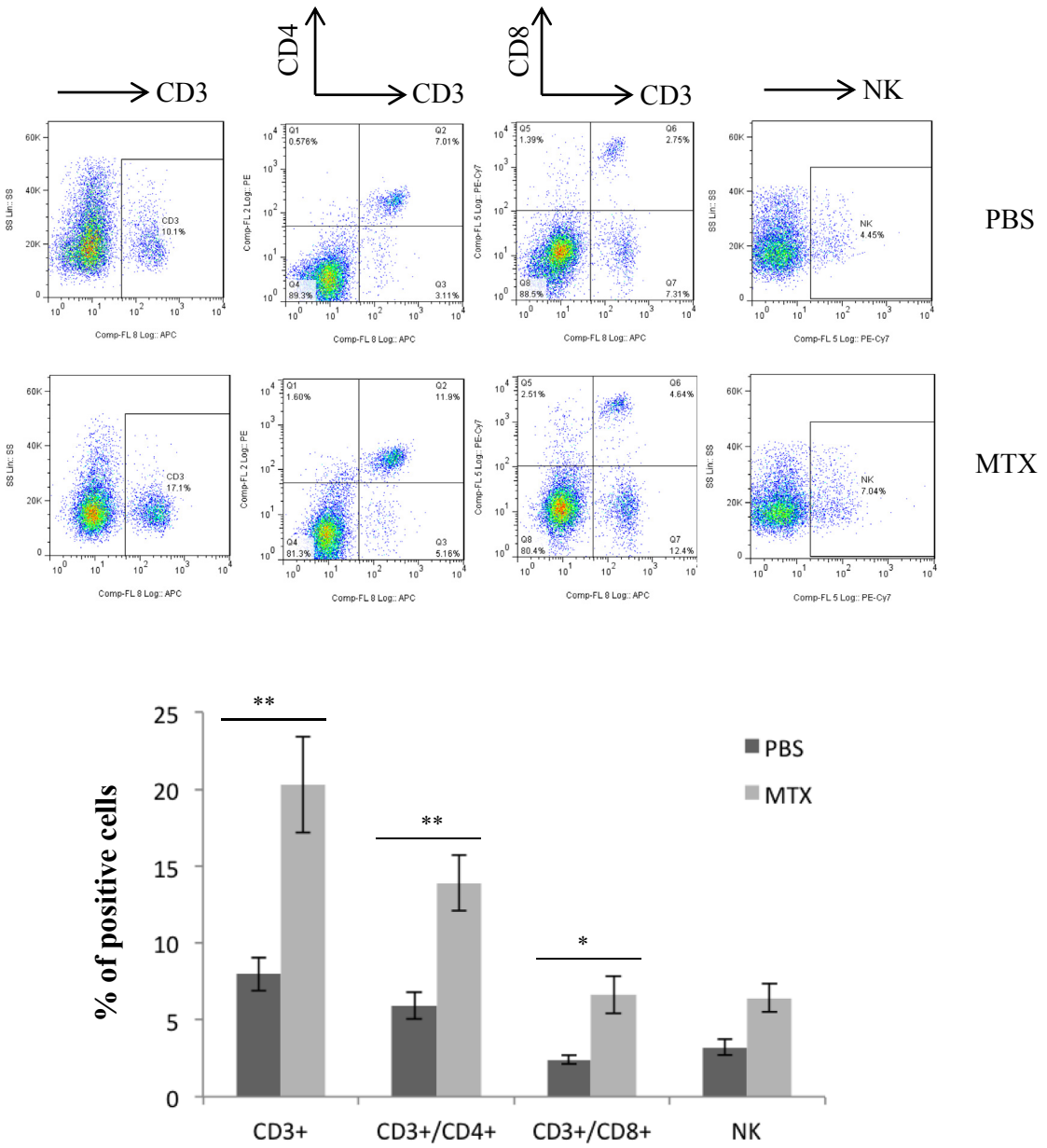

Fig. 3 – Characterization of splenic leukocytes. Mouse spleen cells were isolated from animals after vaccination and establishment of CRLM, labeled with fluorescence conjugated antibodies, and subjected to flow cytometry analysis. Upper panel: Representative flow cytometry profiles of splenic T cell and NK cell subsets. Lower panel: Total CD3+, CD4+, and CD8+ T cells were increased in spleens from MTX-treated cell vaccinations ($n = 5$ per group, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$). (Color version of figure is available online.)

overwhelmed by excessive tumor burden before antitumor immunity could be established.

The liver is generally considered to have a unique environment dominated by immune suppressive function,[32,33] which may favor tumor growth *in situ*. Interactions between the host environment and the tumor determine tumor cell behavior, levels of growth factors and nutrients, and tumor angiogenesis.[34] Our data revealed increased tumor-infiltrating leukocytes within the CRLM in response to vaccination only with MTX-treated colon cancer cells. Furthermore, the lymphocytes were found to be activated. Thus, there was an association between increased lymphocyte trafficking to the tumor and clinical response. It is also worth noting that NK cell populations increased in tumor tissues, thus MTX-induced cell death may generate not only an adaptive immune response, but also an innate immune response in this murine metastatic colon cancer model.[35,36]

MTX was initially approved to treat acute myeloid leukemia,[37] and it has been also used to treat a limited number of cancer types including breast cancer and prostate cancer.[38,39] Concerns over its possible oncogenic effect in multiple sclerosis have been raised, however, the current results, along with others,[19,20,29] highlight the potential application of MTX in colon cancer treatment. To our knowledge, this is the first

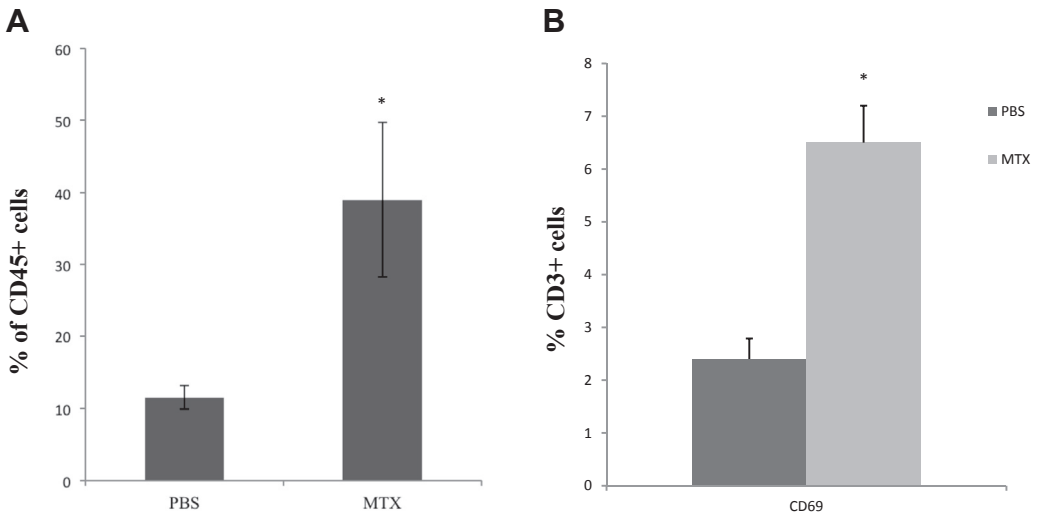

Fig. 4 – Evaluation of tumor-infiltrating leukocytes in CRLM. Tumor cells were isolated from resected livers and evaluated by flow cytometry analysis. (A) The total number of tumor-infiltrating leukocytes (CD45+) in CRLM was increased in animals that received a vaccine consisting of MTX-treated CT26 cells. (B) Although the total number of infiltrating CD3+ cells was not different between the groups, there was a significant increase in infiltrating CD3+ CD69+ (activated T cells) in the MTX-treated vaccine animals ($n = 5$ per group, $^*P \leq 0.05$).

report to examine the immune-stimulatory effects of MTX-treated CT26 cells in CRLM. The concept of stimulating ICD as a strategy to increase antitumor immune responses have led to MTX, along with other ICD-inducing chemotherapeutics, to be studied in multiple phase I and II clinical trials.[40]

The current experiments serve as a proof of principle that MTX-induced cell death in colon cancer primaries may prime the patient with a protective immune response against systemic disease. This is reflected both in an increase in the splenic lymphocyte population and in the tumor microenvironment. Certainly, additional studies will need to be performed to further evaluate the extent of ICD generated with this strategy, the exact phenotype of the tumor-infiltrating lymphocytes, and the role of regulatory cells and suppressive influences in the microenvironment, circulation, and bone marrow. A potential strategy to mimic these findings in a clinical relevant fashion may involve direct-tumor injection of MTX as an *in vivo* vaccination. Clearly, further study and validation of the current findings in additional human cell lines and colon tumors will be required.

There are additional limitations to the study. A potential disadvantage of the vaccination strategy is generation of a nonspecific immune response. ICD effects are measured through surrogate biomarker responses[14,41,42] or through evaluation of lymphocyte surface markers and functional assays[12,43,44] for antitumor effects. These approaches are not completely predictive, nor do they provide an *in vivo* assessment of a tumor-specific immune response.[41,42] For this reason, we evaluated tumor burden as a clinically relevant endpoint, although certainly additional studies with syngeneic but irrelevant tumors as additional negative control groups are warranted. Furthermore, other colon cancer cell lines will be useful to test in this model, although they would need to be in another strain or species as the only established syngeneic Balb/c colon cancer cell line is CT26 and the impact of MTX treatment on antitumor immunity requires an immunocompetent model.[45,46] Furthermore, the splenic injection model is a reproducible, efficient, and consistent model that is clinically relevant to determine the antitumor immune responses in a syngeneic murine model, although it is limited in that it does not represent the entire metastatic process. Thus, it is possible that neoantigens could be exposed, or that additional mechanisms of immunoediting occur during the process of spontaneous metastases. These would need to be studied in longitudinal spontaneous metastatic models or clinical trials. In addition, this model was established in female Balb/c mice, and thus gender effects were not determined in this study.

Despite these limitations, it is provocative that an MTX-treated colon cancer cell vaccine was associated with increased immune cell proliferation in the spleen and T cell activation within CRLM leading to significant decreases in metastatic tumor burden, warranting further evaluation in a disease with limited immunotherapy treatment options.

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Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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