Combining Oncolytic HSV-1 with Immunogenic Cell Death-Inducing Drug Mitoxantrone Breaks Cancer Immune Tolerance and Improves Therapeutic Efficacy

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Abstract

Although antitumor activity of herpes simplex virus 1 (HSV-1) ICP0 null oncolytic vectors has been validated in murine breast cancer models, oncolytic virus treatment alone is insufficient to break immune tolerance. Thus, we investigated enhancing efficacy through combination therapy with the immunogenic cell death–inducing chemotherapeutic drug, mitoxantrone. Despite a lack of enhanced cytotoxicity in vivo, HSV-1 ICP0 null oncolytic virus KM100 with 5 μmol/L mitoxantrone provided significant survival benefit to BALB/c mice bearing Her2/neu TUBO-derived tumors. This protection was mediated by increased intratumoral infiltration of neutrophils and tumor antigen-specific CD8+ T cells. Depletion studies verified that CD8+, CD4+, and Ly6G-expressing cells are essential for enhanced efficacy of the combination therapy. Moreover, the addition of mitoxantrone to KM100 oncolytic virus treatment broke immune tolerance in BALB-neuT mice bearing TUBO-derived tumors. This study suggests that oncolytic viruses in combination with immunogenic cell death–inducing chemotherapeutics enhance the immunogenicity of the tumor-associated antigens, breaking immunologic tolerance established toward these antigens. Cancer Immunol Res; 1(5); 309–19. ©2013 AACR.

Introduction

Oncolytic viruses are therapeutically useful viruses that selectively infect and kill cancerous tissues without causing harm to normal tissues (1). To target herpes simplex virus 1 (HSV-1) replication to tumor cells, viral proteins that are essential for virus replication in normal cells but dispensable in tumor cells are inactivated or deleted (2). HSV-1–based oncolytic viruses have several features that make them attractive oncolytic viruses. G207 was the first oncolytic HSV-1 evaluated in clinical trials to treat patients with glioma in the United States (3). It was previously shown that HSV-1 vectors harboring mutations in the immediate early gene ICP0 (infected cell protein 0), which encodes a protein responsible for overcoming aspects of the host IFN response (4, 5), function as oncolytic vectors, particularly in cancer cells with a decreased capacity to respond to IFN (6). Initial studies in various murine cancer models showed that the antitumor activity of the HSV-1 ICP0 null oncolytic virus KM100 correlates with the immunogenicity of the tumor model used (6,7).

The use of chemotherapeutics has been shown to improve the therapeutic efficacy of oncolytic viruses (8–10). Often, new combination treatments are based on viral sensitizers that enhance virus replication in tumor cell lines (11, 12) or the use of chemotherapeutics such as cyclophosphamide that dampen the antiviral immune response and prolong virus replication within the tumor (13). Here, we investigated an alternative approach using a chemotherapeutic drug that exerts the dual effect of causing cytotoxicity and enhancing the immunogenicity of dying tumor cells. It is now appreciated that the immunosuppressive tumor milieu of suppressive cytokines and immune inhibitory cells exhausts effector T cells, leading to establishment of peripheral tolerance to tumor-associated antigens (TAA; refs. 14-16). Chemotherapeutic agents inducing immunogenic cancer cell death, such as mitoxantrone (MTX) elicit potent anticancer immune responses (17–19).

Both in murine models (17, 18) and in human patients with cancer (20), antitumor immune responses induced by cancer cells undergoing immunogenic cell death are associated with better clinical response. Although oncolytic virus replication within the tumor can be immunogenic (21, 22), this study investigated the effect of combining MTX with oncolytic KM100 to enhance the antitumor immune response. In vitro, the combination of KM100 with MTX failed to increase virus replication, cytotoxicity, or cell death beyond single treatment alone. In vivo, however, the combination of KM100 with MTX provided significant survival benefit when administered locally to nontolerized HER-2/neu...
subcutaneous tumors. Furthermore, we found that the addition of MTX modified the nature and kinetics of immune cell infiltration into the tumor following oncolytic virus treatment. Extension of the study in a tolerized TAA model showed that the combination treatment can break immunologic tolerance and that CD8+ T cells are critical mediators of this response.

Materials and Methods

Cell culture

Human osteosarcoma cells (U2OS; American Type Culture Collection) were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS. TUBO is a cloned cell line generated from a spontaneous mammary gland tumor from a BALB-neuT mouse and highly expresses HER-2 protein on the cell membrane (23). Growth of TUBO cells in BALB/c mice and generation of HER-2-specific CD8+ T cells validate the origin of these cells. TUBO cells were maintained using DMEM with 10% FBS. All media contained 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). All cell lines were grown at 37°C under humidified conditions.

Viruses

KM100gfp (ICP0212 VP161814) is an HSV-1 recombinant that fails to express the ICP0 protein and expresses a mutant version of VP16 lacking the C-terminal transactivation domain (24). KM100 was propagated and titered on U2OS cells in the presence of 3 mmol/L hexamethylene bisacetamide (Sigma). KM100 was purified and concentrated via sucrose cushion ultracentrifugation.

Cell culture treatments

TUBO cells were plated in 12-well plates at a density of 1 × 105 cells/mL. The next day cells were either mock- or KM100-infected at a multiplicity of infection (MOI) of 10 plaque-forming units (pfu) per cell. After 1 hour of infection, medium containing different amounts of MTX (Sigma) was added back. Viral titer samples were harvested 2 days after infection.

HMGB1 ELISA

The supernatants of treated TUBO cells were drawn from each well of a 12-well plate. Cells and debris were pelleted by centrifugation at 2,500 rpm for 10 minutes, and the supernatant was collected. Samples were immediately frozen at −80°C. Samples were used to detect the level of HMGB1 using an ELISA kit developed by Shino Test Corporation (IBL International, GmbH). ELISA was conducted following the manufacturer’s protocol outlined for normal sensitivity format of the assay.

Cell metabolism and membrane integrity

TUBO cells were plated in 96-well plates at a density of 1 × 105 cells/mL. The next day cells were either mock- or KM100-infected at an MOI of 10. After 1 hour of incubation, maintenance medium containing 0, 1, 2, and 5 μmol/L MTX was added back. Cell metabolism and membrane integrity were assessed using AlamarBlue and 5-carboxyfluorescein diacetate acetoxyethyl ester (CFDA-AM), respectively (Invitrogen). Cells were incubated simultaneously with AlamarBlue (5% v/v) and CFDA-AM (4 μmol/L) for 30 minutes at 37°C, after which fluorescence was read using a Safire fluorescence plate reader. Data were analyzed relative to uninfected controls and corrected for background fluorescence.

Western blotting

Whole-cell extracts were prepared by scraping cells and pelleting at 14,000 rpm for 15 minutes. Cell pellets were resuspended in radioimmunoprecipitation assay buffer and lysed for 20 minutes at 4°C. Whole-cell lysates were clarified by centrifugation at 13,000 × g for 10 minutes at 4°C. Protein quantification was carried out using the Bradford Assay Kit (Bio-Rad Laboratories). Twenty micrograms of protein samples were loaded onto 15% SDS-PAGE gels, resolved, and transferred onto polyvinylidene difluoride membranes (Millipore) at 100 V for 1 hour. After 1 hour of blocking at room temperature, membranes were kept in 5% skimmed milk in TBS with rabbit anti-mouse activated caspase-3 (Cell Signaling Technology), rabbit anti-mouse LC3-B (Cell Signaling Technology), or goat anti-β-actin (Santa Cruz Biotechnology) at 4°C overnight. The next day, the blots were incubated for 1 hour with 5% skimmed milk in TBS–Tween20 (0.1%; TBST) containing either horseradish peroxidase (HRP)–conjugated rabbit immunoglobulin G (IgG; Cell Signaling Technology) or goat anti-human actin (Santa Cruz Biotechnology) primary antibodies at 1:1,000 dilution for 1 hour at room temperature, followed by incubation with the appropriate HRP-conjugated secondary antibodies for 1 hour and visualization by chemiluminescence.

KM100 and MTX combinatorial treatment in mice

Mice were maintained at the McMaster University Central Animal Facility, and all procedures were carried out in full compliance with the Canadian Council on Animal Care and approved by the Animal Research Ethics Board of McMaster University (Hamilton, Ontario, Canada). The BALB-neuT breeding colony was a generous gift from Dr. Jay Berzofsky (National Cancer Institute). Six- to 7-week-old BALB/c (Charles River Laboratories) and transgenic BALB-neuT mice were used for subcutaneous injection of 5 × 105 and 1 × 106 TUBO cells, respectively. To minimize experimental variability, low-passaged TUBO cells were used for subcutaneous injections. Palpable tumors formed routinely 12 to 14 days after injection. Tumors were treated by local administration of three 50-μL doses of either 2 × 107 total pfu KM100 (7), 1 (1.3 mg/kg) to 5 μmol/L MTX (6.5 mg/kg; ref. 17), or the combination of both every 36 hours. Tumors were measured every 3 days, and fold changes of the tumor volumes were calculated relative to the tumor volume at the start of treatment (7). Mice having tumors of 10 mm in length and width (volume of 525 mm3) were classified as the endpoint.
Isolation of tumor-infiltrating immune cells

Tumor-infiltrating immune cells were isolated as previously described (25). Briefly, tumors were digested in a mixture of 0.5 mg/mL collagenase type I (Gibco), 0.2 mg/mL DNase (Roche), and 0.02 mg/mL hyaluronidase (Sigma) prepared in Hank’s buffered saline (10 mL/250 mg of tumor). The digested material was passed successively through 70- and 40-μm nylon cell strainers and lymphocytes were purified using mouse CD45.2-positive selection by magnetic separation (EasySep; STEMCELL Technologies, Inc.). The purified cells were split into two samples. One sample was used for direct surface staining with different surface markers, and the other was used for peptide restimulation to quantify the HER-2–specific CD8+ T cells. Samples were stained using the near IR fluorescent reactive dye to gate viable cells. CD8+ T cells were restimulated by incubation for 4 hours (in the presence of brefeldin A; BD GolgiPlug) with the LELTVYPANSLFL peptide, corresponding to the BALB/c immunodominant epitope of rat HER-2 (HER-2 aa 63–77, 1 μg/mL), and stained for intracellular IFN-γ as described by Sobol and colleagues (7). Data were acquired using a FACSCanto flow cytometer (BD Biosciences) and analyzed with FlowJo (TreeStar).

Antibodies

The following monoclonal antibodies (mAb) were used in flow cytometry assays: anti-CD16/CD32 (clone 2.4G2) to block FcRs, anti-CD3 (clone 17A3), anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-Gr1 (RB6-8C5), anti-CD11b (M1/70), anti-F4/80 (BM8), anti-CD11c (HL3) for detecting cell surface markers and anti-IFN-γ (clone XMG1.2; all from BD Biosciences) for intracellular staining. Immunodepletion studies were conducted with the mAbs anti-CD4 (GK1.5), anti-CD8β (clone 53.5.8), Ly6G (clone 1A8; antineutrophil; University of California, San Francisco, San Francisco, CA), and rat isotype IgG (University of California, San Francisco). Purified mAbs (250 μg in 500 μL saline) were injected intraperitoneally 3 days apart during the treatment period and then once a week thereafter at a maintenance dose of 200 μg antibody. The maintenance dose of Ly6G-depleting antibody was 200 μg/treatment administered twice a week. The efficiency of specific depletion of lymphocyte subsets was measured by flow cytometry.

Data analysis

For each statistical analysis used, normality of the distributions and variance assumptions were tested before running the statistical tests. For analyzing the statistical significance of the difference in Kaplan–Meier survival between treatments, the log-rank (Mantel–Cox) test was used. The Kruskal–Wallis test was used to determine the statistical significance of the differences in tumor volumes and tumor-infiltrating immune cells. The null hypothesis was rejected for P values less than 0.05. All data analyses were conducted using GraphPad Prism.

Results

MTX does not enhance oncolytic virus–mediated cytotoxicity in vitro

To investigate whether immunogenic death–inducing drugs enhance oncolytic virus activity, we compared the therapeutic efficacy of KM100 + MTX with single treatments in vitro and in vivo. Our previous studies used a HER-2/neu TAA model within the FVB background, for which a continual cell line has yet to be established, precluding in vitro studies. For these studies, we used a cell line (TUBO) generated from a spontaneous mammary gland tumor from a BALB–neuT mouse that is transgenic for the rat neu oncogene expressed under the control of the mouse mammary tumor virus promoter (26). KM100 by itself does not show any significant cytotoxicity in TUBO cells in vitro (Fig. 1), consistent with the lack of an appreciable viral burst (Supplementary Fig. S1). Increasing doses of MTX alone, from 1 to 5 μmol/L, induced TUBO cell cytotoxicity, assessed by cytopathic effect (Fig. 1A), cell metabolism (Fig. 1B and C), and membrane integrity (data not shown). Combination of MTX with KM100 failed to increase cellular toxicity beyond treatment with MTX alone (Fig. 1) and did not increase KM100 replication (Supplementary Fig. S1).

MTX induces immunogenic apoptosis (17) and autophagic cell death (19). To investigate whether the combination of KM100 + MTX induces a different type of cell death compared with either MTX or KM100 treatment alone, protein samples harvested at different time points after treatment were assessed for apoptotic or autophagic cell death. Caspase-3, endogenously expressed as a 35 kDa protein, is cleaved by initiator caspases, forming activated proteins of 17 and 12 kDa. As evidenced in Fig. 2A, TUBO cells showed increased activated caspase-3 as well as additional morphologic and biochemical signs of apoptotic death (data not shown) upon treatment with the positive control staurosporine. TUBO cells treated with 5 μmol/L MTX showed hallmarks of apoptosis starting from 12 hours posttreatment and showed a maximum level of activated caspase-3 at 24 hours (Fig. 2C). KM100 treatment alone did not show any apoptotic cell death above mock treatment at any of the time points. To verify that TUBO cells show immunogenic cancer cell death in response to MTX, cells were treated and supernatant was collected after 24 hours of treatment. Mock treatment and KM100 did not show detectable levels of HMGB1, whereas both MTX and KM100 + MTX showed active release of HMGB1 in the supernatant (Fig. 2E). Similar to results depicted in Fig. 1, combination treatment mirrors the effect of MTX alone.

Cells undergoing autophagy show increased Atg8/LC3-II lipidation. Although LC3 has several homologs in mammals, LC3B is most commonly used for autophagy assays. LC3B is initially synthesized in an unprocessed form, prol.C3, which is converted into a processed form lacking amino acids from the C terminus, LC3B-I (16 kDa), and is finally modified into the phycoerythrin (PE)-conjugated form, LC3B-II (14 kDa). LC3B-II is the only protein marker that is reliably associated with completed autophagosomes. An increase in the ratio of LC3B-II to either actin or LC3B-I protein level is used as an indicator of increased initiation of autophagy (27). Bafilomycin, rapamycin, and serum starvation were used as positive controls in TUBO cells to measure autophagic cell death (Fig. 2B). Increased LC3B-II/LC3B-I ratios were observed 6 hours posttreatment with MTX. KM100 alone did not induce...
appreciable autophagy, and combination treatment did not enhance the level of autophagy beyond treatment with MTX alone.

Taken together, these in vitro data show that while MTX has cytotoxic activity on breast cancer cells, due in part to increased apoptosis and autophagy, this drug does not directly enhance virus replication or virus-mediated cellular toxicity.

**MTX significantly increases the survival benefit of oncolytic virus treatment in a nontolerized TAA model**

In the in vivo tumor context, enhanced or diminished activity of the immune system on the tumor microenvironment and angiogenesis, expression or repression of secreted factors (i.e., cytokines and chemokines) and toxicity to normal cells and organs contribute to the outcome of a given therapy (8). Although there was no additive or synergistic effect of combining MTX with KM100 in vitro, we hypothesized that by administering MTX along with KM100, we may improve the therapeutic efficacy of the oncolytic virus in subcutaneous TUBO tumor-bearing mice.

Preliminary dose escalation studies determined that 5 µmol/L MTX was the optimal dose in which efficacy was observed in the absence of toxicity (data not shown). Next, we compared the effect of administering KM100 and MTX simultaneously or at different time points and found that there was no difference between the two treatment regimens (data not shown). Thus, subsequent experiments used 1 or 5 µmol/L MTX delivered alone or simultaneously with KM100.

Figure 3 shows the percentage survival and relative fold change in tumor volumes of subcutaneous tumors treated with three consecutive doses of PBS, KM100 alone, MTX alone, or KM100 + MTX (5 µmol/L). Untreated control tumor-bearing mice had a median survival of 12 days, with all mice reaching endpoint within the study period. Treatment with KM100 or MTX alone led to 33% or 22% survival, respectively, with a median survival of 15 days. Only the KM100 + 5 µmol/L MTX–treated mice had statistically significant higher survival (69%) compared with control mice, using the log-rank (Mantel–Cox) test. Mice whose tumors on the left flank regressed were rechallenged on the right flank; however, no tumors formed on the right flank, suggesting that these mice had developed long-standing antitumor adaptive immune responses. Although the combination treatment with 1 µmol/L MTX + KM100 showed a trend of higher survival with lower changes in
tumor volume, there was no significant survival benefit compared with untreated control mice (Supplementary Fig. S2).

Combination therapy increases infiltration of tumor-specific CD8⁺ T cells within the tumor

Given that both KM100 (6, 7) and MTX (19) stimulate the adaptive immune response, we hypothesized that the combination treatment would lead to increased immune cell infiltration within the tumor. To test this hypothesis, the immune cell profile was evaluated after administration of single or combination treatments. Tumors were isolated at days 7 and 10 posttreatment, as differential growth patterns of tumors within the treatment groups were evident at this time (Fig. 3B).

At days 7 and 10 posttreatment, there was a substantial population of Gr-1⁺/CD11b⁺ cells infiltrating tumors in mice treated with KM100 + 5 μmol/L MTX compared with those treated with KM100 or MTX alone (Fig. 4). Moreover, there was an increase in the Gr-1⁺/CD11b⁺ population at day 10 compared with day 7. Gr-1⁺ tumor-infiltrating cells were gated to distinguish the CD11c⁺ (tumor dendritic cells; TDC) or F4/80⁺ (tumor-associated macrophages; TAM) populations. The number of both TDCs and TAMs in tumors was lower following combination treatment compared with those treated with MTX alone (Supplementary Fig. S3).

The total number of CD4⁺ tumor-infiltrating lymphocytes (TIL) showed variability within and between treatment groups, whereas the total number of CD8⁺ TILs was similar across treatment groups, particularly at day 7 (Fig. 4). Mice treated with KM100 + 5 μmol/L MTX showed an early increase in the number of HER-2–specific CD8⁺ TILs secreting IFN-γ by day 7 compared with those treated with either drug alone (Fig. 4). The variability within the combination treatment group stems from the observation that 4 of 5 mice showed similarly elevated levels of HER-2–specific CD8⁺ TILs, but 1 mouse showed negligible levels (data not shown). Indeed, we noted a correlation in the percentage of mice with elevated HER-2–specific CD8⁺ TILs within the tumor at day 7 with the percentage of mice that survive a given treatment regimen (data not shown).

CD8⁺ and CD4⁺ T cells are critical for the therapeutic effect of KM100 + MTX

Given that there were higher numbers of tumor antigen-specific CD8⁺ T cells as well as CD11b⁺/Gr-1⁺ cells in KM100 + 5 μmol/L MTX–treated tumors, we selectively depleted T cells and neutrophils to evaluate which cell population(s) contributes to the enhanced efficacy of this treatment combination. Depletion was conducted using anti-CD8, anti-CD4, or anti-Ly6G mAbs. The Ly6G-specific mAb has been shown to deplete only neutrophils in mice (28). CD4⁺ T-cell depletions were included in the study because these cells contribute helper functions that modulate activity of both antigen-presenting cells (APC; macrophages, dendritic cells) and T cells such as cytotoxic CD8⁺ T cells. Mice were treated with mAbs and blood was analyzed at different time points to ensure target cells were depleted. It was also verified that depletion of a specific cell population did not affect other cell populations studied (data not shown). Tumor-bearing mice left untreated had a median survival of 9 days, with no survivors at the end of the study period, whereas KM100 + 5 μmol/L MTX–treated mice showed a survival rate of 80% at the end of the 40-day observation period (Fig. 5). Mice treated
Combination therapy breaks immune tolerance

In a previous study, we found that KM100-mediated induction of HER-2-specific CD8+ T cells only occurs within a nontolerized environment (7). To test whether combination therapy can break tolerance, we used a tolerized HER-2/neu TAA model (TUBO cells in BALB/c neuT mice). Although 5-fold fewer cells were implanted in BALB/c neuT mice, tumors grew aggressively, resulting in no survivors by the end of the study period and an average median survival of 9 days when mice were left untreated (Fig. 6A) or treated with KM100 alone (data not shown). KM100 + MTX treatment provided a significant tumorstatic effect in the early days posttreatment as well as 13% long-term survival in tumor-bearing mice (Fig. 6). To confirm the role of CD8+ T cells in the tolerized TAA model, we carried out depletion experiments. Tumor-bearing mice treated with KM100 + MTX and treated with the isotype control IgG1 (n = 5) showed similar survival as mice treated KM100 + MTX with no depletion (data not shown). CD8+ T cell-depleted mice had statistically significant higher tumor volumes and significantly lower survival compared with mice treated with KM100 + MTX without depleting antibody (Fig. 6).
Discussion

Oncolytic viruses exert their tumor-debulking effect by direct oncolysis of tumor cells, stimulation of the immune response, and/or induction of tumor vascular shutdown. A commonly pursued strategy to enhance clinical efficacy is combining oncolytic viruses with chemotherapeutic drugs. Combination therapy has shown great potential both in animal models and in clinical trials (1, 2). This study tested the immunotherapeutic effect of combining oncolytic virus KM100 and the immunogenic cancer cell–inducing chemotherapeutic drug MTX. MTX is a topoisomerase inhibitor that is used clinically to treat multiple sclerosis, along with limited types of cancer, including metastatic breast cancer. It is one of the four chemotherapeutics found to elicit bona fide immunogenic cancer cell death (29). Cancer cells undergoing immunogenic apoptosis and autophagy after treatment with

![Figure 4. Tumor-infiltrating immune cell population in subcutaneous TUBO tumors. Tumors were treated with three doses of KM100, 5 μmol/L MTX (6.5 mg/kg), and KM100 + 5 μmol/L MTX. Seven and 10 days after treatment tumors were resected and CD45+ immune cells isolated using EasySep magnetic bead–positive selection. Cells were either stimulated using MHC class I–restricted HER-2–specific peptide or directly stained with the indicated surface marker. The average and SD of cell numbers were generated from 5 mice per treatment group. *, P < 0.05; ns, not significant.](image-url)
MTX express various danger-associated molecular patterns (DAMP) that increased uptake of TAAs by APCs, resulting in establishment of antitumor activity by antigen-specific CD8⁺ and CD4⁺ T cells (17, 19). Consistent with studies in other cell types (17, 19), MTX induced an immunogenic apoptotic and autophagic cell death in TUBO cells. Unlike “viral sensitizers,” such as mitomycin-C that function to increase viral burst size and virus-mediated toxicity (12, 30, 31), MTX did not increase the oncolytic activity of KM100 in vitro. However, when combination treatment was extended in vivo, it provided significant survival benefit to tumor-bearing mice both in a nontolerized and a tolerated tumor antigen setting. Moreover, this study shows that the survival benefit achieved by the combination therapy is mediated through stimulation of the antitumor immunity. These observations are consistent with our previous observations that in vitro replication of oncolytic viruses does not necessarily reflect efficacy in vivo (7).

The host immune response plays a significant role in the success of oncolytic virus therapy (21, 22, 32–34). Quantification of tumor-infiltrating immune cells showed higher numbers of CD11b⁺/Gr-1⁺ cells by days 7 and 10 after treatment with KM100 + MTX compared with each treatment alone. Decreased survival of tumor-bearing mice depleted with the neutrophil-specific Ly6G mAb indicates that neutrophil influx plays an important antitumor role in the context of the combination treatment. The role of neutrophils in tumor...
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Figure 6. Antitumor activity of KM100 + 5 μmol/L MTX treatment in the presence or absence of CD8\(^+\) T cells in BALB-neuT mice bearing subcutaneous tumors of TUBO cells. A, Kaplan–Meier survival estimates of tumor-bearing, KM100 + MTX–treated BALB-neuT mice in the presence and absence of CD8\(^+\) T cells. The statistical significance of the survival differences was analyzed using log-rank (Mantel–Cox). B, the tumor volume of mice treated with either KM100 + 5 μmol/L MTX in the presence and absence of CD8\(^+\) T cells. Except the depletion study, the rest of the data are pooled from three independent experiments. The statistical significance of the survival differences was analyzed using the Kruskal–Wallis test. ***P < 0.0005.

Dynamics has yet to be fully characterized (35). However, in the context of oncolytic virotherapy, vesicular stomatitis virus treatment–induced neutrophilia and inflammatory responses within the tumor are essential components of the tumor vascular shutdown (36, 37). It is not known whether HSV-1–based oncolytic viruses induce vascular shutdown, and further study is required to elucidate the mechanisms by which intratumoral neutrophilia contributes to tumor regression.

In contrast, we observed a lower number of antigen-presenting dendritic cells in KM100 + MTX–treated tumors compared with MTX or untreated control tumors. Although tumor-infiltrating dendritic cells can enhance antigen presentation, in the context of chemotherapy there is evidence suggesting that when present in excess, the tumor-infiltrating dendritic cell network traps CD8\(^+\) T cells in an antigen-subversive manner leading to T-cell tolerance (38). We have not sampled secondary lymphoid tissue, thus it is not possible to rule out that tumor-infiltrating dendritic cells in KM100 + MTX–treated tumors may have migrated to the local lymph nodes to present antigen to T cells. Preliminary in vitro feeding assays using KM100 + MTX–treated tumor cells and dendritic cells showed a trend toward higher phagocytosis of tumor cells compared with either KM100 or MTX treatment alone (data not shown).

Although CD4\(^+\) T cells are not required for antitumor activity mediated by KM100 alone (7), the protective effect of KM100 + MTX treatment requires the presence of CD4\(^+\) T cells in addition to CD8\(^+\) T cells. CD4\(^+\) Th cells are involved in the generation of an antibody response (39), recruitment of macrophages, granulocytes, and natural killer cells to the tumor site (40), activation and expansion of CD8\(^+\) T cells (41) and establishment of effective CD8\(^+\) T-cell memory (42–44). Although Th cells can have direct antitumor effects, their major contribution is providing T-cell help for generating and augmenting tumor-specific CTL responses (40). Future studies are required to precisely define the role of CD4\(^+\) Th cells in combination therapy.

Given the critical importance of cytotoxic CD8\(^+\) T cells in direct killing of cancer cells in the context of MHC class I presentation (45), it is not surprising that KM100 + MTX–treated tumor-bearing mice rapidly reached endpoint when CD8\(^+\) T cells were depleted. CD8\(^+\) T cells are essential for the antitumor effects of MTX (19) and KM100 in an immunocompetent, nontolerized model (7). However, in a tolerized mouse model, which best recapitulates the clinical setting, KM100 treatment alone failed to activate tumor-specific CD8\(^+\) T cells, resulting in tumor stasis, but not tumor regression (7). In patients with breast cancer, increased tumor-infiltrating CD8\(^+\) lymphocytic density is significantly associated with improved clinical outcome (46). The combined release of DAMPs from MTX–induced immunogenic cell death (17, 19) and PAMPs (viral nucleic acids and antigens) from infected tumor cells likely enhanced TAA presentation and subsequent activation of CD8\(^+\) T cells to break immunologic tolerance, which was not possible to achieve using either oncolytic virus or chemotherapy alone.

In summary, this study shows that current in vitro screening strategies for selecting combination treatments using oncolytic viruses and standard chemotherapeutic drugs should be revisited in that in vitro measurements of enhanced cytotoxicity or virus replication may not translate in vivo. Moreover, this study shows for the first time that significant immunotherapeutic effect can be achieved by combining immunogenic cancer cell death–inducing drugs with oncolytic HSV-1 virotherapy. Specifically, combination treatment was able to break immune tolerance. The study also underscores that understanding the dynamics of the tumor microenvironment is an essential aspect for improvement of combinatorial therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Dr. Yonghong Wang (McMaster University) for reagents.

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doi:10.1158/2326-6066.CIR-13-0059-T

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