Attenuated Recombinant Vaccinia Virus Expressing Oncofetal Antigen (Tumor-associated Antigen) 5T4 Induces Active Therapy of Established Tumors

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Abstract

The human oncofetal antigen 5T4 (h5T4) is a transmembrane glycoprotein overexpressed by a wide spectrum of cancers, including colorectal, ovarian, and gastric, but with a limited normal tissue expression. Such properties make 5T4 an excellent putative target for cancer immunotherapy. The murine homologue of 5T4 (m5T4) has been cloned and characterized, which allows for the evaluation of immune intervention strategies in "self-antigen" *in vivo* tumor models. We have constructed recombinant vaccinia viruses based on the highly attenuated and modified vaccinia virus ankara (MVA strain), expressing h5T4 (MVA-h5T4), m5T4 (MVA-m5T4), and *Escherichia coli* LacZ (MVA-LacZ). Immunization of BALB/c and C57BL/6 mice with MVA-h5T4 and MVA-m5T4 constructs induced antibody responses to human and mouse 5T4, respectively. C57BL/6 and BALB/c mice vaccinated with MVA-h5T4 were challenged with syngeneic tumor line transfectants, B16 melanoma, and CT26 colorectal cells that express h5T4. MVA-h5T4-vaccinated mice showed significant tumor retardation compared with mice vaccinated with MVA-LacZ or PBS. In active treatment studies, inoculation with MVA-h5T4 was able to treat established CT26-h5T4 lung tumors and to a lesser extent B16.h5T4 s.c. tumors. Additionally, when C57BL/6 mice vaccinated with MVA-m5T4 were challenged with B16 cells expressing m5T4, resulting growth of the tumors was significantly retarded compared with control animals. Furthermore, mice vaccinated with MVA-m5T4 showed no signs of autoimmune toxicity. These data support the use of MVA-5T4 for tumor immunotherapy.

Introduction

There are currently many cancer vaccine immunotherapies being developed using different TAAs[2] as targets (1). A promising new candidate is the h5T4 that was defined by a monoclonal antibody (mAb5T4) raised against human trophoblast glycoproteins (2). 5T4 is expressed by a wide spectrum of cancers but is not detected on most normal adult tissue (3, 4). In colorectal, gastric, and ovarian carcinomas, tumor expression is associated with poorer clinical outcome (5–9). Human 5T4 antigen is a M_r 72,000 transmembrane glycoprotein with the extracellular part comprising two leucine-rich repeat domains, separated by a stretch of intervening hydrophilic amino acids with 7 *N*-linked glycosylation sites and a 44 amino acid cytoplasmic tail (10, 11). 5T4 antigen is concentrated at microvillus projections of the plasma membrane, overexpression in epithelial cells is associated with changes in morphology and increased motility, and the cytoplasmic domain influences actin/cadherin contacts (12). The murine homologue has been cloned and shows 81% amino acid identity (13), and similar biochemical and tissue expression properties (14).

VV and other members of the Poxviridae have been used extensively to induce both antibody and T-cell responses to a variety of antigens including TAAs (15). Reports illustrate that recombinant poxviruses are able to break immune tolerance to specific TAAs in murine models (16) and human clinical trials (17). The highly attenuated VV strain MVA was originally developed as a safe smallpox vaccine and has been used to vaccinate >120,000 people, many of which were very young or elderly, without reported complications (18). MVA was attenuated by extensive passage in chick embryo fibroblast cells. It is replication defective in all of the mammalian cells tested except baby hamster kidney (BHK21) and will not replicate in primary human cells (19–21). MVA has been shown to be an efficacious potential vaccine vector in a variety of infectious disease and tumor therapy models (22). Many of these putative vaccines are being evaluated in clinical trials.

In this report, we demonstrate MVA-h5T4 immunization can protect against, and actively treat, s.c. and i.v. murine tumors expressing h5T4. In addition, recombinant MVA-m5T4 can break tolerance, inducing antibodies, and provide protection to m5T4-positive tumor challenge.

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[2] The abbreviations used are: TAA, tumor-associated antigen; h5T4, human oncofetal antigen 5T4; m5T4, murine homologue of 5T4; MVA, modified vaccinia virus ankara; VV, vaccinia virus; mAb, monoclonal antibody; MOI, multiplicity of infection; pfu, plaque forming units; HRP, horseradish peroxidase; FACS, fluorescence-activated cell sorter; ZP, zona pellucida.

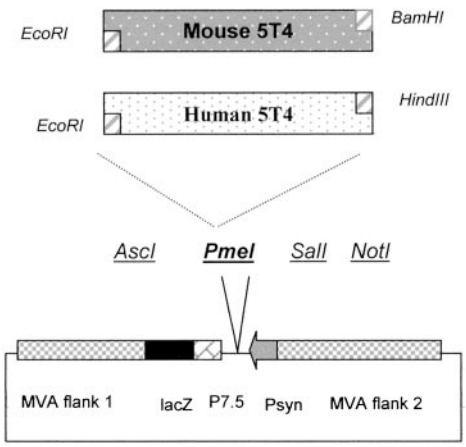

Fig. 1. MVA-5T4 constructs. Pox viral transfer plasmids encoding human and mouse 5T4 were constructed using cDNA ligated into the *PmeI* site of the pox transfer plasmid pLW-22.

Materials and Methods

Mice. Female C57BL/6 ($H2^b$) and BALB/c ($H\text{-}2^d$) mice, ages 8–10 weeks, were obtained from Charles River. Female C57BL/6 ($H2^b$) and BALB/c ($H\text{-}2^d$) mice, ages 8–10 weeks, were used for fertility studies.

Generation of 5T4-transfected CT26 and B16 Tumor Cell Lines. Cell lines CT26 and B16F10 are from a chemically induced BALB/c colon carcinoma (23) and a spontaneous C57BL/6 melanoma, respectively (24). A B16 cell line expressing h5T4 (B16.h5T4) was isolated by transfection using a pCMVα_{neo} vector encoding h5T4 cDNA (11). CT26 cells expressing h5T4 and B16 cells expressing m5T4 were produced using pIRESneo (25) and 5T4 cDNA constructs. Transfected cells were selected using G-418, from 24-h post-transfection at a final concentration of 400 μg/ml for CT26 cells and 1000 μg/ml for B16 cells. Cloned, transfected CT26 and B16 cell lines were screened for expression by FACS or immunohistochemistry using mAb 5T4 or polyclonal rabbit antimouse 5T4 serum raised against a m5T4 extracellular domain-human IgGFc fusion protein prepared as described previously (14, 26). The rabbit was immunized with six s.c. injections of 100 μg m5T4-IgFc fusion protein and the prebleed used as a control. Primary antibodies were detected with appropriate second layer conjugates (see below).

The CT26 pIRESneo h5T4 cell lines were maintained in RPMI 1640 (Dutch modification) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (all from Sigma-Aldrich) and G-418 (400 μg/ml). The B16.CMVα h5T4 and B16 pIRESneo m5T4 were maintained in DMEM and supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Sigma-Aldrich), and G-418(1000 μg/ml; PAA Labs).

Cloning and Production of the MVA-5T4 Constructs. The full-length human or mouse 5T4 cDNA sequences were inserted into the MVA transfer vector plasmid pLW-22 (27). This has MVA flanks, and the insertion site is in the deletion II region that is downstream of a strong synthetic promoter (Fig. 1). Restriction enzyme and sequencing analysis identified transformants containing inserts. Monolayers of 90–95% confluent BHK-21 cells were infected with wild-type MVA. Plasmid DNA/Lipofectin mixture was distributed dropwise over the MVA-infected BHK-21 plate for 5 h at 37°C and then washed. Forty-eight h after transfection, the cells were subjected to several rounds of freeze-thaw/vortexing and used to infect fresh BHK-21 cells. Two days later, recombinant viral plaques or foci expressing the 5T4 protein were identified by immunostaining with mAb5T4 monoclonal antibody or polyclonal rabbit antimouse 5T4. High titer viral stocks were purified by ultra centrifugation through a 36% sucrose cushion as described previously (28). BHK-21 cells were maintained in MEM Eagle, with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum.

SDS-PAGE and Western Analysis. Expression of MVA-5T4 viruses was analyzed using near confluent chick embryo fibroblast cells ($\sim 5 \times 10^5$) infected at a MOI $\sim$1.0 and harvested after 3 days. Cell lysates were prepared in Laemmli buffer to give a final concentration of 1.25×10^3 cells/μl of which 20 μl/well was loaded on a precast Novex 10% Tris-Glycine gel; molecular weight markers were from Roche. Proteins from the gel were then transferred to Hybond-C ECL membrane (Amersham Biosciences) using transfer buffer, 25V (constant) for 2 h. The membrane was blocked (PBS, 0.05% Tween 20, and 5% nonfat dried milk powder). After rinsing briefly with washing buffer (PBS and 0.05% Tween 20), the membrane was incubated with either mAb antihuman 5T4 antibody supernatant (1:20) or polyclonal rabbit antimouse 5T4-IgG Fc (1:1000) for 1 h at room temperature in PBS. After washing, the membranes were incubated for 1 h in either rabbit antimouse HRP (1:1000) or goat antirabbit HRP (1:1000; both antibodies from DAKO), washed, and ECL reagent mix (Amersham; 0.125 ml/cm^2) applied for 1 min followed by exposure to Kodak X-ray film.

***In Vivo* Protection Assays.** Groups of 7 or 10 mice were inoculated i.v. or i.m. with recombinant virus 1×10^7 pfu at days 0 and 21 for MVA-m5T4 or days 0, 21, and 42 for MVA-h5T4 (+/− 1 day). Approximately 21 days after inoculation, BALB/c mice where challenged i.v. with CT26.h5T4 (5×10^5 tumor cells). On day 14 after tumor challenge, BALB/c mouse lungs were removed and stained by inflating them with a solution of Indian ink. Approximately 21 days after inoculation, C57BL/6 mice were challenged s.c. with B16.m5T4 (1×10^5) or B16.h5T4 (5×10^5). Tumors were measured with blinding to the challenge starting on day 3. The product of perpendicular tumor dimensions was determined essentially every other day until reaching 1.24 cm^2. At least two separate experiments were performed in each tumor challenge scenario.

***In Vivo* Active Treatment.** At day 0, BALB/c mice were inoculated i.v. with 5×10^5 tumor cells to establish pulmonary tumors deposits (pseudo-metastases). C57BL/6 mice were inoculated s.c. with 1×10^5 B16.h5T4 tumor cells. On days 3 and 10, groups of 10 mice were inoculated i.v. with 1×10^7 pfu MVA-LacZ, MVA-h5T4, and in some cases, PBS. The short-term study harvested the lungs from challenged BALB/c mice that were stained by inflation with India ink. The long-term study followed BALB/c mice for up to 60 days by daily palpitation of the abdomen. s.c. tumors in C57BL/6 mice were measured from day 3 as above. At least two

separate experiments were performed in each active treatment model.

Antibody (Isotype) Response to h5T4 and m5T4 Antigens. Mouse and human 5T4-IgG Fc fusion proteins, consisting of the extracellular domains of h5T4 or m5T4 and the Fc of human IgG1, were used in ELISA to detect anti-5T4 serological responses. The fusion proteins were produced by cloning into the eukaryotic expression vector sig-*p*Ig plus (Ingenious; R&D Systems), yielding stable expression in transfected Cos-7 cells, and purification using wheat germ agglutinin and protein G affinity chromatography (14, 26). Mouse and human 5T4-IgG Fc fusion proteins were used to coat ELISA plates at 1.0 μg/ml and 0.5 μg/ml, respectively, in 0.1 M (pH 9.5) carbonate buffer. Plates were washed with PBS containing 0.05% (v/v) Tween 20 between steps. Nonspecific binding was blocked by incubation with 2% caesin for 1 h at 37°C. Duplicate or more doubling dilutions of sera (1:250–1:128,000) were analyzed with detection using HRP-labeled rabbit-antimouse immunoglobulin (Dako; 1:1,500) and acidified 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma). To detect the specific mouse IgM and IgG isotypes, HRP-labeled anti-IgG1, IgG_{2a}, $IgG_{2b,}$ IgG3, and IgM (ImmunoKontact) were used (1:2,000) and developed with TMB. Antibody analyses were conducted in C57BL/6 mice given MVA-m5T4 twice (days 0 and 21) and MVA-h5T4 three times (days 0, 21, and 42), with the sera taken 7 days after the last injection; experiments were conducted on pooled sera of 5–10 animals. Similar studies in BALB/c mice used sera taken 7 days after the last injection, ELISA was conducted on sera of individual animals, and results were averaged.

FACS Analysis. Aliquots of 2×10^5 cells in FACS buffer [PBS, containing 0.1% (w/v) sodium azide and 0.2% (w/v) BSA] were incubated with mouse mAb h5T4 or control mouse IgG at 4 μg/ml or rabbit polyclonal anti-m5T4, or prebleed at 1:200 for 45 min, followed by fluorescein-conjugated secondary antibodies (Dako; rabbit antimouse $F(ab_2)$ or swine antirabbit immunoglobulins at 1:20) on ice in the dark. FACS buffer was used to wash the cells and to dilute the antibodies.

Cytotoxic T-Cell Assay. Spleen cells from BALB/c mice immunized previously with MVA constructs or PBS were restimulated *in vitro* with either human 5T4 antigen fusion protein or MVA-h5T4, or control antigen/virus to analyze for the presence of specific CTL. The mice were immunized with MVA-h5T4 or PBS i.v. on days 0, 21, and 42, and spleens taken at 4 and 5 months for *in vitro* stimulation with protein stimulation or MVA, respectively. Erythrocytes in spleen cell suspensions were removed by lysis with 0.1 M ammonium chloride buffer. Normal splenocytes, at 5×10^6/ml, in complete medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 10 μM 2-mercaptoethanol) were incubated with 5T4 fusion protein (50 μg/ml of h5T4-IgG Fc or hIgG) or infected with MVA (MVA-h5T4 or MVA-LacZ, MOI of 10) for 1 h at 37°C. These stimulator cells were washed and added to the autologous primed responder splenocytes at a ratio of 2:1 in a 10-ml final volume and cultured for 5 days. The specific CTL activity of these effector populations was tested *versus* CT26.wt, CT26.h5T4, or CT26.wt infected with MVA-LacZ (overnight at MOI of 10) target cells. Target cells were labeled with ^{51}Cr (0.1 mCi of ^{51}Cr) in normal growth medium for 1 h and then washed three times. Cytotoxicity assays were in complete medium at E:T ratios of 100:1 to 12.5:1 with incubation for 4 h in a V-bottomed, 96-well microtiter plate, and supernatants were counted by Packard Top Count Instrument. Maximum ^{51}Cr release was obtained by lysis of control wells with 2% Tween 20 and spontaneous ^{51}Cr release determined from target wells with only medium. Lysis was calculated according to the following formula: % Specific Lysis = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

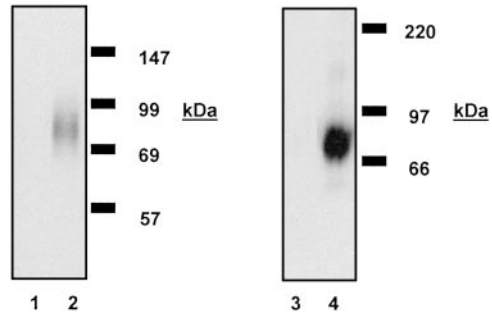

Fig. 2. MVA expression of murine and human 5T4 antigens. Western blots of samples transferred from 10% unreduced SDS-PAGE: Cell lysates from BHK-21 infected with MVA-LacZ (*Lane 1*) or MVA-m5T4 (*Lane 2*) detected with polyclonal rabbit antimouse 5T4-IgG Fc and MVA-LacZ (*Lane 3*) or MVA-h5T4 (*Lane 4*) detected with mAb5T4.

Fertility. The effects of MVA-m5T4 on the fertility of mice were studied. Groups of 5 female mice (BALB/c and C57BL/6) were inoculated i.v. with MVA-m5T4, MVA-LacZ (1×10^7 pfu), or PBS at approximately days 0, 21, and 42. The C57BL/6 mice were mated at day 10, after the last vaccination, and the number of live births and litter size recorded. Similar studies were performed for the BALB/c mice but in addition, numbers and weight of progeny at weaning were recorded for each group. The effect of MVA-m5T4 vaccination on fertility was also studied in this way at days 30 (C57BL/6), 45 (BALB/c), or 60 (C57BL/6) after the last vaccination.

Toxicity. Long-term toxicity was assessed in groups of 5 BALB/c female mice vaccinated with MVA-m5T4, MVA-h5T4, MVA-LacZ (1×10^7 pfu), or PBS, at days 0, 21, 42, and 200. A vet examined the mice on days 148, 311, and 656, and the experiment was terminated on day 660.

Statistical Analysis. Survival in protective and active treatments was analyzed with standard Kaplan-Meier plots (29). Mann-Whitney test (30) was used to compare the effect of the different treatments in the protection experiments or short-term active treatment in BALB/c mice where mice were killed at the same time.

Results

Characterization of MVAs Expressing Murine and Human 5T4 Antigens. The recombinant MVAs encoding the murine and human 5T4 antigens were validated for specific expression by Western blot analysis of unreduced SDS-PAGE of lysates from infected BHK21 cells. Fig. 2 shows that a broad

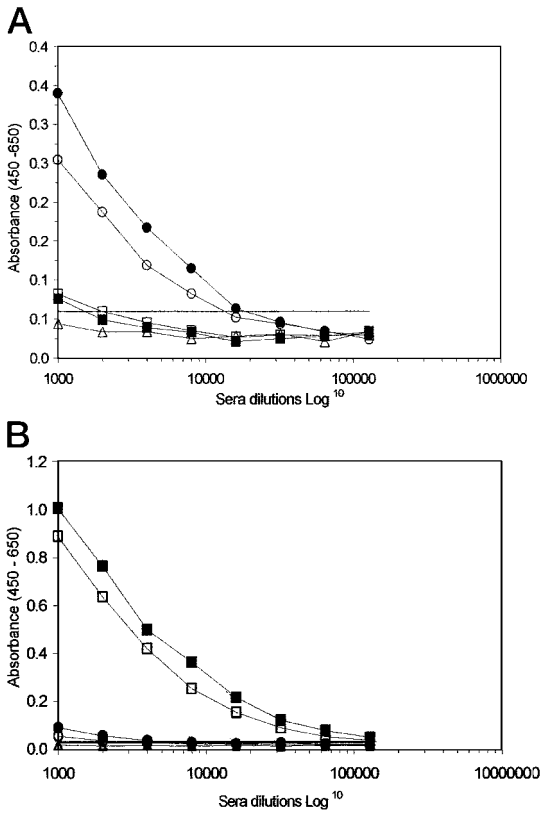

Fig. 3. Immunization with MVA-5T4 induces specific antibodies. BALB/c mice were vaccinated at days 0, 21, and 42, and sera tested for mouse 5T4 (*A*) or human (*B*) 5T4 activity in ELISA at approximately days 28 and 49. 2 × MVA-m5T4, ●; 3 × MVA-m5T4, ○; 2 × MVA-h5T4, ■; 3 × MVA-h5T4, □; 3 × MVA-LacZ, △; titer line.

band (molecular weight M_r 72,000–85,000) is specifically detected by mAb5T4 with MVA.h5T4 but not MVA-LacZ-infected cells (Fig. 2, *Lanes 3* and *4*). The polyclonal anti-m5T4 serum specifically identifies similar murine 5T4 molecules (Fig. 2, *Lanes 1* and *2*). The Western blot 5T4 bands identified are typical of the highly glycosylated human 5T4 molecules (4, 10), and a similar glycosylation pattern has been shown for the murine molecules (13, 14).

Immunogenicity in Mice of Murine and Human 5T4 Delivered by MVA. The ability of the recombinant MVAs encoding human and murine 5T4 antigens to stimulate immunity *in vivo* was tested in two strains of mice. This was assessed by the serological response measured *versus* human and murine 5T4 fusion proteins in ELISA. Initial experiments were in BALB/c and C57BL/6 mice that received i.v. MVA vaccination, with boosts after 21 days and 42 days. Fig. 3 shows BALB/c data for ELISA *versus* m5T4-IgGFc and h5T4-IgGFc of sera taken at 7 days after the second and third immunization with MVA encoding m5T4, h5T4, or LacZ. The LacZ vaccinated animals showed no activity against either mouse or h5T4-IgGFc fusion proteins. As expected, MVA-h5T4 elicits a strong antibody response *versus* h5T4 after two or three immunizations (>128,000 titer expressed as twice the level of MVA-LacZ; Fig. 3*B*). There is evidence of induction of a low level of cross-reactive antibodies to m5T4

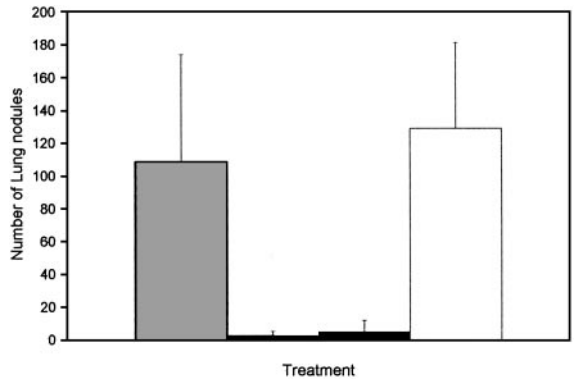

Fig. 4. Vaccination with MVA-h5T4 (i.v. or i.m.) induces protective immunity to CT26.h5T4 tumor challenge. BALB/c mice were vaccinated with 1×10^7 pfu MVA-h5T4 (i.v) ■, MVA-h5T4 (i.m) ▨, MVA-LacZ ▩, and PBS □ at days 0, 21, and 42, and i.v challenged with 5×10^5 tumor cells at day 63. Fourteen days later pulmonary tumor nodules were enumerated in a blinded fashion. The average numbers of nodules obtained using 7 mice/group are shown. Several repeat experiments showed the same level of protection; *bars,* ±SD.

after two or three vaccinations (Fig. 3*A*). The data presented in Fig. 3*A* clearly demonstrate that vaccination with MVA-m5T4 is able to break tolerance and produce an antibody response *versus* m5T4 after two or three immunizations (respective titers 20,000 and 11,000). A low level of cross-reactivity with h5T4 is also detectable (Fig. 3*B*). Such experiments in C57BL/6 mice gave similar results. The influence of route (i.m and i.v.) and number of immunizations (1–3) was also investigated. Irrespective of immunization route, two vaccinations with MVA-m5T4 were sufficient to elicit the maximum titer of anti-m5T4 antibodies, whereas three immunizations with MVA-h5T4 could show marginal improvement by the i.m. route (not shown). In summary, vaccination with MVA-m5T4 can break tolerance to the m5T4 but unlike some murine melanoma self-antigens (31), MVA-h5T4, while highly immunogenic, does not induce a significant antibody response to the m5T4.

MVA-h5T4 Induces Protective Immunity to i.v. and s.c. Tumor Challenge, and Active Immunotherapy of Established Tumors. The efficiency of MVA as a vaccine vector for the induction of a protective immune response to 5T4 antigen was first assessed in BALB/c mice challenged with autologous CT26 tumor cells expressing h5T4 antigen. Mice were vaccinated i.m. or i.v. with MVA-h5T4, MVA-LacZ, or PBS and then given CT26.h5T4 tumor cells i.v. The number of tumor nodules in the lungs was assessed at 14 days after challenge. Fig. 4 shows that the number of tumor nodules in the lungs is significantly lower after vaccination with MVA-h5T4 i.m. or i.v. compared with MVA-LacZ treatment ($P = 0.0002$ and $P = 0.0004$, respectively); there is no difference seen in the number of lung nodules between the MVA-LacZ- and PBS-treated animals. Thus, MVA-h5T4 vaccination can give protection against tumor growth.

The next experiments were designed to test whether MVA-h5T4 vaccination could show efficacy *versus* established tumors. BALB/c mice were inoculated with CT26.h5T4 i.v. and immunized with MVA-h5T4 i.v. on days 3 and 10 to study

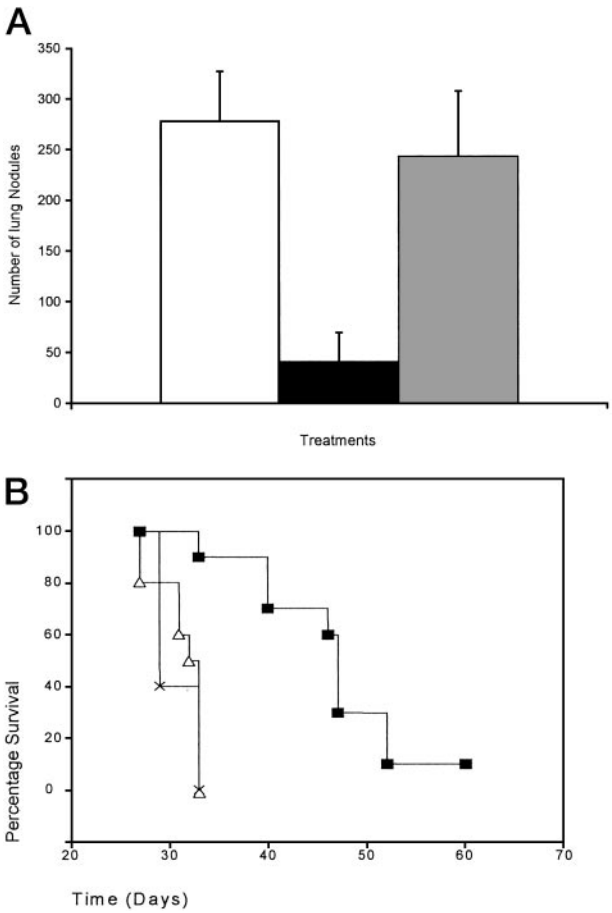

Fig. 5. Active immunotherapy of established pulmonary tumor. BALB/c mice were inoculated with 5×10^5 CT26.h5T4 tumor cells. Three and 10 days after tumor inoculation, mice were inoculated with 1×10^7 pfu i.v. with MVA-h5T4 ■, MVA-LacZ ▨, or PBS □, and 14 days later pulmonary tumor nodules enumerated. The average numbers of metastases obtained using 10 mice/group are shown in *A*. Several repeat experiments showed the same level of active therapy. In *B*, mice were followed for survival for up to 60 days, ■, MVA-h5T4 (i.v); △, MVA-LacZ; ×, PBS. The survival curve shown was obtained using 5–10 mice/group. A repeat experiment showed the same level of active therapy; *bars,* ±SD.

short-term active treatment. As can be seen from Fig. 5*A* there is a significant reduction in the number of tumor nodules in MVA-h5T4 compared with MVA-LacZ- ($P = 0.003$) or PBS-treated mice. In a longer-term study, the BALB/c mice were treated similarly and followed for up to 60 days (Fig. 5*B*). Mice receiving treatment with i.v. MVA-h5T4 displayed a significantly increased survival when compared with control groups inoculated with MVA-LacZ ($P = 0.0001$) or PBS ($P = 0.0002$). Median survival was 47 days for MVA-h5T4, 32 days of MVA-LacZ, and 29 days for PBS. Thus, in the CT26.h5T4 lung tumor model, MVA-h5T4 could provide both protection and active therapy.

The efficacy of the MVA vector for induction of a protective immune response to h5T4 was assessed in a second tumor model. Here, C57BL/6 B16 melanoma cells expressing h5T4 were given to a s.c. site in mice immunized previously with either MVA-h5T4 or MVA-LacZ i.v. or MVA-h5T4 i.m. and growth of tumors measured. As can be seen from Fig. 6,

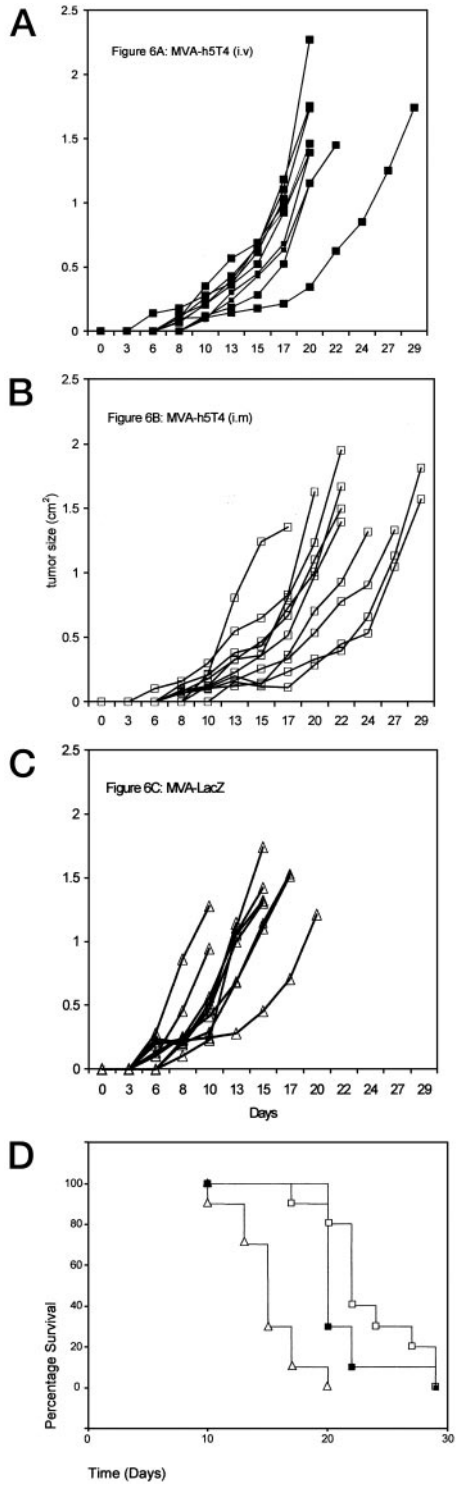

Fig. 6. Vaccination with MVA-h5T4 (i.v. or i.m.) induces protective immunity to tumor challenge with B16.h5T4. C57BL/6 mice were vaccinated on days 0, 21, and 42, and challenged s.c. 21 days later with 5×10^5 B16.h5T4 tumor cells, 10 mice/group. Starting on day 3, the product of perpendicular tumor diameters was determined approximately every other day until reaching 1.24 cm^2. Several repeat experiments showed the same level of protection. The data are displayed as a tumor growth curves (*A–C*) or as a Kaplan-Meier survival curve (*D*); treatment with MVA-h5T4 (i.m.) □ or (i.v.) ■ significantly delays the development of the tumors, or prolongs the survival when compared with inoculation with MVA-LacZ △.

A–C, treatment with MVA.h5T4 (i.m. or i.v.) delays the development of the B16.h5T4 tumors when compared with immunization with MVA-LacZ; the differences are statistically significant for i.v. and i.m. MVA-h5T4 vaccination compared with MVA-LacZ (Mann-Whitney comparing time to 1 cm^2; i.m. $P = 0.00067$ and i.v $P = 0.0002$, respectively). The data are also displayed by Kaplan-Meier plot (Fig. 6*D*), and there is significantly improved survival for mice immunized with MVA-h5T4 compared with MVA-LacZ immunized mice by either i.m. or i.v. routes ($P = 0.00001$ and $P = 0.0001$, respectively). Median "survival" was 20 days for MVA-h5T4 (i.v.), 22 days for MVA-h5T4 (i.m.), and 15 days for MVA-LacZ.

Therefore, protection is established in the B16 tumor model, and experiments to test active therapy in the latter were also conducted. C57BL/6 mice were inoculated with B16.h5T4 s.c. and received either MVA-LacZ or MVA-h5T4 i.v. at days 3 and 10 with tumor growth followed until the tumors reached 1.24 cm^2. Mice that received treatment with MVA-h5T4 displayed a marginally significantly increased "survival" compared with the control animals inoculated with MVA-LacZ ($P = 0.007$). Median survival was 21 days for MVA-h5T4 (i.v) and 16 days for MVA-LacZ (not shown).

Therefore, in two different mouse strains with different routes of tumor challenge, immunization with MVA-h5T4 can deliver both protection and therapy of murine tumors expressing the human 5T4 antigen.

MVA-m5T4 Induces Protective Immunity to Tumor Challenge in a Self-Antigen Model. B16 melanoma cells, which do not express m5T4, were transfected with m5T4 cDNA to provide a self-antigen tumor model. To evaluate the efficacy of MVA as a vaccine vector for the induction of a protective immune response to 5T4 in this model, C57BL/6 mice were inoculated with MVA-m5T4 (i.v.) on days 0 and 21, and animals challenged with B16.m5T4 s.c. around day 42. The growth of B16.m5T4 tumors was significantly delayed or failed in animals vaccinated with MVA-m5T4 when compared with MVA-LacZ immunization. Kaplan-Meier survival plots are shown in Fig. 7; MVA-m5T4 induced statistically significant protective immunity ($P = 0.00024$). Median survival was 43 days for MVA-m5T4 (i.v) and 25 days for MVA-LacZ.

Preliminary analysis of active therapy was also performed. C57BL/6 mice were inoculated with B16.m5T4 s.c. and received either MVA-LacZ, MVA-m5T4 i.v., or PBS at days 3 and 10, and were followed until the tumors reached 1.24 cm^2. Mice receiving treatment with MVA-m5T4 display increased survival when compared with a control group inoculated with PBS ($P = 0.0424$), but this is not statistically significant when compared with MVA-LacZ ($P = 0.3629$). Median survival was 27 days for MVA-m5T4 (i.v), 25 days for MVA-LacZ, and 23 days for PBS (data not shown).

Lack of Detection of CTL to h5T4 in MVA-h5T4-vaccinated Mice. In some tumor protection models it has been possible to demonstrate CTL recognizing the TAA. This was investigated in BALB/c animals that had been immunized three times with MVA-h5T4 or control animals that received PBS. *In vitro* restimulation of spleen cells from MVA-h5T4 *in vivo* primed animals with either MVA-LacZ- or MVA-h5T4-infected autologous lymphocytes was able to generate significant CTL activity *versus* MVA- (LacZ or h5T4) infected CT26 cells targets (*e.g.,* E:T, 50:1 gave 59% and 35% specific cytotoxicity, respectively) but not uninfected CT26 targets (7% and 10% specific cytotoxicity, respectively). Spleen cells from PBS control mice stimulated with MVA-h5T4-infected stimulators did not lyse CT26, CT26.h5T4, or CT26 infected with MVA-h5T4. In addition, splenocytes from the control or immunized animals were restimulated *in vitro* with autologous lymphocytes alone, loaded with h5T4-IgFc fusion protein or hIgG. When the effector populations generated were tested for cytotoxicity *versus* CT26, CT26.h5T4, or CT26 infected with MVA-LacZ, no cytotoxicity was seen. Thus, vector-specific CTLs can be easily detected after MVA restimulation *in vitro,* but no anti-h5T4 cytotoxicity was found. 5T4 CTLs may be relatively infrequent compared with MVA activity and, therefore, not detectable by chromium release assay. However, it is possible that other types of immunity may account for the observed tumor protection or therapy.

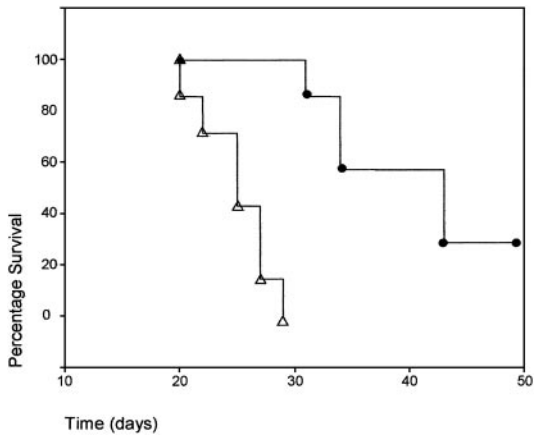

Fig. 7. Vaccination with MVA-m5T4 (i.v.) delays the development of tumors when challenge with B16.m5T4. Groups of 7 C57BL/6 mice were vaccinated at days 0 and 21. and challenged s.c. 21 days later. Kaplan Meier survival curves (maximum allowed tumor size) for MVA-m5T4 ● and MVA-LacZ △ immunized mice are presented.

CTL generation is generally favored by a Th1 type response. Some insight into the nature of the Th response may be inferred from the presence/levels of different IgG subclasses against the 5T4 antigen in the serum of the vaccinated mice. The levels of IgM and IgG subclasses against either human or mouse 5T4 antigen were measured after three or two MVA vaccinations. No significant IgM levels were detected in MVA-m5T4- or MVA-h5T4-injected mice. Titers of anti-h5T4 or anti-m5T4 IgG1, IgG_{2a}, IgG_{2b}, and IgG3 responses in C57BL/6 mice were of the order (×1000): 20, 2.5–4, 64, 4–22, and 8–10, 1–1.5, 30–32, 1.5–15, respectively. For BALB/c mice the results were respectively (×1000): 2000, 320, 160, 200, and 15, 13, 2.5, and 3. The presence of either IgG_{2a} or IgG_{2b} and IgG1 is consistent with a mixed Th1 and Th2 response.

MVA-m5T4 and MVA-h5T4 Has No Toxicological Effect on Mice. Any use of MVA with a self-antigen as the immunogen might lead to deleterious autoimmunity. In a long-term

Table 1 MVA-m5T4 does alter fertility

A. Inoculation with MVA-m5T4 does not effect fertility of C57BL/6 mice[a]

	10 day study			30 day study	60 day study
	MVA-m5T4	MVA-LacZ	PBS	MVA-m5T4	MVA-m5T4
No. of pregnancies	4/5	4/5	3/5	4/5	5/5
No. live births	24	30	18	22	33
Average litter size	6	7.5	6	5.5	6.6

B. Inoculation with MVA-m5T4 does not effect fertility of BALB/c mice or the health of their pups[b]

	10 day study			45 day study
	MVA-m5T4	MVA-LacZ	PBS	MVA-m5T4
No. of pregnancies	5/5	4/5	4/5	4/4
No. live births	30	23	22	24
Average litter size	6	5.75	5.5	6
Ratio F:M	13:11	14:5	11:11	15:9
Average weights	10.4 g	11.2 g	10.1 g	11.4 g

[a] C57BL/6 mice were inoculated with MVA-m5T4, MAV-LacZ, or PBS at 0, 21, and 42 days; the mice were then mated at day 10 after last inoculation. MVA-m5T4 groups were also mated at days 30 and 60 after last inoculation.
[b] BALB/c mice were inoculated with MVA-m5T4, MAV-LacZ, or PBS at 0, 21, and 42 days; the mice were then mated at day 10 after last inoculation. An MVA-m5T4 group was also mated at 45 days after last inoculation.

study, mice immunized four times with MVA-m5T4 (days 0, 21, 42, and 200) were examined by a vet at days 148, 311, and 656. All of the mice appeared healthy in relation to coat condition, mobility, and weight. There were no signs of wasting or respiratory problems. There were no obvious differences between mice that have received PBS and those injected with the recombinant MVA expressing mouse or human 5T4. Four immunized mice died of natural causes on days 639 and 655 (MVA-m5T4), day 639 (MVA-h5T4), and on day 547 (MVA-LacZ).

MVA.m5T4 Has No Adverse Effect on Fertility in Mice. Because in humans 5T4 is an antigen expressed by trophoblast of the placenta the influence of breaking tolerance to m5T4 on pregnancy was investigated. Mice vaccinated with MVA-m5T4, MVA-LacZ, or PBS were mated at day 52 after vaccinations at 0, 21, and 42. For C57BL/6 animals there were no differences in the number of live births or average litter size between these groups. Data from animals mated at 30 and 60 days after final vaccination with MVA-m5T4 did not show any difference from the latter groups (Table 1*A*). Similar studies for BALB/c animals also reported the number of progeny surviving to weaning as well as their weight and sex. As seen in Table 1*B* there were no differences in the three different vaccination groups mated at day 10; the day 45 post-MVA-m5T4 vaccination mated progeny were similar. No difference in the average weights on weaning, or male to female ratio or any other defects were detected.

Discussion

In this report, the use of a recombinant MVA expressing a novel human TAA, 5T4-oncofetal antigen, for cancer immunotherapy has been evaluated. We have shown that vaccination (i.v. or i.m.) with MVA-h5T4 induces both protective immunity and active tumor therapy in BALB/c and C57BL/6 mouse models. The data from the C57BL/6 self-antigen B16 melanoma model shows that vaccination with MVA-m5T4 can induce protection to tumor challenge with a trend for active therapy.

MVA-h5T4 does not induce a significant antibody response against the m5T4 antigen, whereas MVA-m5T4 induces a serological response to the autologous m5T4 self-antigen but no significant activity *versus* the human 5T4 antigen. This is consistent with MVA-m5T4 immunization breaking tolerance or *de novo* activation in an otherwise unstimulated repertoire. Studies of m5T4 expression by RNase protection (13) and immunohistochemistry (14) have confirmed the limited expression in adult tissues, which is similar to that described for human expression (3). An important consideration is the consequence for induction of immunity (breaking tolerance) to an oncofetal antigen with some (albeit restricted) expression in the adult and the possibility of antigestational effects. Indeed, breaking tolerance to ZP protein ZPB in female rabbits using a recombinant myxoma virus followed by purified protein boosts renders 80% of the animals infertile (32). Similar studies in mice using the ZP3 glycoprotein have shown that female mice infected with a recombinant ectromelia virus encoding ZP3 were infertile for 5–9 months (33). The analysis of fertility of female mice vaccinated with m5T4-MVA showed no evidence of any influence on fecundity or the health of the progeny. In addition, the vaccinations showed no long-term toxicity in the adult animals supporting use of 5T4 as an immunotherapy target.

Several tumor immunotherapy models have shown that CD8 and/or CD4 T cells are essential for antitumor activity *in vivo*. For example, immunization with VV or adenovirus vectors expressing HPV E6 and E7 generated protective CD4- or CD8-dependent tumor immunity, depending on the mouse model used (34). In MUC1-transgenic mice (35), MUC1 peptide vaccination induces CD8 T-cell-dependent tumor rejection. Others have reported the relevance of antibodies of the IgG_{2a} isotype mediating antibody-dependent cellular toxicity as an important protective mechanism against virus-induced tumors (36). In various melanoma TAA models, evidence for the prime role of CD8 (37) or CD4 (16) T-cell-mediated components of immunity have been documented. In immunizations based on murine immunoglobulin idiotypes for treatment of syngeneic lymphoma, anti-idiotypic antibodies (38) or alternatively specific T-cell responses (39) can provide protection against tumor challenge.

TRP-1 is a TAA expressed by B16 melanoma cells, and immunization with VV-TRP-1 protects against tumor challenge. It also induces autoimmune vitiligo in the C57BL 6 mice, with high titer IgG antibodies but no evidence of CTL recognizing TRP-1 (16). Furthermore, VV-TRP-1-vaccinated C57BL/6 CD8 knockout mice are still able to provide for tumor protection and exhibit vitiligo. This work established the critical importance of CD4 cells in generating tumor immunity. Other studies have shown that immunity generated against poorly immunogenic proteins can display a pronounced dependency on CD4 cells (40, 41).

The precise combinations of humoral and cellular immunity, which are optimal for antitumor therapy, are difficult to

define and may vary for different tumor types and TAA targets. In this study, although immunization with 5T4-MVA can induce anti-MVA-specific CTL after *in vitro* restimulation with virus infected cells, no h5T4-specific (or m5T4 not shown) CTL activity was seen. We also failed to detect h5T4-specific CTL when MVA-h5T4-primed lymphocytes were restimulated with the h5T4 fusion protein. The possibility exists that there are an excess of vector-specific T cells after the multiple vaccinations, which make the detection of a relatively low frequency of 5T4-specific effectors more difficult.

Although we were unable to demonstrate CTL *versus* 5T4 antigen, generation of the latter is usually the result of a CD4 Th1 helper-type response. The presence of IgG2a and/or Ig2b isotype antibodies to 5T4 antigen in specifically vaccinated mice suggests the potential for such a CTL response. Specific IgG1 antibodies were also detected, which is consistent with a mixed Th1 and Th2 response. Other studies using the MVA vectors for generating tumor immunity examined cytokine profiles, which are consistent with Th1 responses (42). It remains a possibility that the observed antibody responses have been driven by a T-independent mechanism with class switching promoted by bystander cytokine release derivative from the vaccinia-induced response (43). If the latter is the case then the antibodies induced may be responsible for the observed tumor protection and therapy. Additional experiments should evaluate the T-cell responses measured by ELISPOT and different cytokine release assays (44). The latter may influence tumor immune mechanisms but not be detectable in cytotoxicity assays (45). Interestingly, more recent studies using a modified MVA-h5T4 in the CT26 models have indicated that antitumor effects are CD8 independent.[3]

Many models show protection from tumor challenge when mice are prevaccinated with the TAA; however, it is rare that simple vaccination with a vector expressing the TAA without the aid of immunological cofactors, *e.g.,* IL-12 and B7.1 (46), can treat established tumors. In this report, MVA-h5T4 was effective at treating both CT26 lung and B16 s.c. tumors. The B16.m5T4 tumor model showed evidence for autologous MVA-m5T4 protection, but active therapy was not optimal. It will be important to investigate additional strategies to maximize therapy in the autologous vaccination model. These should include methods to enhance the efficacy of VV-based tumor vaccines, *e.g.,* coexpression of immune enhancer molecules and heterologous prime boost regimens (17, 47, 48). Alternative strategies to enhance tumor immunity include using anti-CD40 antibodies (49), CTLA-4 blockade and depletion of CD25+ regulatory cells (50), and or direct expression of TAA in dendritic cells (51).

Because 5T4 is expressed at the surface, it is a potential target for both T cell and antibody-mediated cell killing. Antibody-dependent mechanisms may also be advantageous considering the numerous reports of HLA downregulation in human tumor cells, which allows immune escape from CTL effectors (52). Parallel approaches aimed at exploiting 5T4 antibody delivery of therapy have yielded encouraging results (53, 54).

In summary, the data from these preclinical studies in mice have provided support for the implementation of a clinical trial of a modified form of the MVA-h5T4 vaccine for therapy of late-stage colorectal cancer.

[3] R. Harrop and M. W. Carroll, unpublished observations.

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