Myeloid-Derived Suppressor Cells as a Vehicle for Tumor-Specific Oncolytic Viral Therapy

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Abstract

One of the several impediments to effective oncolytic virus therapy of cancer remains a lack of tumor-specific targeting. Myeloid-derived suppressor cells (MDSC) are immature myeloid cells induced by tumor factors in tumor-bearing hosts. The biodistribution kinetics of MDSC and other immune cell types in a murine hepatic colon cancer model was investigated through the use of tracking markers and MRI. MDSCs were superior to other immune cell types in preferential migration to tumors in comparison with other tissues. On the basis of this observation, we engineered a strain of vesicular stomatitis virus (VSV), an oncolytic rhabdovirus that bound MDSCs and used them as a delivery vehicle. Improving VSV-binding efficiency to MDSCs extended the long-term survival of mice bearing metastatic colon tumors compared with systemic administration of wild-type VSV alone. Survival was further extended by multiple injections of the engineered virus without significant toxicity. Notably, direct tumor killing was accentuated by promoting MDSC differentiation towards the classically activated M1-like phenotype. Our results offer a preclinical proof-of-concept for using MDSCs to facilitate and enhance the tumor-killing activity of tumor-targeted oncolytic therapeutics.

Cancer Res; 73(16); 5003–15. ©2013 AACR.

Introduction

Tumor-secreted factors have been shown to promote the abnormal differentiation and accumulation of myeloid progenitor cells, which in turn promote tumor progression and metastases. Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid cells critical to the development of tumor-induced immune tolerance (1, 2). Classically, they have been described as CD11b+Gr1+ cells (3) in murine models, which can be further characterized into monocytic (Gr1–Ly6ClowCD115+) and granulocytic (Gr1–Ly6ChighCD115+) subsets (4–6).

MDSCs are enriched when immature myeloid cells develop abnormally in the bone marrow of tumor-bearing hosts (7, 8), at which time MDSCs are released into circulation, accumulating in lymphoid organs (7) and ultimately migrate from these locations to tumors, a process that is mediated by multiple tumor-secreted inflammatory factors, including granulocyte macrophage colony—stimulating factor (GM-CSF; ref 6), macrophage colony stimulating factor (5), SCF (8), S100A8/A9 (9), VEGF (10), IL-1β (11), and chemokines, for example, CCL2 (12), CCL5 (our unpublished results). Once MDSCs localize within the tumor microenvironment, they can mediate immune suppression through multiple pathways: that is, production of arginase (13), inducible nitric oxide synthase (14), reactive oxygen species (15), and suppressive cytokines including interleukin (IL)-10 and TGF-β (5) or via the activation and recruitment of regulatory T cells (Tregs; refs. 5, 16). They also differentiate into more mature, tumor-associated macrophages, which promote angiogenesis and lymphangiogenesis (17, 18).

Vesicular stomatitis virus (VSV) is an oncolytic rhabdovirus that infects mammalian cells. VSV preferentially replicates within and lysses tumor cells due to the tumor cell's inability to mount an appropriate interferon response, which, in normal cells, interferes with viral reproduction, enabling clearance of the virus (19, 20). VSV effectively prolongs survival in mice with metastatic cancer when injected intratumorally (21). A major drawback to this type of therapy in humans is the inability to treat multifocal diseases or inaccessible tumors. Although systemic administration of the virus would allow for dissemination to occult metastases, the treatment through systemic injection is limited by toxicity from high doses of VSV (22). Our group and others have observed that these doses lead to neuro-pathic changes in mammals (22–24).

Because of their unique capability to specifically migrate to tumors, we explore the possibility of using monocytic, Ly6C–
MDSCs (heretofore referred to as MDSCs) as vectors to deliver tumor-specific therapies. Treatments loaded into MDSCs could be directly targeted to the tumor sites, increasing intratumoral, while decreasing extratumoral, dosages, thus preventing systemic side effects and increasing the therapeutic index. We further hypothesize that viral-transduced MDSCs can switch from the protumor functional M2 phenotype to antitumor, M1 response, due to viral pathogen mediated inflammatory response. We also showed that MDSCs exhibited much greater tumor tropism when compared with a variety of other immune cell types (25, 26) and that treatment with VSV-loaded MDSCs, compared with systemic viral therapy, significantly prolonged survival in tumor-bearing mice. This survival benefit was further enhanced through repeated administration of virus-loaded MDSCs. Interestingly, we showed synergistic tumor killing by both the oncolytic virus and the MDSCs themselves, which, after viral interaction, exhibit an M1-like phenotype that promotes tumor killing.

**Materials and Methods**

**Experimental animals**

BALB/c and C57BL/6 mice were purchased from Jackson Laboratories. Animal experiments were carried out in accordance with the guidelines of Mount Sinai School of Medicine (New York, NY).

**Antibodies and flow cytometry**

Anti-Ly6C-FITC, anti-Ly6C-PE, anti-CD11b-APC, anti-Gr-1-PE-Cy5, anti-CD45.1-biotin, anti-Thy.1.2-FITC, and isotype-matched monoclonal antibodies were purchased from eBioscience. Anti-Arg-Biotin was purchased from Abcam and anti-iNOS-FITC was purchased from BD Biosciences. PKH26 was purchased from Sigma-Aldrich. Flow cytometric analyses were conducted using FACS_Canto II and FACS DiVa software (BD Biosciences).

**Isolation of monocytic MDSCs**

BALB/c and C57BL/6 mice were injected subcutaneously with 5 × 10^5 MCA26 colon cancer cells and 5 × 10^5 Lewis lung carcinoma (LLC) cells, respectively. Mice were sacrificed when tumors reached 1 × 1 cm^3. Splenocytes and bone marrow were processed to single-cell suspensions. Red blood cells were lysed with ACK lysis buffer (Gibco). MDSCs were enriched by Percoll density gradient (GE Healthcare). Fraction 2 cells were stained, in the presence of FcR blocking Ab, with Ly6C-FITC, bound to anti-FITC microbeads (Miltenyi), sorted via AutoMACS cell sorter (Miltenyi).

**Mouse models of hepatic and lung metastases**

MCA26 is a BALB/c-derived, chemically induced colon carcinoma with low immunogenicity (27). This cell was obtained from MD Anderson Cancer Center (Houston, TX) on 1994. It has been implanted in BABL/c mice. Our lab has routinely conducted MHC class I, II staining and HLA typing and DNA fingerprinting using short tandem repeat to avoid contamination for every 6 to 12 months. Pathologic analysis of tumor tissue had been preformed and diagnosed as colon adenocarcinoma at a regular basis during the experiments. A metastatic colon cancer model was generated as described previously (28). LLC is a C57BL/6-derived stable lung cancer cell line obtained from American Type Culture Collection, 2011. Intrahepatic lung cancer metastases were similarly inoculated using 7 × 10^5 LLC cells in C57BL/6 mice. Diffuse lung metastases were generated with the injection of 2 × 10^5 LLC cells via tail vein. Pathologic analysis had been preformed to confirm the lung adenocarcinoma developed in the lung from experimental animals during the studies.

**Cell migration comparison**

MDSCs were isolated as above from CD45.1 C57BL/6 mice. Cytokine-induced killer (CIK) cells were isolated per established protocols (29) from CD45.1 mice. Tumor-specific T cells were isolated from the spleens of tumor bearing CD45.1 C57BL/6 mice via staining by anti-Thy.1.2-FITC, and separated using anti-fluorescein isothiocyanate (FITC) microbeads via an AutoMACS cell sorter. Activated T cells were isolated similarly and cultured in the presence of IL-2 (Peprotech) at 200 U/mL for 3 days. Macrophages were isolated by culturing bone marrow of naïve CD45.1 mice for 7 days in the presence of macrophage colony-stimulating factor (Peprotech) at 30 ng/mL, followed by harvesting attached cells. Dendritic cells were generated by culturing bone marrow of naïve CD45.1 mice for 7 days in the presence of 1% GM-CSF–conditioned medium (from J558L cell line) followed by harvesting non-adherent cells. Monocytes were isolated from percoll fraction 2 from the bone marrow of naïve CD45.1 mice. Cells were adoptively transferred, via tail vein, to CD45.2 C57BL/6 mice bearing intrahepatic LLC tumors (14 days after tumor implantation). Mice were sacrificed after 72 hours for analysis.

**Migration of PKH26-labeled cells**

For PKH26 analysis, MDSCs were stained with PKH26 per manufacturer’s instructions and adoptively transferred via tail vein into BALB/c mice bearing intrahepatic MCA26 colon cancer (14 days after inoculation). Organs were homogenized and immune cells were isolated, stained for MDSC markers, and analyzed by flow cytometry. Murine organs were also fixed with Optimum Cutting Temperature compound (Tissue-Tek), sectioned, and stained with Perl’s Prussian blue to assess the presence of iron.

**Feridex labeling and uptake**

MDSCs were cocultured for 4 hours with Feridex (ferumoxide, Berlex) at 11.2 mg/mL and sent to inductively coupled plasma mass spectrometry (ICP-MS) to determine total iron content. The percentage Feridex uptake was then determined based upon the amount of iron detected in the cells by ICP-MS versus the total concentration of Feridex added during incubation. Cytospins were prepared by diluting 2 × 10^4 cells in 300 μL PBS. The cells were spun on glass slides using a Cytospin 3 centrifuge (Shandon). Perl’s Prussian blue staining was conducted by fixing the samples with 4% paraformaldehyde for 10 minutes followed by incubation with 2% K3[Fe(CN)6] in 2% HCl. Slides were counterstained with nuclear fast red and dehydrated in ethyl alcohol. Images were acquired with a...
Nikon microscope using specialized software (SOFT, Diagnostic Instruments).

**In vitro MRI of Feridex-labeled cells**

Phantoms were prepared by adding known numbers of Feridex-labeled MDSCs into 0.2 mL 2% agarose gel, mixed, and snap frozen. Phantoms were imaged at 9.4 Tesla using a 89 mm bore system operating at a proton frequency of 400 MHz (Bruker Instruments). Multi-echo gradient echo (GRE) sequences were applied with the following parameters: TR = 29.1 ms, TE = 5.1 ms to 10 ms (n = 5), 30 slices, flip angle = 30°, number of signal averages = 8, in-plane resolution = 0.098 mm², and 100% z-rephasing gradient. R2* maps were generated on a pixel-by-pixel basis using Matlab (R2007b; The Mathworks). The signal intensity associated with each pixel was normalized to the SD of adjacent noise before linear fitting of the signal-to-noise ratio versus echo time (TE). For the GRASP sequence, the z-rephasing gradient was reduced to 50%.

**In vivo detection of Feridex-labeled cells by MRI**

Tumor bearing BALB/c mice (n = 7) were treated with 5 × 10⁶ Feridex-labeled MDSCs via tail vein. MR images of the liver, spleen, and tumor were obtained before injection and over 1 week after injection. All in vivo MRI imaging was conducted as follows. Respiratory gating (SA Instruments, Inc.) was used. R2 and R2* mapping was conducted on a pixel-by-pixel basis using Matlab. To account for tumor growth over the 4-day interval, signal-to-noise ratios (SNR, where SNR = signal intensity divided by the SD of the noise) were divided by the tumor area (mm²) for all data obtained using GRE sequences. At various time points, representative mice were sacrificed, saline perfused, and their organs were harvested. A section of tissue was stained for iron using Perl’s Prussian blue and the remaining tissue was weighed. The liver was sent to relaxometry and the tumor and spleen to ICP-MS for determination of iron content.

**Relaxometry**

Dose–response curves were generated by spiking ex vivo tissue homogenate with known concentrations of Feridex (0–1 mmol/L Fe, n = 6). The transverse relaxation times (T2) were determined at 60 MHz (40°C) using a Bruker Minispec spectrometer (Bruker Medical GmbH). T2 values were calculated based on a monoexponential fit of echo amplitude versus time.

**Recombinant vesicular stomatitis virus vectors and Transwell assay**

The construction of rVSV-GFP and rVSV(MΔ51)-M3 have been described (30, 31). A total of 1 × 10⁶ tumor cells were cultured for 24 hours in the lower chamber of a 24-well Transwell plate (Corning Costar). MDSCs were isolated as previously described and placed into VP-SFM medium (Gibco) on ice with rVSV-MDSC at various multiplicity of infection (MOI) for 4 hours followed by thorough washing with cold medium. A total of 1.5 × 10⁷ cells were placed in the top chamber of the same Transwell plate (0.4 μm pore size), incubated for 24 hours, and analyzed with a Leica DMRA2 fluorescent microscope.

**Therapeutic protocols**

Eight-to-nine days after hepatic tumor implantation, when the tumor size reached 5 × 5–6 × 6 mm², 5 × 10⁶ monolytic MDSJs or Ly6C- cells passively loaded with rVSV-GFP in VP-SFM for 4 hours on ice at an MOI of 300 in the presence of polybrene (hexadimethrine bromide, Millipore), washed with ice-cold PBS 3 times to remove free virus, and then were injected via tail vein. 5 × 10⁶ palque forming units rVSV-GFP was resuspended in 250 μL PBS before transfer. Anti-VSV monoclonal antibody was incubated at 2 μg/mL on ice in the presence of rVSV-GFP or rVSV(MΔ51)-M3 (MOI300) and polybrene for 1 hour, followed by addition of 5 × 10⁴ MDSJs or Ly6C- cells and incubation on ice for an additional hour. Cells were washed 3 times with ice-cold PBS before transfer. Some mice were kept for survival and some were terminated at 96 hours after MDSC therapy and saved for histology, quantitative PCR (qPCR), TCID₃₀ and stained for VSV-G antigen (Alpha Diagnostic).

**FACS analysis of viral binding**

A total of 2.5 × 10⁷ MDSJs were combined with rVSV-GFP in the presence or absence of antibody as described above at viral MOIs of 0, 3, 10, 30, 100, 300, and 1,000. Cells were left in culture at 37°C in RPMI for 72 hours before staining and flow cytometric analysis.

**TCID₃₀ analysis of viral binding**

Organs and cells were lysed, serially diluted, and incubated with BHK21 cells at 37°C in VP-SFM medium for 72 hours. Cells were then examined under light microscopy for cytopathic effects (CPE). TCID₃₀ concentration was determined using the Spearman–Karber method.

**qPCR**

RNA was isolated from organs using TRIzol (Invitrogen) per manufacturer’s specifications. qPCR was conducted using RT² Real-Time SYBR Green/ Rox PCR Master Mix (SA Biosciences) on an ABI PRISM 7900HT (Applied Biosystems) using the following primer sequences: 5'-TCTTGTGTTCTCGAGTT-GG-3' and 5'-AACAGGAGGATGCAGCATTT-3'.

**Cytotoxicity assay**

MDSCs cultured in the presence or absence of VSV-G antibody and rVSV(MΔ51)-M3 (MOI300) in the presence of polybrene as described above and coincubated with LLC tumor cells at 12.5:1, 25:1, 50:1, and 100:1 for 4 hours. Supernatants were collected for measurement of lactate dehydrogenase (LDH) release (CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit; Promega). Specific killing (in percentage) was calculated as experimental LDH release/maximum LDH release.

**Statistical analysis**

One-way ANOVA with Bonferroni *post hoc* tests was used to evaluate the significance associated with change in R2* as a
function of time after injection. Student one-sided t test was used to compare the differences in tumor sizes and weights as well as FACS data, qPCR, and TCID<sub>50</sub> results. The log-rank test was used to determine the significance of survival data.

Results

MDSCs exhibit stronger tumor tropism than other immune cell types

In multiple murine models, MDSCs are present at high numbers at tumor sites (5, 6, 8, 11). We hypothesized that adoptive transfer of MDSCs should lead to preferential accumulation of MDSCs within tumor tissue, making them an excellent vector for oncolytic virus delivery. Previous studies have used different immune cell types for oncolytic virus delivery, including TCR transgenic and IL-2 producing T-cells (25), CIKs (29, 32), as well as macrophages (26). In an attempt to determine which subset of cells possesses the strongest tumor tropism, we conducted a series of experiments in which CD45<sup>+</sup> MDSCs, CIKs, tumor-specific T-cells, IL-2-activated T-cells, monocytes, macrophages, and dendritic cells were adaptively transferred into CD45<sup>2</sup> mice bearing intrahepatic LLC tumors, simulating metastatic lung cancer (Fig. 1). At 72 hours after treatment, the mice were sacrificed and CD45<sup>1</sup> cells present in various organs were quantified. We observed a significantly higher number of MDSCs migrating to tumor sites when compared with spleen and liver. We also found that MDSCs exhibited significantly greater tumor tropism than CIKs, activated T-cells, normal T-cells, monocytes, macrophages, and dendritic cells. The results suggest that MDSCs possess a greater potential for use in the delivery of tumor-specific therapies than other immune cell types analyzed.

Using novel MRI techniques to track the kinetics of MDSC migration and localization in a hepatic tumor model

To determine the in vivo migration patterns of MDSCs longitudinally within individual mice, it is necessary to use diagnostic imaging. Recently, superparamagnetic iron oxide particles (SPIO), such as Feridex, an SPIO currently used for in vivo clinical cell tracking, have been used to label multiple cell types for MRI detection (33, 34). This technique enables longitudinal tracking of SPIO-labeled MDSCs, as a function of time after injection, without the need to sacrifice tumor-bearing mice. MRI also enables one to pinpoint the specific locations within each organ to which the labeled cells migrate.

MDSCs were isolated and purified from tumor-bearing mice (5). More than 96% of this population was CD11b<sup>+</sup> Gr-1<sup>+</sup> (Supplementary Fig. S1), the classical markers of MDSCs. We created MRI phantoms of known iron and MDSC concentrations in agar and imaged via MRI (Supplementary Fig. S2A). These phantoms show a linear relationship between cell number and signal loss (Supplementary Fig. S2B). Prussian blue staining on cytospin samples confirmed the majority of MDSCs cocultured with Feridex stained positive for iron (Supplementary Fig. S2C), indicating that MDSCs can be effectively labeled with Feridex and imaged by MRI.

Feridex-labeled MDSCs were administered to BALB/c mice previously implanted intrahepatically with MCA26 cells, simulating hepatic metastases of colon cancer. Mice received MRIs before adoptive transfer, then daily (n = 7) for one week. Representative T2<sup>-</sup>-weighted images (GRE) and GRASP images are shown in Fig. 2A and B at different...
magnifications. At 72 hours after transfer, increased signal loss around the periphery and within vascular structures inside the tumor was observed. GRASP imaging has been developed to increase the accuracy of labeled cell detection (35) and a good correlation between GRE and GRASP is observed. The relative change in the R2*/C3 values, indicates maximum uptake of the cells into the lesion at 72 hours after injection (Fig. 2C).

Mice were sacrificed at various time points and their organs were harvested. Iron concentration was quantified within tumor and spleen via mass spectrometry. Relaxometry, an NMR technique, which is able to distinguish superparamagnetic iron from physiologic iron, was then used to determine the Feridex concentrations within the liver tissue where there is a high concentration of endogenous iron (36). Although iron levels were high in the tumor (38 ± 2%ID), minimal spleen (13 ± 0.6%ID), and liver uptake (0.5 ± 1%ID) was observed at 72 hours (Fig. 2D). Perl's staining was used to confirm the presence of iron in transferred cells within tumors at 72 hours after injection (Fig. 2E). These methods reliably show that the adoptively transferred MDSCs targeted tumors, reaching peak concentrations 72 hours after transfer, localizing peripherally and perivascularly, and some penetrating inside the tumor and intratumoral vasculature.

**Confirmation of MRI findings through FACS analysis of PKH26-labeled MDSCs**

We next used flow cytometry to confirm the MRI findings. In this experiment, MDSCs were labeled with the membrane dye, PKH26, and adoptively transferred to BALB/c mice bearing intrahepatic MCA26 tumors. Representative mice were sacrificed each day and MDSC were reisolated from liver, lungs, bone marrow, spleen, lymph nodes, blood, and tumors (Fig. 3A). FACS analysis showed PKH26 signal increased in the tumor 4-fold over background at 48 hours after after transfer, peaking at 8 times the background signal at 72 hours after transfer (Fig. 3B and C). Little increase in PKH26 signal was observed in other organs. Notably, the liver showed no signal increase despite its proximity to the tumor. Circulating MDSCs decreased in correspondence to tissue dissemination, with blood levels reaching their nadir by 48 hours after transfer (Fig. 3D).

![Figure 2.](image-url) The use of MRI to follow in vivo migration of adoptively transferred MDSCs to tumor sites. Feridex-labeled Ly6C⁺ MDSCs were transferred to mice bearing intrahepatic MCA26 tumors in their livers (n = 7). A, representative T2-weighted images of the entire mouse cross section are shown from before adoptive transfer (Pre) and then daily after transfer, up to 4 days, in both GRE (top) and GRASP (bottom) modalities. B, representative close-up view of the tumor section reveals signal loss in multiple cuts peripherally as well as in a vascular distribution at 72 hours after transfer (GRE, black; GRASP, white; /C3, tumor). C, MRI data was then analyzed and R2* quantification was conducted pixel-by-pixel to quantify signal loss as a function of time. Data is presented as an increase in signal loss over baseline (pretreatment values) at the designated time points. D, mice were sacrificed, saline perfused, and tumor, liver, and spleen were removed. The presence of Feridex was shown to be significantly higher in the tumor than the spleen via ICP-MS [40 ± 2% injected dose (ID) vs. 19 ± 0.3%ID, 48 hours and 38 ± 2%ID vs. 13 ± 0.6%ID, 72 hours] as well as in the liver via relaxometry (0.5 ± 1%ID, 72 hours) and results were compared with the known amount of Feridex injected into the mice based on the standard curve determined in Supplementary Fig. S2B (**, P < 0.001). E, tumors were resected 72 hours after transfer and stained with Perl's Prussian blue to indicate the presence of Iron oxide (positive results indicated by arrows).
Figure 3. Confirmation of MDSC migration to tumor sites by flow cytometric analysis. PKH26-labeled Ly6C+ MDSCs were transferred to mice bearing MCA26 tumors in their livers. Representative mice were sacrificed daily for 3 days and spleen, bone marrow, lymph nodes, liver, lung, and tumor were resected and homogenized. Immune cells were isolated by Percoll fractionation, stained with antibodies against Ly6C, and analyzed via FACS for PKH26 positivity (n = 3 per time point). A, representative FACS plots are shown comparing the spleens, livers, and tumors (tumor-infiltrating leukocyte, TIL) from tumor-bearing mice receiving no MDSC transfer (control, top) with those from tumor-bearing mice sacrificed 3 days after PKH26-labeled MDSC transfer (3 day, lower panels). B, the total number of MDSCs present was determined for each of the harvested organs. C, data are also displayed as fold-increase over background signal (based on mice that did not receive PKH26 labeled cells). D, the number of circulating-labeled MDSCs was also determined by sacrificing representative mice at the designated time points followed by cardiac aspiration. Leukocytes were prepared and the total number of Ly6C+ “PKH26” cells was determined by flow cytometric analysis (n = 3 per time point).

Transfer of oncolytic viruses from MDSCs to tumor cells in vitro and in vivo and prolonged survival in treated tumor-bearing mice

We next determined whether MDSCs could effectively deliver oncolytic viruses. MDSCs were incubated with rVSV-GFP, a VSV vector expressing GFP (30), at varying MOIs. Free virus was washed away and cells were added to the top chamber of a Transwell plate, with MCA26 cells seeded in the bottom chamber. After 24 hours, monolayers were examined for CPE and GFP expression. MDSCs at all MOIs expressed high levels of GFP with minimal CPE, indicating that they had taken up and translated the viral genome without being lysed at 24 hours (Fig. 4A, first 2 columns). Tumor cells exhibited both CPE and GFP expression (Fig. 4A, columns 5 and 6). Although GFP expression was high at all MOIs, CPE after 24 hours was most extensive at MOI 300. Similar results were obtained using 4T1 breast cancer and LLC cells, showing applicability of VSV for multiple tumor models (Fig. 4A columns 3, 4 and 7, 8).

To test the efficacy and safety of VSV-MDSCs in vivo, we again used the intrahepatic colon cancer model. Mice were injected with 5 × 10⁶ VSV-MDSCs 6 days after tumor implantation. Twelve days later, mice were sacrificed, and tumors measured and weighed. Mice receiving VSV-MDSCs had significantly smaller tumors than controls, both dimensionally (Fig. 4B) and by weight (Fig. 4C).

To test whether VSV-MDSCs can maintain tumor specificity, we repeated the previous migration experiment using PKH26-labeled VSV-MDSCs. We were able to show a similar pattern of migration to tumors at 72 hours after transfer, as seen with MDSCs lacking virus (Supplementary Fig. S3), indicating that infection of MDSCs with VSV does not alter their migratory kinetics.

Having shown that VSV-MDSCs inhibit tumor growth, our next goal was to assess their effect on survival. Although there were no long-term survivors, the median survival in mice treated with VSV-MDSCs was significantly increased compared with controls (Fig. 4D). We also observed a survival advantage when comparing VSV-MDSC–treated mice to those treated with MDSCs alone (P < 0.0002) or Ly6C-negative control cells loaded with VSV (P < 0.002). This confirmed that the survival benefit was due to the tumor-targeting abilities of MDSCs and the oncolytic effects of VSV.

Finally, we tested whether VSV-MDSCs were superior to a similar dose of peripherally administered rVSV-GFP. Through TCID₅₀ assays, we determined the amount of VSV delivered by 5 × 10⁶ VSV-MDSCs to be no more than 5 × 10⁹ TCID₅₀ of VSV (data not shown). Systemic administration of an equivalent
MOIs were used for the antibody-conjugated VSV-MDSCs (significantly more VSV-positive cells were observed even when lower passive coupling at various MOIs. More importantly, significantly increased survival following this treatment was significantly increased compared with PBS controls, Ly6C⁺ cell fraction, acquired during MDSC isolation and passively loaded with VSV at MOI: 300 (P < 0.002; n = 5), or an equivalent amount of free VSV-GFP virus (P < 0.0001; n = 10).

The therapeutic efficacy of VSV-MDSC treatment using a second-generation rVSV conjugated to MDSCs via a specific anti-VSV

To enhance viral loading of MDSCs, we tested whether a non-neutralizing monoclonal antibody directed against the VSV G-protein could enhance VSV binding by bridging the virus to the Fc receptors present on MDSCs.

We tested this theory by binding rVSV-GFP to the optimal amount of antibody followed by addition of MDSCs. When analyzed by FACS, MDSCs with antibody-bound virus showed increased VSV staining (Fig. 5A) and GFP expression (Supplementary Fig. S4) compared with VSV-MDSCs generated by passive coupling at various MOIs. More importantly, significantly more VSV-positive cells were observed even when lower MOIs were used for the antibody-conjugated VSV-MDSCs (P = 0.003; Fig. 5A). TCID₅₀ assays were conducted on BHK-21 cells using VSV-MDSCs generated with or without antibody at a virus:MDSC ratio of 300. A 70-fold viral titer increase was observed when comparing antibody-conjugated VSV-MDSCs with passively coupled VSV-MDSCs (P < 0.01; Fig. 5B), thus confirming that significantly more VSV associates with the MDSCs through antibody conjugation.

We also sought to use a second-generation recombinant VSV to combat the robust host antiviral immune response, which limits viral replication in tumors. This recombinant VSV has been engineered to express the murine gammaherpes virus M3 protein, which binds a variety of chemokines with high affinity, leading to a significant delay in viral clearance (31).

The novel rVSV(M₅₁)-M₃ [VSV(M₃)] has also been engineered with an altered viral matrix protein, improving its safety profile by attenuating its ability to inhibit cellular protein synthesis, thus rendering the virus more susceptible to the interferon response of normal cells (19). There are no significant differences between these 2 forms of VSV in terms of viral replication (31).

We tested BALB/c mice bearing hepatic tumors with MDSCs conjugated to VSV(M₃) via Fc receptor-bound anti-VSV [MDSC+Ab+VSV(M₃)], MDSC+Ab+VSV(M₃) treatment led to long-term survival in 4 of 15 (26.3%) mice. The median survival following this treatment was significantly increased compared with PBS controls, Ly6C⁻ cells antibody conjugated to VSV(M₃), or systemic injection of VSV(M₃) (Fig. 5C). Mice treated with MDSC+Ab+VSV(M₃) also survived significantly longer than mice treated with MDSCs antibody conjugated to rVSV-GFP virus [MDSC+Ab+VSV(GFP)] as well as MDSCs passively conjugated with VSV(M₃) [VSV(M₃)-MDSC], indicating that enhanced survival can be attributed to both the improved viral vector and the improved viral loading onto...
MDSCs via antibody conjugation. Similar therapeutic effects were obtained in a lung metastatic tumor model (Fig. 5D). In the lung metastasis model, we also observed an additive benefit for multiple injections of viral loaded MDSCs. Mice treated with 4 doses of MDSC+Ab+VSV(M3) survived significantly longer than mice administered the same treatment just once (P = 0.008). We also further confirm that MDSCs exposed to virus exhibited enhanced tumor killing when compared with those exposed solely to antibody alone. We concluded that the antibody did not affect MDSCs (Supplementary Fig. S7). These results further support the use of viral loaded MDSCs in treating multiplets tumor types in various organs.

Confirmation of superior tumor-specific delivery of VSV-MDSCs compared with systemic viral therapy using immunohistochemical staining, TCID<sub>50</sub>, and qPCR

To confirm that MDSC-mediated delivery of VSV exhibits greater tumor specificity than systemic viral treatment, representative mice from each treatment group were sacrificed at 96 hours after treatment and their tumors and organs were harvested. Staining for VSV-G was conducted (Fig. 6A high power and Supplementary Fig. S5 low power). Tumors from mice treated with various MDSC-mediated therapies, including MDSC+Ab+VSV(M3), MDSC+Ab+VSV(GFP), and VSV(M3)-MDSCs, exhibited more extensive staining than tumors from controls or those treated with free virus. Necrosis was observed within the tumors of mice treated with MDSC+Ab+VSV(M3) but not other MDSC-targeted viral therapies, indicating that this method of treatment leads to the most robust tumor cell death at this time point. Intense staining was observed in the spleens and lungs, and to a lesser degree in the livers, from mice treated with free VSV(M3), but not in the mice treated with MDSC-targeted therapy. Central nervous tissue was also examined for VSV-G staining as a surrogate sign of potential neuropathic toxicity. Free VSV treatment resulted in brain positivity that was not showed among MDSC-targeted VSV groups, indicating that MDSC-targeted therapy is safer than free virus therapy, likely due to the tumor-specific tropism of MDSCs and the inability of the MDSCs to cross the blood brain barrier. Pathologic
examination of the central nervous tissue of mice in which long-term survival had been achieved showed no abnormalities in the myelination, neuron density, or morphology of the cerebral cortex or cerebellar Purkinje cell concentration within the cerebellum. In addition, there were no signs of ischemic damage or tissue necrosis (Supplementary Fig. S6).

To determine whether the positive staining represented viable VSV, organs from mice receiving free VSV(M3), VSV(M3)-MDSC, and MDSC+Ab+VSV(M3) were harvested and TCID\textsubscript{50} assays were conducted (Fig. 6B). Tumors in mice receiving MDSC+Ab+VSV(M3) showed significantly more virus and viral RNA in the tumor than in other organs as well as more virus and viral RNA in the tumor than in mice treated with VSV-MDSCs or free virus (*, \(P = 0.05\); **, \(P = 0.03\); ***, \(P = 0.01\)).

**MDSCs acquire an M1-like phenotype with inherent tumor killing ability upon exposure to VSV(M3)**

MDSCs have classically been shown to promote an M2-like, protumor environment. Recently, we showed a plasticity in MDSC phenotype wherein, under certain conditions, MDSCs can exhibit M1-like characteristics (37). We used FACS to measure the M1 marker, inducible nitric oxide synthase (iNOS, Fig. 7A), and the M2 marker, arginase 1 in mice receiving MDSC+VSV(M3) and MDSC+Ab+VSV(M3) showed significantly more viral RNA than those receiving free VSV(M3) and MDSC+Ab+VSV(M3)-treated mice, viral RNA was more prevalent in tumors than in the spleens, livers, lungs, or brains. These may indicate the viral replication or more viruses taking up by the tumor due to the MDSC-mediated oncolytic viral targeting.
Conversely, infection with VSV caused a decrease in Arg-unmanipulated MDSCs to those cocultured with the anti-VSV antibody and VSV(M3) (Fig. 7C). To further show that MDSCs exposed to VSV(M3) exhibited an M1-like phenotype, we compared direct tumor lysis by T-cells with VSV(M3). We found that MDSCs, after viral exposure, efficiently killed tumor cells at multiple MDSC/tumor ratios (Fig. 7D). These data suggest that MDSCs, when exposed to virus, can switch from an M2 (Arg+) to an M1-like phenotype (iNOS+ and tumor lysis activity), which further promotes tumor killing.

Discussion

Previously, macrophages, T-cells, and NK cells have been used as vectors for tumor-specific delivery of oncolytic virus (25, 26, 29, 32). Most of these immune cells migrate to lymphoid organs or to the liver (Fig. 1A). While OT-II (CD4+ VQA-specific TCR transgenic) cells used as tumor-specific T cells worked well in a proof-of-principle study (25), for clinical use, generation and expansion of tumor specific T-cells is expensive and requires genetic manipulation (i.e., TCR transgene) making their use less practical. When comparing tumor-targeting ability, we found that MDSCs were superior to other immune cell types analyzed and exhibited more specific tropism. Moreover, we showed that MDSCs penetrated into well-established tumors (tumors implanted for more than 14 days and larger than 1 cm3) by multiple approaches, e.g., MRI and fluorescent labeling (Fig. 1 and 2). Most other immune cells tested preferentially migrated and homed to the spleen or liver.

CD34+ mesenchymal stem cells (MSC) have also been used for tumor targeting. They migrate into lung, tumor, bone marrow, spleen and liver. Their use for the purpose of oncolytic virus delivery proves problematic. Upon injection, MSC accumulate in the lung and do not migrate to tumors until 11 days after injection (40), at which time virally infected cells will likely have been cleared by the host immune response. Furthermore, MSC cannot efficiently penetrate into tumors and predominantly reside within the border zone of stromal cells and tumor tissues (41). Most importantly, MSC possess the ability to differentiate into different cell types, for example, adipocyte, stromal fibroblast, osteoblast etc, potentially facilitating tumor progression (40–42). In contrast, oncolytic virus-loaded MDSC acquired M1-like antitumor phenotype without promitumor growth activity and infiltrate tumors preferentially.
We showed via MRI that MDSCs accumulated at the tumor periphery as well as perivascularly. To our knowledge, this is the first time the in vivo destination for MDSC migration has been shown via direct imaging. The use of Feridex to image cell migration offers advantages over immunologic labeling techniques, including the ability to follow migration longitudinally in the same individual in vivo, which could translate well to humans. It has previously been shown that a single SPIO-labeled cell can be identified in vivo via MRI (38). However, with our equipment we are reliably able to detect 125 cells when identifying dendritic cells (36), which are similar in size to MDSCs and seem to take up Feridex similarly based on our observations (data not shown). Because of its dextran coat, Feridex remains intact and detectable longer than observations (data not shown). Because of its dextran coat, Feridex remains intact and detectable longer than observations (data not shown).

In summary, both in humans and mice, MDSCs have been found to increase in the presence of virtually every form of cancer, including colon cancer (44), renal cell carcinoma (46), breast cancer (47), melanoma (44), and hepatocellular carcinoma (48). Despite their well-documented protumor characteristics in tumor-bearing hosts, we showed that MDSCs, when loaded with oncolytic virus, can be used as a Trojan horse for the treatment of cancer and possibly a variety of other diseases.

References


Myeloid-Derived Suppressor Cells as a Vehicle for Tumor-Specific Oncolytic Viral Therapy

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Cancer Res 2013;73:5003-5015. Published OnlineFirst March 27, 2013.

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