Inhibition of tumor growth by recombinant vaccinia virus expressing GA733/CO17-1A/EpCAM/KSA/KS1-4 antigen in mice

Jan Zaloudik,¹ Weiping Li,¹ Lutz Jacob,¹ Marie Paule Kieny,²
Rajasekharan Somasundaram,¹ Bruce Acres,² Hong Song,¹ Tianqian Zhang,¹
Jian Li,¹ and Dorothee Herlyn¹

¹The Wistar Institute, Philadelphia, Pennsylvania 19104, USA; and ²Transgene S.A., Strasbourg, France.

The human colorectal carcinoma (CRC)-associated GA733 antigen (Ag), also named CO17-1A/EpCAM/KSA/KS1-4, has been a useful target in passive immunotherapy of CRC patients with monoclonal antibody (mAb) and in active immunotherapy with anti-idiotypic antibodies or with recombinant protein. These approaches have targeted single epitopes (monoclonal anti-GA733 antibodies and anti-idiotypic antibodies) or extracellular domain epitopes (recombinant protein), primarily by B cells. To determine whether a reagent that induces immunity to a larger number of both B- and T-cell epitopes might represent a superior vaccine, we analyzed the capacity of full-length GA733 Ag expressing multiple potentially immunogenic epitopes and encoded by recombinant vaccinia virus (VV GA733-2) to induce humoral, cellular, and/or protective immunity in mice. VV GA733-2 induced Ag-specific antibodies that reacted predominantly to unknown epitopes on the Ag and lysed Ag-positive CRC targets in conjunction with murine peritoneal macrophages as effector cells. Immunized mice developed Ag-specific, proliferative and delayed-type hypersensitive lymphocytes. VV GA733-2 inhibited growth of ras-transformed syngeneic tumor cells expressing the human GA733 Ag in mice. These results suggest the potential of VV GA733-2 as a candidate vaccine for patients with CRC, possibly in combination with recombinant GA733-2-expressing adenovirus, which has been shown to induce cytolytic antibodies and T cells as well as tumor protective effects in mice. The combined vaccine approach may be superior to the use of either vaccine alone in patients who are pre-immune to both viruses.

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The 40-kDa glycoprotein GA733/CO17-1A/EpCAM/KSA/KS1-4 was originally defined by monoclonal antibodies (mAbs) GA733 and CO17-1A, which bind to different epitopes on this antigen (Ag).^{1–3} The Ag, referred to hereafter as GA733 Ag, is primarily expressed on colorectal carcinomas (CRCs), gastric, and pancreatic carcinomas. Approximately 85% of the metastatic CRC lesions from various patients, and >80% of the cells within a lesion, are positive.^{4–7} Thus, GA733 is a suitable target for active immunotherapy of these cancers. Although the Ag is also expressed on some normal tissues, such as gastrointestinal, lung, breast, and thyroid tissues,^{4–7} the density of the Ag is higher on colonic tumor tissues than on normal colon tissues as determined *in vitro*,¹ *ex vivo*,^{8,9} and *in vivo*.¹⁰ GA733 is not shed into the patient's blood, which may facilitate tumor targeting by Ab and/or T cells.¹¹ Passive immunotherapy of cancer patients with mAb CO17-1A in doses up to 10 g has been well tolerated.¹² A phase II trial with 189 patients randomized to treatment with mAb CO17-

1A or placebo control and a median follow-up of 7 years has demonstrated a significant ($P < .01$) increase in survival of the mAb-treated patients.¹³

In approaches to active immunotherapy targeting the GA733 Ag, patients with cancer mounted humoral^{14–18} and cellular (delayed-type hypersensitive and helper T cells^{17,18}) immune responses to the Ag following the administration of alum-precipitated anti-idiotypic antibodies (Ab2) that functionally mimic the CO17-1A or GA733 epitope. These immune responses may underlie the clinical responses observed in some of the treated patients.^{14,18}

The GA733 Ag expresses multiple potentially immunogenic epitopes and in animals, has elicited more effective antitumor immunity than Ab2 mimicking only one epitope on the Ag.¹⁹ Patients with gastrointestinal carcinoma immunized with alum-precipitated GA733-2E (extracellular domain) protein derived from recombinant baculovirus²⁰ showed enhanced humoral and cellular immune responses compared with Ab2-treated patients.²¹ These clinical trial data clearly demonstrate that induction of active specific immunity to the Ag is possible.

Here, we tested the ability of a recombinant vaccinia virus (VV) expressing full-length GA733 Ag²² (VV GA733-2) to induce humoral, cellular, and protective immunity in mice.

Address correspondence and reprint requests to: Dr Dorothee Herlyn, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA. E-mail: dherlyn@wistar.upenn.edu

Unlike GA733-2E, which expresses only extracellular domain epitopes, VV GA733-2 expresses both extracellular and intracellular domain epitopes of the Ag, thus increasing the number of potentially immunogenic epitopes. Importantly, intracellular replication of VV leads to endogenous Ag expression, which favors CTL induction. Moreover, the GA733 gene product is amplified on replication of the viral vector, thus increasing the Ag load. Whether viral replication also induces cytopathic effects that diminish the yield of Ag presented remains unknown. However, virus-lysed host cells will likely attract immune and Ag-presenting cells due to local inflammation, necrosis, and apoptosis associated with the self limiting viral infection.

We find that VV GA733-2 elicits tumor cytotoxic antibodies and proliferative and delayed-type hypersensitive lymphocytes. VV GA733-2 also prevents growth of syngeneic Ag-positive tumor cells in mice. The high amino acid sequence homology between the mouse and human GA733 Ag in normal tissues,²³ and the similarity in normal tissue distribution²⁴ makes the mouse a relevant model for experimental active immunotherapy against the GA733 Ag.

Materials and methods

Tumor cells

Balb/c 3T3 cells (a gift of Pierre Chambon, Strasbourg, France) were transfected with the ras oncogene in plasmid pVEJB²⁵ using CaPO₄ precipitation. Cells with the transformed phenotype (loss of contact inhibition) were isolated and grown. Clones were tested for tumorigenicity in Balb/c mice. A tumorigenic clone was transfected with the human GA733-2 cDNA²² and selected with G418 (Gibco BRL, Grand Island, NY). FACS analysis revealed H-2k^d expression in ~98% of the transfectants and GA733 Ag expression in ~86% of the cells *in vitro* (not shown). GA733 Ag expression in transfectants injected *in vivo* followed by *ex vivo* culture was comparable to Ag expression by the cells cultured *in vitro* only (not shown), emphasizing the *in vivo* stability of the transfected Ag. CT-26-GA733-2 and CT-26-ASEN (control) transfectants have been described.²⁶ Murine P815 mastocytoma cells, murine Yak natural killer target cells, and human SW1116 CRC cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Melanoma cell line WM9 has been described.²⁷ Murine cell lines were grown in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum (FBS), whereas human cell lines were grown in Leibovitz L-15 medium supplemented with 10% FBS.

Antibodies and Ags

mAbs CO17-1A, GA733, and M77 bind to different epitopes on the GA733 Ag.¹⁻⁴ Anti-CRC mAb CO-029, which binds to an unrelated CRC Ag, has been described.²⁸ Native and recombinant GA733-2E proteins, the latter derived from recombinant baculovirus, were used in *in vitro* lymphocyte stimulation and enzyme-linked immunosorbent assay (ELISA), respectively, and have been described.²⁰

Recombinant VV and adenovirus (Ad) expressing GA733 Ag

The GA733-2 cDNA was subcloned as an *Sma*I-*Pst*II fragment into a plasmid under the control of the VV promoter pH5R. VV GA733-2 was generated by homologous recombination of this plasmid in chicken embryo fibroblasts with the genome of VV Copenhagen.²⁹ As control VV, VV Copenhagen strain²⁹ was used. Ad GA733-2 has been described.³⁰

Immunization and tumor challenge

Six- to 8-week-old female Balb/c mice were used in all immunizations. For antibody induction, seven mice per group were immunized by tail scarification with 10⁶ to 10⁸ plaque-forming units (PFU) of VV GA733-2 or VV on days 1, 8, and 38. Sera were obtained on days 28 and 60. For induction of proliferative lymphocyte responses, five mice per group were immunized with 10⁷ PFU per mouse of VV GA733-2 or VV on days 1, 8, and 38. Two weeks after the last immunization, mice were sacrificed and regional lymph nodes were removed for lymphocyte proliferation assays. For induction of cytolytic T-lymphocyte responses, mice were immunized once or three times (on days 1, 30, and 60) with 10⁷ PFU of VV GA733-2 or VV and regional lymph node lymphocytes were collected 2 weeks later. For induction of delayed-type hypersensitivity (DTH) responses, mice (six per group) were immunized with 10⁷ PFU of VV GA733-2 or VV once and DTH reaction was determined after 7 days.

Because previous tumor protection studies using murine colon carcinoma CT-26 cells expressing the GA733 Ag revealed significant growth inhibition by low doses of control VV (results not shown), Balb/c 3T3-ras-GA733-2 transfectants were used in the tumor growth inhibition studies. The minimum number of GA733-2-ras transfectants required to induce s.c. tumors in 100% of the animals was approximately 1×10⁶ cells per mouse (not shown). To prevent tumor induction, mice (10 per group) were immunized with 10⁷ PFU of VV GA733-2 or VV on days 1, 8, and 38 and injected s.c. with 10⁶ 3T3-ras-GA733-2 tumor cells on day 52. To inhibit growth of established (vascularized) tumors, mice (eight per group) were injected s.c. with 10⁶ tumor cells on day 0 and immunized with VV GA733-2 or VV on days 5, 12, and 43. In both tumor growth inhibition experiments, tumor volumes in cubic millimeters (maximal width×length×height) were measured every 3 days with a caliper.

Ab binding assays

Binding of serum antibodies of immunized mice to tumor cells was determined in mixed hemadsorption assay (MHA) using sheep red blood cells (SRBCs) sensitized with mouse anti-SRBC antibody to which goat anti-mouse IgG had been bound.²⁰ Results were expressed as percent tumor cells binding SRBC. Binding of serum antibodies to GA733-2E protein was tested in ELISA using peroxidase-labeled goat anti-mouse IgG (ICN Biomedicals, Costa Mesa, CA) and substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) to detect antibody binding.

Figure 1 VV GA733-2 elicits Ag-specific cell-binding antibodies in mice. Seven mice per group were immunized by tail scarification with 10^6 to 10^8 PFU of VV GA733-2 or 10^8 PFU VV on days 1, 8, and 38. Sera were obtained on day 60. Binding of sera to GA733 Ag-positive SW1116 cells was tested in MHA. Sera from VV GA733-2 immunized mice showed significant ($P < .05$) binding to SW1116 cells compared with control mice immunized with VV at all serum dilutions tested. Data are given as mean $\pm$ SD of seven mice per group.

Ab binding inhibition assays

Inhibition of mAb 17-1A, GA733, M77, or CO29-10 binding to SW1116 CRC cells by the sera of VV GA733-2- or VV-immunized mice was determined in inhibition radioimmunoassay (RIA). 125 I-labeled mAb at concentrations showing $\sim 50\%$ maximal binding (10,000 cpm of 125 I-17-1A; 2,500 cpm of 125 I-GA733, M77 or CO29-10 per well of microtiter plate) was added to SW1116 cells preincubated with various dilutions of sera from immunized mice for 1 hour at room temperature. Binding inhibition of 125 I-labeled mAbs by either pre-immune or post-immune sera was determined relative to buffer controls.

Antibody-dependent cell-mediated cytotoxicity (ADCC) assays

Sera from immunized mice were analyzed for ADCC reactivity as described.³¹

Lymphocyte stimulation and proliferation assay

Lymphocyte proliferation assays were performed as described.²⁶ Regional lymph nodes from immunized mice were removed and cell suspensions were prepared. Cells were stimulated at 1×10^5 /well/0.2 mL of RPMI-1640 medium (Gibco BRL; supplemented with 10% FBS, 50 μ g/mL gentamycin, 2×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, and 2 mM L-glutamine) with different concentrations (20 and 5 μ g/mL) of native full-length GA733 Ag or with 10^5 UV-irradiated splenocytes infected with 10^7 PFU of VV GA733-2 or VV for 3 days. Full-length, rather than extracellular domain, GA733 Ag was used for these studies to detect potential activation of T lymphocytes by intracellular epitopes present in the VV GA733-2 vaccine. Triplicate cultures were then pulsed overnight with 1 μ Ci/well of [3 H]TdR and harvested by a Harvester 96 system

(Tomec, Orange, CT). Radioactivity bound to the cells was determined in a Matrix 96TM direct beta counter (Packard, Meriden, CT). Results are expressed as stimulation index (SI).

$$SI = \frac{\text{cpm (experimental cultures)}}{\text{cpm (control cultures)}}$$

Lymphocyte cytotoxicity assay

For CTL induction, lymphocytes were stimulated with P815 cells infected with 100 PFU/cell of either VV GA733-2 or Ad GA733-2 and irradiated with 10,000 rad (cesium source). Infected P815 cells expressed the GA733 Ag, whereas infected CT26 and 3T3-ras cells did not as determined by FACS analysis (not shown). Target cells included uninfected P815 cells, P815 cells infected with VV GA733-2, Ad GA733-2 or the corresponding control viruses; CT26-GA733-2 or CT-26-ASEN cells; or Yak cells. Balb/c 3T3-ras-GA733-2 cells could not be used as targets because of high spontaneous 51 Cr release (see below) of the labeled cells.

Lysis of target cells by mouse lymphocytes was determined *in vitro* using a standard 51 Cr-release assay.

DTH assay

To determine DTH responses, immunized mice were challenged intradermally (i.d.) with 1 μ g of native GA733 protein into the left ear and with 1 μ g of bovine serum albumin (BSA) into the right ear. Increase in ear thickness was measured at 18, 24, and 48 hours after challenge.

Figure 2 VV GA733-2 elicits Ag-specific antibodies binding specifically to recombinant GA733-2E protein in mice. Mice (7 per group) were immunized with 10^6 PFU of VV GA733-2 or VV as described in the Figure 1 legend and sera were collected at the indicated days. Binding of sera (diluted 1:50) to GA733-2E extracellular domain protein was determined in ELISA. Sera from experimental mice showed significantly ($P < .05$) higher binding to GA733-2E compared with sera from control mice on days 28 and 60. The experimental sera did not bind to BSA (not shown). Data are given as mean $\pm$ SD of seven mice per group.

Figure 3 VV GA733-2 elicits Ag-specific ADCC-reactive antibodies in mice. Mice (seven per group) were immunized with 10^6 PFU of VV GA733-2 or VV as described in Figure 1 legend. ADCC reactivity of sera (at optimal dilution 1:200) against GA733 Ag-positive SW1116 CRC cells was tested in a 3-day [3 H]thymidine-incorporation assay using thioglycolate-elicited murine peritoneal macrophages as effector cells. Specific lysis by sera of VV GA733-2-immunized mice on day 60 post-immunization was significantly ($P < .05$) higher than the corresponding pre-immunization value and the value obtained in VV-immunized mice on day 60. Sera did not significantly lyse Ag-negative WM-9 melanoma cells (not shown). Data are given as mean $\pm$ SD of seven mice per group.

Statistical analyses

Experimental and control values were compared using the Student's *t* test. Differences between experimental and control values were considered significant at $P < .05$.

Results

Antibody responses in VV GA733-2-immunized mice

Sera of mice immunized three times with VV GA733-2 showed significant binding ($P < .05$) to GA733 Ag-positive

Figure 4 VV GA733-2 elicits Ag-specific lymphoproliferative responses in mice. Mice (five per group) were immunized with 10^7 PFU per mouse of VV GA733-2 or VV on days 1, 8, and 38. Two weeks after the last immunization, mice were sacrificed and regional lymph nodes were removed and stimulated with native GA733 protein for 3 days. Lymphocyte proliferation was tested in [3 H]thymidine-incorporation assay. Lymphoproliferative responses of mice immunized with VV GA733-2 and stimulated with 20 µg/mL native GA733 protein were significantly ($P < .05$) higher than those of control VV-immunized mice stimulated with the same amount of protein. Lymphocytes from both experimental and control mice proliferated in response to VV (positive control) stimulation. Data are given as mean $\pm$ SD of five mice per group.

SW1116 cells in MHA compared with sera of VV-immunized control mice (Fig 1). SW1116 cells, which naturally express GA733 Ag at a density of $\sim 10^6$ molecules per cell,^{2,32} were used as targets instead of 3T3-ras-GA733 transfectants on which Ag density is higher ($>10^6$ molecules/cell), potentially biasing interpretation of the results. There was no virus dose dependency (10^6 , 10^7 , 10^8 PFU) of the immune response. Antibody titers were $>1:12,800$. These antibodies did not bind to Ag-negative WM-9 melanoma cells in MHA or to the CO17-1A, GA733 or M77 epitopes in inhibition RIA (not shown). ELISA analysis revealed significant ($P < .05$ vs. control sera) binding of the elicited antibodies to GA733-2E protein (Fig 2), but not to BSA (not shown). Serum antibodies elicited by VV GA733-2 mediated significant ($P < .05$) lysis of GA733 Ag-positive SW1116 cells compared with control sera (Fig 3) or to WM-9 target cells (not shown).

Presence of proliferative and absence of cytolytic lymphocyte responses in VV GA733-2 immunized mice

Mice were immunized three times with VV GA733-2 or VV and regional lymph node lymphocytes were stimulated with native GA733 protein or with splenocytes infected with VV GA733-2 or VV, followed by UV irradiation. Comparison of the proliferative activities of lymphocytes from VV GA733-2 and control immunized mice revealed Ag-specific significant ($P < .05$) proliferation only when native GA733 protein was used as stimulant (Fig 4). Stimulation of lymphocytes with VV-infected splenocytes (positive control) induced proliferative lymphocyte responses in experimental and control mice, which were of similar magnitude

Figure 5 VV GA733-2 elicits Ag-specific DTH responses in mice. For induction of DTH responses, mice (six per group) were immunized with 10^7 PFU once and DTH reaction was determined after 7 days. Mice were challenged i.d. with 1 µg of native GA733 protein into the left ear and with 1 µg of BSA into the right ear. Increase in ear thickness was measured at 18, 24, and 48 hours after challenge; values are shown for the 18 hour time point. DTH reactions of VV GA733-2 immunized mice to challenge with native GA733 Ag were significantly ($P < .05$) higher than the responses to BSA challenge or the responses of VV immunized mice challenged with GA733 Ag. Data are given as mean $\pm$ SD of six mice per group.

Figure 6 VV GA733-2 inhibits growth of Balb/c 3T3-ras-GA733-2 tumor cells in mice. Mice (10 per group) were immunized with 10^7 PFU of VV GA733-2 or VV on days 1, 8, and 38, and injected s.c. with 10^6 3T3-ras-GA733-2 tumor cells on day 52. Tumor volumes (maximal width $\times$ length $\times$ height) were measured with a caliper. Control mice and 5 of 10 experimental mice were sacrificed on day 30 because of large tumor burden. Tumor growth was significantly ($P < .05$) inhibited by VV GA733 compared with VV on day 30. Five of the 10 experimental mice were tumor free on day 100. Ratios are numbers of animals with tumors to total number. Data are given as mean $\pm$ SD of 10 mice per group.

compared to the Ag-specific proliferative lymphocyte responses observed in experimental mice after lymphocyte stimulation with GA733-2 protein (Fig 4) or VV GA733-2-infected splenocytes (not shown).

For CTL induction, mice were immunized once or three times, and immune lymphocytes were stimulated with GA733-2E or P815 cells infected with VV GA733-2 or Ad GA733-2. Syngeneic virus-infected splenocytes were not used as stimulators because they did not induce Ag-specific lymphoproliferative immune responses (see above). Lysis of various target cells (uninfected P815 cells or P815 cells infected with VV GA733-2, Ad GA733-2 or the corresponding control viruses; CT26-GA733-2 or CT-26-ASEN cells; or Yak cells) was determined. However, no Ag-specific CTL responses were detected in any of the mice immunized with VV GA733-2. In positive control experiments, lymphocytes from VV GA733-2-immunized mice stimulated with VV GA733-2-infected P815 cells significantly lysed VV or VV GA733-2-infected P815 target cells compared with P815 cells. However, lysis of the VV GA733-2-infected target cells was no greater than lysis of VV-infected target cells (88% and 75% specific lysis of P815 cells infected with VV and VV GA733-2, respectively; 20% lysis of uninfected P815 cells). Thus, anti-VV CTL, but not GA733 Ag-specific CTL responses were obtained.

DTH responses

Mice were immunized once with VV GA733-2 or VV and challenged i.d. with native GA733 Ag or BSA. Increase in ear thickness was measured at 18, 24, and 48 hours after challenge. DTH reactions of VV GA733-2-immunized

mice to challenge with native GA733 Ag were significantly ($P < .05$) higher than the responses to BSA challenge or the responses of VV-immunized mice challenged with GA733 Ag (Fig 5).

Inhibition of tumor growth

In mice immunized three times with VV GA733-2 and challenged with 3T3-ras-GA733-2 cells 12 days after the last immunization, tumor growth was significantly ($P < .05$) inhibited compared with control mice (Fig 6). The tumors grew exponentially in all 10 control mice, which were sacrificed on day 30 of the experiment because of large tumor burden (>1 cm maximal diameter). In contrast, only 5 of 10 experimental mice showed tumor growth on day 30, and these 5 mice were also sacrificed because of large tumor burden. The 5 surviving mice still showed no tumor growth on day 100 when the experiment was terminated. However, VV GA733-2 was unable to inhibit 5-day established tumors in mice ($P > .05$; not shown).

Discussion

The GA733-2 Ag expressed in recombinant VV is a promising vaccine reagent in mice. VV GA733-2 induced humoral, cellular, and protective immunity specific for the Ag in mice, whereas no significant immunity was induced by the control virus. VV GA733-2 induced antibodies that specifically bound to and lysed Ag-positive tumor cells in ADCC. Antibody epitope analysis revealed that none of the three epitopes (CO17-1A, GA733, and M77) tested was recognized by VV GA733-2-immunized mice. Thus, the humoral immune responses to VV GA733-2 included primarily antibodies directed to CO17-1A-, GA733- and M77-unrelated epitopes. In contrast, mice immunized with human CRC cells produced antibodies to the CO17-1A and GA733 epitopes.^{1,2} Therefore, immunologic presentation of the GA733 Ag expressed by human tumor cells differs from Ag presentation in the VV vector.

VV GA733-2 induced Ag-specific cellular immune responses consisting of proliferating and DTH-reactive lymphocytes in mice. However, GA733 Ag-specific CTL responses were not detected, although immunized mice produced strong anti-VV CTL responses. This was surprising because after infection of host cells with recombinant viruses, the foreign Ag is endogenously produced by the cells and usually presented to host immune lymphocytes in the context of major histocompatibility (MHC) class I Ag.³³ Thus, CTL, which may play an important role in the control of cancer³⁴ and which recognize endogenously produced Ag, usually in association with MHC class I molecules,³⁵ are likely to be induced by recombinant vaccines employing viral vectors. Indeed, several studies have shown that recombinant VV expressing tumor-associated Ag can induce Ag-specific CTL responses in experimental animals.³⁶⁻⁴⁸ The GA733 Ag expresses amino acid sequences that could potentially associate with murine H-2k^d (amino acid residues 173-181, 185-193, and 88-96). Furthermore, Ad GA733-2 induced GA733 Ag-specific CTL responses.³⁰ It is possible that following VV GA733-2

immunizations in mice, the GA733 CTL epitopes were not functionally expressed by Ag-presenting cells or not adequately presented to T cells.

VV GA733-2 prevented the growth of GA733 Ag-positive, syngeneic tumor cells but not growth of established tumors. In contrast, Ad GA733-2 inhibited growth of established tumors in mice, although murine CT-26 colon carcinoma cells were used in that study;³⁰ those cells were not used in the present study because their growth was nonspecifically inhibited by control VV. Because Ad GA733-2, but not VV GA733-2, elicited CTL responses in mice, these responses may be crucial for the inhibition of established tumors.

Our results obtained *in vitro* with cells from mice immunized according to protocols optimized for induction of humoral and cellular immunity suggest that GA733-2 Ag-specific proliferative and/or DTH-reactive lymphocytes, and/or ADCC-reactive antibodies contribute to tumor growth inhibition *in vivo*. The DTH-reactive lymphocyte responses most likely are mediated by T-helper type 1 (Th1) cells,^{49,50} which may have activated effector cells of the innate immune system, such as monocytes/macrophages, natural killer cells, neutrophils, and/or eosinophils at the tumor site, leading to tumor destruction. In addition, viral infection may have activated the innate immune system.

Recombinant VV expressing various tumor-associated Ags have been shown to control tumor growth in laboratory animals. Tumor growth inhibition was demonstrated with prophylactic immunizations,^{36,37,39,41-44,46,48,51-60} with therapeutic immunizations,^{38,40,45,61-64} or with both prophylactic and therapeutic immunizations.^{42,44,46,57-59} In some of those studies, costimulatory molecules or cytokines were used to enhance vaccine effects.^{44,45,59,64} These studies have suggested a role for antibodies,^{36-41,43,47,48,51-53,60,63,64} CTL,³⁶⁻⁴⁸ and delayed-type hypersensitive T cells,^{36,37,46,53} although the role of these factors was not directly demonstrated by *in vivo* cell depletion or adoptive cell transfer. The mouse models targeting human carcinoembryonic Ag (CEA) and MUC-1 with VV vaccines,^{36,39-41,43,45-48,52,63} similar to the human GA733 model used in the present study, include Ags that are expressed by tumors and by normal tissues. Mice express homologues of these human Ags on their normal tissues^{23,65,66} and thus provide a clinically relevant model. VV CEA has induced CTL and clinical responses in patients with CRC.⁶⁷⁻⁷⁰

The VV GA733-2 construct described here is a candidate vaccine for patients with CRC. However, a concern pertaining to the use of recombinant VV vaccines in patients is whether previous exposure to the virus (most patients >50 years of age are seropositive for VV) negatively affects induction of Ag-specific immunity by recombinant VV,⁷¹ although repeated immunization of CRC patients with VV CEA induced immune responses in the majority of patients.⁷¹

VV GA733-2 may be especially valuable for combined vaccinations of cancer patients with Ad GA733-2, which induces humoral, cellular, and protective immune responses in mice.³⁰ Sequential immunization with different recombinant viruses has shown superior antitumor effects compared with immunizations with a single virus,^{38,39} due possibly to

the enhanced boosting of the tumor Ag-specific compared with virus-specific immune responses when different viruses are used in combination. The combined vaccine approach is particularly attractive for patients with cancer who are pre-immune to Ad and/or VV. Moreover, VV GA733-2 and Ad GA733-2 vaccines differ in their capacity to induce CTL, DTH, and protective responses against established tumors in mice, further pointing to the need to evaluate the combined effect of these reagents.

A clinical trial with Ad GA733-2 is in preparation and if promising, will be followed by a trial combining both Ad GA733-2 and VV GA733-2. We have already demonstrated that active immunotherapy of CRC patients with Ab2 mimicking the CO17-1A or GA733 epitope or with extracellular domain GA733-2E protein, all precipitated with alum, induced epitope-specific humoral, cellular, and, possibly, protective immunity.^{15-18,21} However, single-epitope Ab2 vaccines and extracellular domain GA733-2E protein are expected to induce less potent antitumor immunity compared to full-length Ag vaccines, which express multiple, potentially immunogenic epitopes. Thus, Ab2 mimicking the GA733 epitope²⁶ and extracellular domain protein GA733-2E¹⁹ did not protect mice against challenge with CT26-GA733-2 tumor cells, in marked contrast to the significant antitumor effects of VV GA733-2 described here and Ad GA733-2 described previously,³⁰ which both express full-length GA733 Ag.

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