Intravenous Injection of MVA Virus Targets CD8<sup>+</sup> Lymphocytes to Tumors to Control Tumor Growth upon Combinatorial Treatment with a TLR9 Agonist

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Abstract

Effector T-cell access to tumor tissue is a limiting step for clinical efficacy of antigen-specific T cell–based immunotherapies. Ectopic mouse tumor models, in which a subcutaneously (s.c.) implanted tumor is treated with s.c. or intramuscular therapeutic immunization, may not be optimal for targeting effector T cells to an organ-borne tumor. We used an orthotopic renal carcinoma model to evaluate the impact of injection routes on therapeutic efficacy of a Modified Vaccinia virus Ankara viral vector expressing the human mucin 1 tumor–associated xeno-antigen (MVA-MUC1). We show that intravenous (i.v.) administration of MVA-MUC1 displayed enhanced efficacy when compared with s.c. injection. Therapeutic efficacy of MVA-MUC1 was further enhanced by i.v. injection of a TLR9 agonist. In all cases, infiltration of tumor-bearing kidney by CD8<sup>+</sup> lymphocytes was associated with control of tumor growth. Biodistribution experiments indicate that, following i.v. injection, MVA-encoded antigens are quickly expressed in visceral organs and, in particular, in splenic antigen-presenting cells, compared with those following s.c. injection. This appears to result in a faster generation of MUC1-specific CD8<sup>+</sup> T cells. Lymphocytes infiltrating tumor-bearing kidneys are characterized by an effector memory phenotype and express PD-1 and Tim3 immune checkpoint molecules. Therapeutic efficacy was associated with a modification of the tumor microenvironment toward a Th1-type immune response and recruitment of activated lymphocytes. This study supports the clinical evaluation of MVA-based immunotherapies via the i.v. route. Cancer Immunol Res; 2(12); 1163–74. ©2014 AACR.

Introduction

Modified Vaccinia virus Ankara (MVA) is a double-stranded DNA poxvirus derived from a Turkish smallpox vaccine strain through more than 570 passages in primary chicken embryo fibroblasts (1). Consequently, it has lost nearly 30 kb of genomic information and is unable to complete its replication cycle in most mammalian cells. MVA has several features that render it a good vector for targeted immunotherapy of cancer: (i) an excellent safety profile in humans (1); (ii) a large amount of foreign DNA (up to 20 kb) can be integrated into the MVA genome without loss of infectivity; (iii) viral DNA remains in the cytoplasm and, therefore, gene expression is cytoplasmic; and (iv) MVA has the ability to induce both humoral and cellular responses against the encoded foreign antigens (2, 3). On the basis of promising preclinical results, clinical trials for cancer immunotherapy have been and currently are being conducted using recombinant MVA that is injected subcutaneously (s.c.; ref. 1).

Nevertheless, similar to most cancer immunotherapies, translation of preclinical efficacy to clinical benefit has remained below expectations (4). The generation of robust cellular immunity to tumor-associated antigens requires the induced tumor-specific T cells to traffic to and enter the tumors (5). T-cell infiltration is associated with patient survival in many cancers (6–8), and it is now accepted that one of the major challenges of immunotherapy is to target T cells to the tumors (9). In this context, most preclinical therapeutic cancer models, which are based on ectopic (s.c.) implantation of tumor cell lines from various tumor types, are poor representative of the clinical situation. One of the differences is the tumor microenvironment at the anatomic site of injection resulting from the implanted tumor cells. For example, the implantation of colorectal adenocarcinoma cells under the skin would not recreate a tumor microenvironment reflecting that of spontaneous colon cancer. In addition, there is now evidence to suggest that the immunization route influences the subsequent migratory capacity of primed, antigen-specific T cells (10–12). Hence, s.c. or intramuscular (i.m.) immunizations of immunotherapeutic drugs may not be optimal for directing...
the primed, antigen-specific T cells to visceral organ tumor tissue.

To address these issues, we used a BALB/c orthotopic renal carcinoma (RenCa) model based on the subcapsular kidney implantation of renal carcinoma cells expressing the human xeno-antigen mucin 1 (MUC1; RenCa-Muc1). Renal cell carcinoma (RCC) accounts for nearly 3% of adult cancers and is thus the 10th most common malignancy in developed countries (13). Furthermore, the MUC1 antigen is detected in more than 80% of human primary renal carcinoma (14). This pathology is characterized by the absence of early warning signs and resistance to radiotherapy and chemotherapy. As a result, one third of the patients with RCC display distant metastasis at the time of diagnosis, and metastatic relapse occurs in 40% of patients after nephrectomy. These patients face a poor prognosis and have few therapeutic options (15).

We evaluated the effect of intravenous (i.v.) administration of MVA expressing the MUC1 xeno-antigen on mouse survival and immune-cell trafficking in the orthotopic RenCa-MUC1 model. In comparison with s.c. injection, the i.v. administration of MVA-MUC1 significantly increased the survival of mice bearing RenCa-MUC1 kidney tumors. This was associated with an early detectable cellular immunity to MUC1 and comparatively more infiltration by CD8⁺ T cells. CD8⁺ T-cell infiltration was observed in various organs, including the kidneys, irrespective of the presence of tumor or the expression of MUC1 by MVA. Upon i.v. injection, a reporter gene encoded by MVA was expressed more rapidly in organs and antigen-presenting cells (APC) from the spleen, in comparison with that following s.c. injection. Antibody-mediated depletion revealed the importance of CD8⁺ T cells in this model. Infiltrating T cells were mostly of the memory phenotype and displayed evidence of T-cell exhaustion. Coinjection of a Toll-like receptor 9 (TLR9) agonist significantly increased the survival of mice bearing RenCa-MUC1 kidney tumors, which was associated with changes in the pattern of expression of inflammatory genes. This study shows that i.v. injection and combination with a TLR9 agonist improve the therapeutic efficacy of an MVA-MUC1 vaccine in an orthotopic model of renal carcinoma. When translated to the clinic, this observation could constitute a significant advance in antigen-specific cancer immunotherapy.

Materials and Methods

Mice and cell line

Six-week-old BALB/c mice (Charles River Laboratories) were housed in a pathogen-free animal facility and acclimated for 1 week. In vivo experiments were performed in full compliance with the CEE (European Economic Community) directive 2010/63 of September 22, 2010, relating to the protection of animals used for experimental purposes and in compliance with the French law (décret n° 2013-118 of February 1, 2013) and approved by the Comité National de Réflexion Ethique sur l’Experimentation Animale (CNREEA, Ethical Committee TG number 93).

The mouse renal carcinoma cell line was obtained in January 2010 from the ATCC (CRL-2947; batch C58045446) and not authenticated. Cells were transfected with the MUC1 expression plasmid pHMG-ETAtm (16) and pY3 (hygromycin B resistance; ATCC). Resulting RenCa-MUC1 cells were cultured in a 5% CO₂ atmosphere in DMEM supplemented with 10% fetal bovine serum, gentamicin (40 μg/mL), and hygromycin B (0.6 mg/mL). RenCa-MUC1 cells were tested for MUC1 expression by Western blot analysis and for Mycoplasma and nucleic acids from rodent viruses (RapidMAP-27; Taconic) by real-time PCR, most recently in August of 2013.

Constructs

MVA-MUC1 plasmid was generated by introducing a modified sequence of the human MUC1 cDNA (NCBI Nucleotide database, #NM_002456.5) under the control of the pH5R promoter into the natural deletion II of the parental virus MVA-TGN33.1 (17). The “empty” MVA-TGN33.1 vector without any inserted transgene is designated control MVA.

Orthotopic renal carcinoma model

Mice were anesthetized with isoflurane, and a lateral incision was made in the left flank of each mouse. Ten thousand RenCa-MUC1 cells in 30 μL of PBS were injected into the subcapsular space of the left kidney with a 0.3-mL insulin syringe and a 30-gauge needle. The incision was then sewn closed with suture thread. On days 1 and 8 following grafting, mice were administrated s.c. (flank) or i.v. (caudal vein) with 5 × 10⁷ pfu (plaque forming units) of MVA-MUC1 or control MVA. Where indicated, on days 2 and 9, mice were treated s.c. or i.v. with 10 μg of class B CpG oligonucleotide ODN1826 (InvivoGen). In some experiments, CD4⁺ and CD8⁺ T cells were 95% depleted by two intraperitoneal injections at 1-day interval (200 μg per intraperitoneal injection) of rat anti-mouse CD4 mAb (clone GK1.5) or anti-mouse CD8 mAb (clone 53.6.72), or isotype control rat IgG (Rockland/Tebu-bio).

Biodistribution

To compare the biodistribution of MVA after s.c. versus i.v. injection, BALB/c mice were injected s.c. or i.v. with 5 × 10⁷ pfu of MVA-luciferase, MVA-GFP, or control MVA in 100 μL. Six hours later, animals were perfused and their lungs, livers, spleens, and axillary and inguinal lymph nodes were sampled and processed for luciferase detection (18) and flow cytometric analysis of GFP expression by their APCs.

Antibodies and flow cytometry

To characterize the infiltration of renal tumors by immune cells and to assay gene reporter expression by APCs, lymphoid and nonlymphoid organs were cut into small pieces, transferred into PBS-containing C-type tubes (Miltenyi Biotec), and mechanically dissociated (GentleMACS; Miltenyi Biotec). Cells were passed through filters (pore size, 70 μm), washed twice in PBS, and prepared for immunofluorescence staining and flow cytometric analysis. Living cells were labeled using Live/Dead near-IR (Invitrogen). Fc receptors were blocked with 1 μg of mouse anti-CD16/CD32 (clone 93; ebioscience), and cells were stained for 30 minutes at 4 °C in various panels, depending on the immunophenotyping with the following mAbs: anti-CD45-PE/Cy7, anti-CD45-PerCP, anti–CD45-FITC (clone 30-F11), anti–B220-PE (clone RA3-6B2), anti–CD3-PE, anti–CD5-

**Immunohistochemistry**

Mouse formalin-fixed paraffin-embedded tissue sections were deparaffinized and rehydrated. Epitopes were retrieved by boiling in a 10 mmol/L citrate buffer, pH 6. Sections were saturated with 3% H2O2 and 10% goat serum to eliminate endogenous peroxidase activity and nonspecific staining, respectively, before incubation for 90 minutes at room temperature. Sections were deparaffinized with 4% paraformaldehyde (Sigma) and mounted in Mowiol (Calbiochem). Images were acquired with an optical microscope 90i (Nikon) equipped with ×40 objective and epifluorescence. Signal was quantified on section scans (Nanozoomer; Hamamatsu) using the Caplaxis software (TRIVBN). The blue pixels defined the tumor section surface, and the red pixels corresponded to the immunostained cell surface. The percentages of positive cells were calculated as (red pixels/blue pixels) × 100.

**ELISpot assay**

Flat-bottomed, 96-well filter plates (MultiScreen HTS; Millipore) were coated overnight at 4°C with IFNγ mAb (15 μg/mL, AN–18; Mabtech). After PBS washing, plates were blocked with 10% heat-inactivated FCS for 1 hour at 37°C. Fifty thousand sorted splenic CD8+ cells (CD8sx T-cell isolation kit II; Miltenyi Biotec) were incubated for 20 hours in complete medium (RPMI-1640, 10% FCS) in the presence of 1 μg/mL peptide or peptide pool arrays. The latter are composed of 25 pools of 12 to 13 peptides covering the MUC1 protein with 11 amino acid polypeptides, overlapping by eight amino acids (19). Upon PBS washing to remove cells, the revelation IFNγ mAb (1 μg/mL, R4-A21-biotin; Mabtech) was added to each well for 2 hours at room temperature. Plates were then washed and developed with ExtrAvidin-alkaline phosphatase (1:5000; Sigma–Aldrich) for 1 hour at room temperature. After washing, substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma) was added and incubated for 5 minutes, washed and air-dried. Spots were counted with a dedicated reader (ImmunoSpot; CTL) and analyzed with ImmunoSpot software.

**Gene expression analysis**

Tumor samples were collected in RNeasy lysis buffer (Qiagen). After tissue dissociation, RNA was extracted using the Qiagen RNeasy Mini Kit. cDNA was reverse transcribed from RNA, and genomic DNA was eliminated with RT2 first strand kit (Qiagen). Expression of several genes was determined by qRT–PCR with SYBR green (Qiagen SYBR Green Mastermix with ROX). Gene expression was normalized against β-actin expression. Real-time PCR cycling conditions were as follows: 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds; 60°C for 1 minute; 95°C for 30 seconds; and 60°C for 15 seconds. Data analysis was conducted according to the manufacturer’s instructions.

**Statistical analysis**

Statistical analyses were performed using Prism (GraphPad software). Statistical significance of survival was evaluated by the log-rank assay. For multiple group comparisons, nonparametric Kruskal–Wallis and Dunn multiple-comparison tests were used. For ELISpot data, statistical significance was evaluated with the DFR(eq) script (20). Differences were considered statistically significant when P < 0.05.

**Results**

**Effects of injection route and coadministration of a TLR9 agonist on the survival of mice bearing orthotopically implanted renal carcinoma tumors**

The route of immunization can affect the subsequent migratory capacity of primed antigen-specific T cells (10–12). Therefore, we compared the s.c. and i.v. routes of immunization with MVA–MUC1 to treat orthotopically implanted RenCa-MUC1 tumors. Immunization with MVA–MUC1 via the s.c. route had no discernible impact on the survival of mice bearing RenCa-MUC1 tumors in comparison with control MVA-treated animals (P = 0.97; Fig. 1A). In contrast, upon i.v. administration of MVA–MUC1, we observed a significant increase in survival compared with animals treated with control MVA (P = 0.02; Fig. 1B).

Toll-like receptor agonists are being developed as adjuvants for immunotherapies to treat cancer and infectious diseases (21). Additional s.c. administration of a TLR9 ligand (ODN1826) did not improve the survival of mice treated with s.c. MVA–MUC1 (P = 0.23; Fig. 1A). In contrast, the addition of i.v. ODN1826 to i.v. MVA–MUC1 significantly improved the survival of animals in comparison with those treated with i.v. MVA–MUC1 alone (P = 0.004; Fig. 1B), with i.v. ODN1826 alone (P < 0.0001), or with control MVA + ODN1826 i.v. (P < 0.0001).

These data indicate a therapeutic advantage of i.v. immunization with MVA–MUC1, which is augmented by the combination with a TLR9 ligand.
Infiltration of kidneys, liver, and lungs by CD3+CD8+ lymphocytes upon i.v. injection of MVA

We postulated that the increased therapeutic effect following i.v. injection of MVA-MUC1 was supported by a localization of immune cells in tumors. We quantified and characterized the CD45+ cellular infiltrate upon s.c. versus i.v. injections of MVA-MUC1. Tumor-implanted kidneys from MVA-MUC1 intravenously treated mice contained more CD45+ cells, T and B lymphocytes than those from subcutaneously treated counterparts (Fig. 2A–C, not significant). Moreover, tumor-implanted kidneys from intravenously treated mice were more significantly infiltrated with CD3+CD8+ T lymphocytes than were the subcutaneously treated counterparts (P < 0.05; Fig. 2E). We next focused on i.v. injection of MVA to further characterize tumor infiltration by CD45+ immune cells upon treatment with different MVA, alone or in combination with the immunostimulating B-CpG, ODN1826. In comparison with kidneys from tumor-free untreated mice, the presence of RenCa-MUC1 tumors did not induce any significant infiltration of CD45+ immune cells (Figs. 2 and 3A–F) with the exception of Treg (CD4+CD25+FOXP3+) lymphocytes (Fig. 3G; P < 0.001). The i.v. injection of control MVA or MVA-MUC1 increased the infiltration of CD45+, CD3+, CD4+, CD8+, and CD19+ cells in tumor-bearing kidneys (Fig. 3A–E). The addition of i.v. administered ODN1826 to i.v. MVA injections significantly increased the infiltration of immune cells, including the immunosuppressive myeloid-derived suppressor cells (MDSC; CD11b+Gr1+; Fig. 3A–F). Of note, i.v. treatment by ODN1826 alone of tumor-bearing animals significantly increased the number of Treg lymphocytes in kidney-bearing tumors (P < 0.01; Fig. 3G). Consistent with these results, results from IHC analyses indicated that tumor cores from MVA-MUC1–treated mice were more infiltrated with CD3+ lymphocytes than were those treated with ODN1826 alone (Fig. 3H and I). This infiltration was similar in invasive margins of tumors (Supplementary Fig. S1).

A significant infiltration of CD45+, CD3+, CD4+, and CD8+ immune cells was also observed in the contralateral kidneys of tumor-implanted mice upon i.v. injection of MVA-MUC1. This effect was not influenced by the addition of ODN1826 (Supplementary Fig. S2). Infiltration of kidneys by immune cells upon i.v. injection of MVA-MUC1 also occurred in tumor-free mice (data not shown). In tumor-bearing animals, we also observed that the lungs and livers were significantly infiltrated with CD45+, CD3+, and CD8+ immune cells upon i.v. treatment with MVA-MUC1 (data not shown).

Importance of CD8+ lymphocytes

The presence of CD3+CD8+ T lymphocytes in kidneys after i.v. injection of MVA-MUC1 suggests an essential role of these cells in the therapeutic activity of MVA-MUC1. The depletion of CD4+ or CD8+ cells before tumor grafting and therapeutic immunization demonstrated that CD8+ T cells were essential for the therapeutic protection against tumor growth observed in this orthotopic tumor model (Fig. 4A). To characterize further the MVA-MUC1–induced CD8+ T cells, we analyzed the anti-MUC1 CD8+ T-cell response following i.v. immunization of tumor-free mice with this vector, using arrays of pools of overlapping 11mer peptides (19), which cover the MUC1 sequence encoded by MVA-MUC1. Using IFNy ELISpot assay, we identified two 11 amino acid peptides (#96 and #118) recognized by sorted CD8+ splenocytes of MVA-MUC1–treated mice (Fig. 4B and inset).

Comparative analyses of s.c. versus i.v. injections of MVA

To better understand the observed antitumor therapeutic differences between i.v. and s.c. injected MVA-MUC1, we injected MVA-GFP s.c. and i.v. at the same dose as in experiments with MVA-MUC1. Six hours after injections,
the percentages of CD8\(^+\) (CD103\(^+\) in tissues) dendritic cells (DC), CD11b\(^+\) DCs, plasmacytoid DCs, macrophages, monocytes, and neutrophils were determined (Fig. 5A–C). Results show that higher percentages of these cells expressed GFP in the spleen following i.v. injection of MVA-GFP, as opposed to s.c. administration. In contrast, it was often the opposite in lymph nodes draining the s.c. injection site, as well as in lungs and livers. As expected (18), tissue distribution analysis using MVA-luciferase indicated that 6 hours following i.v. injection, MVA-luciferase had reached and infected every tissue analyzed, compared with that following s.c. injection (Fig. 5C). The impact of the route of administration on the MUC1-specific T-cell response was also explored. We compared the immune response with MUC1 after s.c. versus i.v. injections of MVA-MUC1. Only one i.v. injection of MVA-MUC1 was sufficient to induce a detectable and significant immune response to MUC1 as determined by IFN\(\gamma\) ELISpot assay (Fig. 5D, left). In contrast, two injections were necessary to observe a significant response via the subcutaneous route (Fig. 5D, right). Surprisingly, the addition of ODN1826 to the respective immunization schemes diminished the specific cellular immune response against MUC1 to background levels.

**Immunophenotyping of kidney-infiltrating lymphocytes**

We next investigated T-cell memory phenotype as well as patterns of chemokine receptor and immune checkpoint markers expression of tumor-bearing kidney-infiltrating CD8\(^+\) and CD4\(^+\) T cells. Splenocytes from matching animals were included for comparison. Following i.v. MVA-MUC1 administration, splenocytes were characterized by a prevalence of CD45RBlow CD62L\(^+\) naive CD8\(^+\) and CD4\(^+\) T lymphocytes (22). In contrast, kidney-infiltrating T lymphocytes were mostly of the CD45RBlow memory phenotype (Fig. 6A). The presence of RenCa-MUC1 tumor cells in kidneys increased the proportion of effector memory CD8\(^+\) and CD4\(^+\) T lymphocytes (CD45RBlowCD62L\(^-\)). Intravenous treatment with MVA-MUC1 further enhanced the proportion of effector memory T lymphocytes. ODN1826 increased the proportion of central memory CD8\(^+\) lymphocytes (CD45RBlowCD62L\(^-\)) and the proportion of naive CD4\(^+\) lymphocytes, but did not modify the memory profile of lymphocytes induced by i.v. immunization with MVA-MUC1 (Fig. 6A). These observations indicate that i.v. injection of MVA-MUC1 increases the proportion of effector memory T cells in the tumor-bearing kidney. This phenomenon is independent of the addition of ODN1826 to the immunization scheme.

Figure 2. Comparative infiltration of tumor-bearing kidneys by CD45\(^+\) cells following s.c. or i.v. treatment with MVA-MUC1. Animals were treated with s.c. or i.v. injection of 5 \(\times\) 10\(^7\) pfu of MVA-MUC1 on days 1 and 8 following tumor implantation. Fifteen days after tumor implantation, kidneys were removed, weighed, mechanically dissociated, numbered, and processed for flow cytometry analysis of CD45\(^+\) cells (A), T CD4\(^-\), T CD8\(^-\) and B lymphocytes (B–E). Results are reported as number of cells per mg of kidney tissue. The mean values \pm SEM (\(n = 11\)) from two independent experiments are represented. Statistical significance was evaluated by Kruskal–Wallis test and Dunn multiple comparison test (*, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\)).
An upregulation of T-lymphocyte receptors specific for chemokines expressed in target tissues following antigen encounter and T-cell proliferation has been described (23). In particular, CCR1, CCR2, CCR3, and CCR5 are necessary to enable an appropriate redistribution of activated T cells (23). Consistent with this, a low proportion of CD8⁺ and CD4⁺ splenocytes expressed these chemokine receptors, whereas, in any analyzed group, most tumor-bearing kidney-infiltrating T lymphocytes were positive for these receptors (Supplementary Fig. S3). In contrast, CXCR3 expression was detected on the surface of approximately 40% of CD3⁺CD8⁺ splenocytes, whereas only about 10% of CD3⁺CD8⁻ lymphocytes from kidneys expressed this receptor (Fig. 6B). Intravenous treatment with MVA-MUC1 significantly increased to about 30% the proportion of CD3⁺CD8⁺ lymphocytes expressing CXCR3 (Fig. 6B). The proportion of CD3⁺CD4⁺ splenocytes and kidney lymphocytes expressing CXCR3 was also low (5%–25%). However, a higher proportion of kidney-infiltrating CD4⁺ lymphocytes from MVA-MUC1-treated animals expressed CXCR3 (Fig. 6B). These results show that i.v. injection of MVA-MUC1

**Figure 3.** Impact of ODN1826 on the infiltration of tumor-bearing kidneys by CD45⁻ cells following i.v. treatment with MVA. Animals were treated as described in the legend of Fig. 1B. Fifteen days after tumor implantation, kidneys were removed, weighed, mechanically dissociated, numbered, and processed for flow cytometry analysis of CD45⁻ cells (A), T CD4⁺, T CD8⁺, B lymphocytes, MDSCs and CD4⁻ Treg lymphocytes (B-G). Results are reported as number of cells per mg of kidney tissue. The mean values ± SEM (n = 10) from two independent experiments are represented. Statistical significance was evaluated by Kruskal–Wallis test and Dunn multiple comparison test (*, P < 0.05; **, P < 0.01; ****, P < 0.001; ***, P < 0.0001). H, in a different set of experiments, kidneys were also processed for quantification of tumor-infiltrating CD3⁺ cells by immunohistochemistry. I, representative images of core tumor section and their infiltration by CD3⁺ cells (magnification, ×40). Scale bar, 50 μm.
immune responses to minimize collateral tissue damage during an immune response. Tumors divert this physiologic process to escape destruction by cytotoxic T lymphocytes. Hence, we monitored the level of expression of PD-L1, Tim3, and LAG3 in our model. Expression of PD-1 on tumor-bearing kidney-infiltrating T lymphocytes was markedly increased upon injection of MVA-MUC1 and/or ODN1826 (Fig. 6C, top, and Fig. 6D). MVA-MUC1 treatment increased the expression of Tim3 on the surface of both CD3+CD8+ splenocytes and tumor-bearing kidney-infiltrating CD3+CD8+ lymphocytes. In contrast, Tim3 upregulation was restricted to CD3+CD4+ tumor-bearing kidney-infiltrating lymphocytes upon ODN1826 treatment (Fig. 6C, middle). LAG3 expression was increased significantly on the surface of CD3+CD8+ splenocytes upon MVA-MUC1 i.v. treatment in comparison with control. Conversely, tumor-bearing kidney-infiltrating CD3+CD8+ lymphocytes from MVA-MUC1–treated animals displayed a lower expression of LAG3. ODN1826 treatment increased the expression of LAG3 on the surface of tumor-bearing kidney-infiltrating CD3+CD8+ lymphocytes. These observations suggest that i.v. injection of MVA-MUC1 is associated with a more exhausted phenotype of T cells infiltrating tumor-bearing kidney.

Analysis of tumor-bearing kidney gene expression

To gain further insight into immune/inflammatory activity in tumors following treatment, we analyzed 84 genes involved in inflammation using qRT-PCR. When compared with normal kidneys, the expression of some genes was modified by the implantation of RenCa-MUC1 tumor cells and not influenced further by treatment of the animals with s.c. injection of MVA-MUC1. However, i.v. treatment with MVA-MUC1 plus ODN1826 (administered s.c. or i.v.) limited the modification of gene expression induced by RenCa-MUC1 tumor cells (Fig. 7A). Among genes whose expression is limited are those encoding chemokines CCL7 and CCL12, and the receptor CCR1, all of which are involved in attracting eosinophils, monocytes, and activated T cells. CCR7 is expressed on many cancer cells and is associated with metastasis to lymph nodes and poor prognosis (24). MVA-MUC1 (i.v.) and ODN1826 treatment also limited the upregulation of the Idol, Nrp3, Tlr4, Tnf, Tlr5, Il6, Il1b, and Casp1 genes, whose products have been described to favor tumor progression through diverse mechanisms (25–34).

A separate set of RenCa-MUC1–induced genes was not modified further by treatment of the animals with s.c. injection of MVA-MUC1 with or without ODN1826 (Fig. 7B). For most of these genes, i.v. injection of MVA-MUC1 with and, in some cases, without ODN1826, amplified the modification of expression induced by tumor cells. Found within this subset are genes coding for chemokines involved in the selective recruitment of DC, T, and NK cells, including CCL5, CCL19, and CXCL11 (35), as well as its corresponding receptor (CXCR3), the gene encoding the B and follicular T helper cells chemotractant CXCL13 (36), the genes encoding the costimulatory molecule CD40L, the senescence/inhibitory receptor KLRG1, the ligand for the death receptor Fas, CD27, which is required for the generation of T-cell memory (37), the genes encoding the immune checkpoint molecules PD-1, CTLA-4, and ICOS, the
genes associated with the Th1 response (IFNγ, IL27RA, STAT4, and TBX-21), as well as the gene encoding lymphotoxin B (Fig. 7B). Data on the remaining genes of our panel are summarized in Supplementary Table S1.

Discussion

We analyzed the impact of the route of injection on the immunotherapeutic activity of MVA-MUC1 in an orthotopic model of renal carcinoma, and report that the i.v. route is superior to the classical s.c. route.

Consistent with work from others on this model (38, 39), the requirement of CD8+ lymphocytes was demonstrated by specific in vitro depletion preceding MVA-MUC1 immunization. We identified two peptides containing H-2d-restricted epitopes indicating that the human MUC1 protein is weakly immunogenic in BALB/c mice, as frequencies of responding T cells were very low (below 10−8). Interestingly, the identified peptides show 88% and 73% homology with the corresponding mouse MUC1 sequence, while the predicted anchor residue positions for the H2-Kd molecules are consistent with those found in the mouse sequence.

Figure 5. Comparative biodistribution and antigenicity of i.v. versus s.c. injection of MVA. A, animals were treated with 5 × 10^7 pfu of MVA-GFP either s.c. (white bars) or i.v. (black bars). Six hours after immunization, indicated organs (DLN, draining lymph nodes) were sampled, mechanically dissociated, and analyzed by flow cytometry to determine GFP expression by the various indicated DC subtypes. B, same as in A, but GFP expression was determined from macrophages, monocytes, and neutrophils. C, same as in A but animals were injected with MVA coding for luciferase and 6 hours after injection animals were perfused and the indicated organs were processed for enzymatic detection of luciferase. D, mice were immunized s.c. (blue) or i.v. (red) at days 0 and 7. Where indicated, ODN1826 was given at days 1 and 8. On days 7 and 14, IFNγ ELISPOT assays were performed using MUC1-specific peptides #96 and #118 for in vitro stimulation. The DFR(eq) method was used to determine statistical significance (*) from background results obtained with an irrelevant peptide. Such background has been subtracted from this representation. The mean values ± SEM (n = 3 or 4) are shown. Representative of two independent experiments.
Intravenous Injection of MVA Targets CD8⁺ T Cells to Tumors

Figure 6. Characterization of tumor-bearing kidney-infiltrating lymphocytes following MVA-MUC1 and ODN1826 i.v. treatments. A, Animals were treated as described in the legend of Fig. 1B. Fifteen days after tumor implantation, kidneys and spleens were sampled, mechanically dissociated, and processed for flow cytometry characterization of the memory profile CD45⁺CD3⁺CD8⁺ and CD45⁺CD3⁺CD4⁺ lymphocytes through surface detection of CD45RB and CD62L. Stacked histograms show the relative proportions of naïve lymphocytes (CD45RBhighCD62L⁺), effector memory lymphocytes (CD45RBhighCD62L⁻), and central memory lymphocytes (CD45RBlowCD62L⁻, black) from spleens (top) and tumor-bearing kidneys (bottom); B, same as in A for the determination of the percentage of CD45⁺CD3⁺CD8⁺ and CD45⁺CD3⁺CD4⁺ lymphocytes expressing CXCR3 from spleens (white) and tumor-bearing kidneys (gray). C, same as in A for the determination of the intensity of expression of PD-1 (top), Tim3 (middle), and LAG3 (bottom) on the surface of CD45⁺CD3⁺CD8⁺ and CD45⁺CD3⁺CD4⁺ lymphocytes from spleens (white) and tumor-bearing kidneys (gray). Medians of fluorescence are represented; nonspecific background from isotype control is subtracted. D, representative example of PD-1 staining on CD45⁺CD3⁺CD8⁺ and CD45⁺CD3⁺CD4⁺ lymphocytes from tumor-bearing kidneys. Isotype staining (gray line), anti-mouse PD-1 staining (black line). The mean values ± SEM (n = 10) from two independent experiments are represented. Statistical significance was evaluated by Kruskal-Wallis test and Dunn multiple comparison test (*, P < 0.05; ***, P < 0.001). Asterisks indicate statistical significant differences from the control group implanted with RenCa-MUC1 tumors.

conserved between the two sequences (40). This observation strengthens the value of this model as the human MUC1 protein cannot be considered as a true xenogenic antigen. It also emphasizes the ability of MVA-based immunization to recall and stimulate low-affinity antigen-specific lymphocyte clones.

It is recognized that T cells generated from antigen-specific immunotherapy must traffic to and infiltrate tumor tissue for optimal efficacy. T-cell infiltration in tumors has been associated with favorable outcome for patients in some of the most prevailing cancers (6–8). In this study, we show that, when compared with s.c. injection, i.v. injection of MVA-MUC1 induces massive infiltration of CD8⁺ lymphocytes in peripheral organs as well as in kidney tumors. This observation is linked neither to the primary site of antigen encounter imprints the generated molecular mechanism rather than a passive diffusion is responsible of this phenomenon. Furthermore, the primary site of antigen encounter imprints the generated antigen-specific T cells with matching migratory capacities (10–12). Hence, the observations described in this article are likely to explain why therapeutic activity is associated with i.v. injection of MVA-MUC1. Results from biodistribution experiments indicate that MVA-encoded antigens were quickly expressed in visceral organs and in APCs from the spleen following i.v. injection of MVA in comparison with s.c. injection. Consistent with this observation, an IFNγ ELISpot study showed that i.v. injection of MVA-MUC1 was better than s.c. injection at inducing quickly detectable cellular immunity specific to MUC1.

Kidney-infiltrating CD8⁺ lymphocytes following MVA-MUC1 i.v. immunization were positive for the exhaustion markers PD-1 and the negative regulator of Th1 cell response Tim3. This suggests that, at this time point, MUC1-specific CD8⁺ T cells may have been exhausted in tumor-bearing
kidneys through engagement of these molecules by their natural ligands PD-L1, PD-L2, and Galectin 9, respectively (42).

Literature concerning the expression of TLR9 on mouse immune cells (43) indicates that the marked increase in the therapeutic activity of i.v.-injected MVA-MUC1 in combination with ODN1826 could be due to an increase in the frequency of MUC1-specific T cells. Surprisingly, our IFNγ ELISpot analysis, using splenic lymphocytes, did not confirm this hypothesis. Pharmacokinetic studies in rodents showed that phosphorothioate bonds oligonucleotides injected i.v. display an elimination half-life of 25 to 50 hours, with the majority of the drug taken up by the liver and kidney (44). In our experiment, i.v. injection of ODN1826 alone did not induce CD8<sup>+</sup> lymphocyte infiltration in kidneys but promoted infiltration of Treg CD4<sup>+</sup> lymphocytes. In contrast, upon combination with i.v.-injected MVA-MUC1, ODN1826 was associated with modifications of the tumor-bearing kidney microenvironment toward an inflammatory response of the Th1 type with recruitment of activated lymphocytes. These observations support a local effect of ODN1826 on kidney TLR9<sup>+</sup> immune cells. This matches the conclusions of others about the importance of the Th1-type immune context (45), while confirming

Figure 7. Inflammatory gene expression analysis in tumor-bearing kidney following MVA-MUC1 and ODN1826 treatments. A, animals were treated as described in the legend of Fig. 1. Applied treatments are indicated above graphs from the top. Fifteen days after tumor implantation, kidneys were sampled and processed for RNA extraction and qRT-PCR analysis. Levels of gene expression among different groups were normalized according to the gene Irf3. Fold-changes in gene expression relative to contralateral tumor-free kidneys of mice injected with RenCa-MUC1 tumors are represented. The set of genes corresponding to those induced by the presence of RenCa-MUC1 tumors, and whose expression is not further modified by s.c. injection of MVA-MUC1, but decreased either by s.c. addition of ODN1826 to s.c. MVA-MUC1 or by i.v. treatment with MVA-MUC1 with or without ODN1826. B, same as in A, but the set of genes corresponding to those weakly induced by the presence of RenCa-MUC1 tumors, and whose expression is amplified by i.v. treatment with MVA-MUC1 especially when ODN1826 is added to the therapeutic scheme. The mean values ± SEM (n = 10) from two independent experiments are represented.
CD8+ lymphocytes infiltration of tumor-bearing kidney as the key factor of i.v. applied viral-based immunotherapy efficiency in this orthotopic model (39). Moreover, we observed that i.v. injection of MVA-MUC1 was sufficient to limit the upregulation of many tumor-promoting genes induced by RenCa-MUC1 tumors.

Recent clinical observations emphasize the good prognostic value of infiltrating CD8+ lymphocytes in various cancers, including renal carcinoma (6–8, 46–48). The clinical version of MVA-MUC1, TG4010, has been shown to increase the survival of patients with metastatic non–small cell lung cancer (49). In this phase IIB trial, TG4010 was injected into patients via the s.c. route in addition to chemotherapeutic standard of care. In light of our preclinical results, patients could benefit from i.v. injection of TG4010. It is now routine to produce constant clinical grade batches of recombinant pox viruses. These replication-competent viruses are injected i.v. and intra-tumorally in clinical studies (50). This, combined with the good safety record of MVA in the clinic (1), makes it reasonable to consider that, after appreciation of the benefit/risk ratio, an MVA vector may receive approval for i.v. injection in humans for advanced cancer therapy.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Intravenous Injection of MVA Virus Targets CD8+ Lymphocytes to Tumors to Control Tumor Growth upon Combinatorial Treatment with a TLR9 Agonist

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