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A persistent virus vector confers superior anti-tumor immunity, compared with a non-persistent vector

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Abstract

Active vaccination strategies using viral vectors often give disappointing protection from tumor development, and usually requires multiple immunizations. These approaches normally use viruses that cause acute infections, as they provoke potent CD8 T cell responses. Persistent virus vectors have not been used in this setting due to the perception that exhaustion of the T cell response occurs and would lead to poor anti-tumor protection. However such exhaustion generally only occurs in high-load virus infections, whereas T cell function is intact in lower-load persistent infections. In fact, CD8 T cell responses in these infections, which are adapted for long-term immune surveillance, have properties that may make them more desirable for long-term anti-tumor immunity. In this report, we show that a persistent gammaherpesvirus vector provides superior protection against melanoma, relative to a non-persistent mutant of the same virus. These data suggest that vaccine vectors derived from persistent viruses may perform better than those from acute viruses at mediating anti-tumor protection.

Keywords

Immune surveillance; Persistent virus; Melanoma; Tumor Immunotherapy; Tumor vaccine; Herpesvirus vaccine

Introduction

Active vaccination is a remarkably effective strategy to prevent infectious disease, however it has been largely unsuccessful in combating tumors. In part this may be due to the necessity of vaccinating after the onset of tumor development. Also the effectiveness of vaccine-induced anti-tumor responses is limited by inhibitory mechanisms elaborated by the tumor or the microenvironment. However another component of the problem may be that suboptimal vectors are used to elicit anti-tumor T cell responses.

Infectious vectors generally induce the strongest T cell responses, and those used to date have been based upon attenuated pathogens that cause self-limited acute infections. A common problem with these vectors is that strong T cell epitopes from the virus are often presented at the expense of weaker tumor-associated epitopes. However this can be overcome by a prime-boost approach, where the priming and boosting vectors share only the epitope derived from the tumor antigen. This approach can yield robust immunity [1]. Acute viral vectors induce a classical memory response, which maintains long-term immunity in the absence of cognate antigen. However this type of vaccination may not be suitable for inducing a population of

cells capable of long-term immune surveillance, preventing tumor recurrence or the outgrowth of micrometastases. This type of immune surveillance is more akin to that seen in certain persistent virus infections, where the host immune response contains the infection, preventing overt disease, despite being unable to fully eliminate the virus.

In some chronic virus infections there is extensive viremia, and this high level of viral antigen can exhaust the T cell response, compromising effector functions and proliferative activity [2]. However in other, lower-load infections, such as those of the herpesvirus family, the response is not exhausted, and dysfunction is restricted to reduced IL-2 secretion or limited Bcl-2 upregulation [3,4]. Antiviral effector activity is maintained, and virus-specific T cells are necessary for long-term containment of the infection. In fact our previous results indicate that memory CD8 T cells from mice bearing a persistent infection upregulate granzyme B faster and clear a secondary infection more quickly than mice which cleared the infection [4]. Therefore if an analogous tumor-specific T cell response could be generated, it may lead to better protection against malignancies.

To definitively test this hypothesis, it is necessary to use a persistent and a nonpersistent virus that are identical except in their ability to persist. This is possible using the murine gammaherpesvirus system. Viral mutants with a non-functional ORF73 gene lack the ability to persist in the host, but can still undergo acute replication [5,6]. While some ORF73 mutants replicate to slightly lower levels during acute infection [6], the mutant we employ replicates to normal levels [5]. Our previous work has shown the induction of antiviral T cell responses is very similar in both ORF73 deleted and revertant viruses [4]. Therefore these viruses offer the ideal system in which to test whether virus persistence alone, rather than other qualities unique to different virus infections, can provide superior antitumor protection. We used B16 tumor growth as a stringent readout system, showing immunization with a persistent virus vector is protective even when administered 6 days after the tumor.

Materials and Methods

Mice, virus, and reagents

Murine gammaherpesvirus (MHV-68), clone G2.4, was originally obtained from Prof. A. A. Nash (University of Edinburgh) [7]. MHV-68 virus containing a frame-shift mutation in ORF73 (FS73) and the revertant virus (FS73R) were obtained from Dr. Stacey Efstathiou (University of Cambridge, UK) [8]. Vaccinia virus expressing the ORF61₅₂₄₋₅₃₁/D^b (p79) epitope of MHV-68 (VV-p79) was obtained from Dr. Peter Doherty (St. Jude Children's Research Hospital, TN) [9]. Intranasal (i.n.) infections with MHV-68 (400 PFU) mice were performed under anesthesia with Ketamine/Xylazine, and this route was used for mice immunized before tumor administration. For intraperitoneal (i.p.) infections with VV-p79 or MHV-68, 2×10^6 PFU was used. C57BL/6 mice were purchased from The National Cancer Institute (Bethesda, MD). The Animal Care and Use Program of Dartmouth College approved all animal experiments.

Tissue preparation, MHC/peptide tetramer, antibody staining, and flow cytometric analysis

Spleen and lymph node cell suspensions were prepared as described previously [10]. MHC/peptide tetramers for the MHV-68 epitope ORF61₅₂₄₋₅₃₁(TSINFVKI)/K^b (p79), were obtained from the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Cells were stained with tetramers for one hour at room temperature as previously described [11]. Samples were analyzed on a FACS Calibur flow cytometer using CellQuest software.

Intracellular staining

Spleen or lymph node cells were incubated with 1 µg/ml of p79 peptide plus 10 U/ml IL-2 and 10 µg/ml Brefeldin A (BFA) in complete medium at 37°C for 5 hours. Cells were stained with anti-CD8 antibody, then fixed and rendered permeable before staining with anti-IFN-γ (XMGI.2), and anti-Granzyme B (clone 100) or isotype control antibody [4].

Construction of B16-p79 cell line

A synthetic oligonucleotide encoding the epitope ORF61_{524–531}(TSINFVKI) (p79) flanked by a Kozak consensus sequence and a stop codon (GTCGACGCCACCATGACCAGTATCAACTTTGTGAAGATATAAGCGGCCGC) was inserted between the SalI and NotI restriction sites of the pFB-Neo vector (Stratagene). This plasmid was transfected into the Bosc23 and PT67 retrovirus packaging cell lines as previously described [12]. B16-F10 melanoma cells were incubated with recombinant retrovirus-containing PT67 supernatant in the presence of 8 µg/ml polybrene. The following day, the medium was changed and replaced with medium containing 1 mg/ml G418 cultured for 9 days to select for transduced cells.

B16 tumor injections

Anesthetized mice were shaved on the flank, then 10⁵ B16 or B16-p79 cells injected intradermally in a volume of 50 µl.

Antigen presentation assay

Presentation of the ORF61_{524–531}/K^b (p79) epitope was measured using the 4943 ORF61_{524–531}/K^b-specific lacZ-inducible T cell hybridoma cell line, described previously [13]. Cells were incubated with 50 U/ml IFN-γ overnight to upregulate MHC class I. On the day of assay, 10⁵ hybridoma cells were incubated with 10⁵ B16 or B16-p79 cells in an overnight culture. The following day, cells were fixed, and the lacZ substrate X-gal added as previous described [13]. LacZ-positive cells were enumerated microscopically.

Results

Construction of B16 expressing an MHV-68 epitope

B16 cells were infected with a recombinant retrovirus containing the p79 epitope from MHV-68 as described in Materials and Methods. Following selection for retrovirus-infected cells, B16-p79 cells were tested to determine if they could present the viral epitope to T cells. B16-p79 cells were incubated with a lacZ-inducible T cell hybridoma recognizing the p79 epitope, and the response measured by counting the number of lacZ positive cells [13]. As a control, wild-type B16 cells were tested under the same conditions. Less than 10 lacZ⁺ cells were detected in the B16 cultures, however >250 lacZ⁺ cells were observed in the B16-p79 cultures, demonstrating these cells presented the p79 epitope to T cells.

Prophylactic vaccination with MHV-68 confers protection from tumor growth

We used these transfected tumor cells to determine if a T cell response directed against the p79 epitope of MHV-68 was capable of restricting tumor growth. mice were immunized against this epitope and challenged with B16-p79 tumor eight days later. Immunization consisted of infection with one of three different viruses, all containing the p79 epitope: (i) recombinant vaccinia virus encoding the p79 epitope (VV-p79), (ii) an MHV-68 mutant virus lacking the ability to persist in the host, due to a frameshift mutation in the ORF73 gene (FS73, [8]), (iii) a revertant MHV-68 virus, which is competent to persist in the host indefinitely (FS73R).

Mice receiving either the persistent or non-persistent strains of MHV-68 were significantly protected against tumor challenge (Fig. 1A), relative to mice treated with HBSS. However protection conferred by VV-p79 was weaker, failing to reach statistical significance. Continued expression of the p79 epitope in vivo was confirmed by the fact that the lacZ-inducible T cell hybridoma was activated by single cell suspensions derived from tumor tissue when tumor-bearing mice were sacrificed (data not shown).

Superior therapeutic vaccination using a persistent virus vaccine

Next we tested whether any of these viral vaccines could prevent the growth of an established tumor. The B16-p79 tumor was injected four days before immunization with virus. In these experiments mice were immunized by the i.p. route, as we reasoned this would elicit a faster immune response, which is probably necessary to restrict growth of rapidly-growing tumor such as B16. Mice treated with VV-p79 were protected no better than control mice receiving HBSS (Fig. 1B). Mice immunized with persistent FS73R virus were significantly protected, however no significant protection was observed with the non-persistent FS73 virus. For a more stringent test of the therapeutic capacity of this vaccination, mice were immunized six days after tumor implantation (Figure 1C). We observed significant protection in FS73R immunized mice, but protection afforded by the non-persistent FS73 virus was not statistically significant. These data indicate that a persistent virus vaccine provides superior protection relative to a non-persistent vaccine.

It was possible that the persistent virus vaccine provided superior protection due to factors other than the T cell response directed towards the p79 epitope. For example systemic levels of IFN- γ or inflammatory mediators may be higher during virus persistence. To test for such non-antigen-specific effects, we injected one group of mice with B16-p79 tumor and another with wild-type B16, then vaccinated with FS73R virus four days later. Both tumors grew at an equivalent rate in control mice injected with HBSS (Fig. 2), however only B16-p79 growth was restricted in mice immunized with FS73R virus, whereas B16 tumor grew normally. Therefore protection conferred by FS73R virus is not due to non-antigen-specific anti-tumor effects.

Larger size and quality of p79-specific response after vaccination with persistent virus

To understand the mechanism behind the level of protection conferred by the different viruses, we determined the frequency of antigen-specific T cells present in each vaccinated group. The frequency and function of p79-specific CD8 T cells was measured in mice that received a therapeutic vaccination, 4 days after tumor injection.

Using intracellular IFN- γ staining, we detected a larger number of IFN- γ ⁺ CD8 T cells in both the lymph node and spleen in mice immunized with the persistent FS73R virus compared with the FS73 virus, reaching statistical significance at 15 days post-infection (Fig. 3A–D). The amount of IFN- γ produced per cell was similar in both groups, as measured by the mean fluorescence intensity (data not shown). In contrast, there was a much smaller response induced by the VV-p79 vaccine (Fig. 3E). Expression of granzyme B in p79-specific T cells was similar on a population level in both FS73 and FS73R vaccinated groups (Figs. 4A–B), although significantly higher levels were detected in FS73R vaccinated mice at 8 days post-infection in the spleen (Fig 4B). However when we gated selectively on granzyme B^{hi} cells, we found a small but consistent and significant increase in the FS73R vaccinated group in both the lymph node (Fig 4C–D) and spleen (data not shown). These data indicate the persistent virus vaccine induces a larger anti-tumor CD8 T cell response, which may have greater cytolytic potential due to higher production of granzyme B.

Discussion

Here we show that prior immunity to both non-persistent and persistent MHV-68 viruses could protect against the growth of B16 melanoma, indicating both viruses induced potent anti-tumor responses. However the persistent virus gave clearly superior protection when given therapeutically, a more clinically realistic situation. Virus was administered on day 4–6 post-tumor injection, and control (uninfected) mice began to succumb to the tumor at approximately 18 days after injection. By this time, virus replication has ceased, and there is no detectable virus persisting for ORF73 mutant viruses, however the revertant (FS73R) virus has established a latent infection [6,8]. The persistence of the virus leads to a larger virus-specific T cell response, and also higher production of granzyme B, which is likely instrumental in killing tumor cells. The relatively weak response to the p79 epitope induced by VV-p79 inoculation is consistent with the fact that exogenous antigens inserted into this vector are often out-competed by a more vigorous response to endogenous epitopes from the virus [14,15].

Protection in these experiments is likely mediated by the combination of a larger CD8 T cell response and improved effector functions in the CD8 T cells elicited by vaccination with the persistent FS73R virus. The difference in magnitude of the CD8 T cell response between the persistent and non-persistent infections is due to the induction of splenomegaly by the persistent virus [4,8]. Studies by ourselves and others have shown that during the establishment of latency in B cells in lymphoid organs, there is a marked expansion of both T and B lymphocytes [16], however splenomegaly is absent when the virus cannot establish latency. Splenomegaly results in a larger absolute number of virus-specific cells during the early stages of MHV-68 infection. Persistence of virus also results in the ability of memory CD8 T cells to more rapidly re-express effector functions such as granzyme B following re-exposure to antigen [4]. The tumor model here could be considered an acute model, in the sense that control of the tumor is the consequence of a T cell response induced during the first two weeks after virus infection. We speculate that CD8 T cell-mediated immune surveillance induced by a persistent virus may also mediate better long-term anti-tumor surveillance to prevent the outgrowth of metastases. Conceptually, long-term control of a persistent infection is analogous to controlling tumor micrometastases that can be contained, but not eliminated by the immune response. In persistent gammaherpesvirus infection, the T cell response contains the virus exceptionally well, as disease is generally associated only with severe immune suppression. It will therefore be interesting to determine whether the success seen in the current model system translates into long-term containment of malignant conditions.

Another recent study harnessed the T cell response against persistent virus infections to counter tumor growth in human subjects. Pule et al. [17] took the approach of transducing T cells *in vitro* with a chimeric antigen receptor that recognized a tumor associated antigen expressed by neuroblastoma cells. The study compared transduced polyclonal T cells and EBV-specific T cells. EBV-specific transduced cells survived longer after transfer back into patients, and maintained proliferative potential and cytotoxicity. All patients received both polyclonal and EBV-specific cells, so it was not possible to test if EBV-specific transduced cells conferred superior protection. However four of eight patients with evaluable tumors experienced tumor regression or necrosis, one of which was a complete remission. These data, together with the data in the mouse model reported here, indicate that persistent viral vectors, or redirected T cells responding to persistent viruses, may offer a novel and effective strategy to induce tumor immunity.

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Figure 1.

Anti-tumor protection afforded by persistent and non-persistent vaccine vectors. B6 mice were injected with B16-p79 tumor cells i.d. on the flank, and immunized with either VV-p79, FS73 or FS73R viruses, or HBSS as a control, as described in Materials and Methods. (A) Immunization 8 days prior to tumor inoculation. (B) Immunization 4 days after tumor inoculation. (C) Immunization 6 days after tumor inoculation. Graphs show survival curves using 8 mice per group. P values shown are relative to HBSS control group.

Growth of B16 and B16-p79 in control mice

Growth of B16 and B16-p79 in immunized mice

Figure 2.

Specificity of protection afforded by persistent vaccine vector. Mice were inoculated with either wild-type B16 or B16-p79 then immunized with FS73R MHV-68 vaccine 4 days later. Survival of (A) naive mice, or (B) FS73R immunized mice is shown. P values shown are relative to the B16 group.

Figure 3.

Enhanced CD8 T cell response after immunization with FS73R vaccine vector. Mice were immunized with the viruses indicated 4 days after inoculation of B16-p79 tumor cells. At the days indicated, draining lymph nodes (A, C) or spleen (B, D) were removed and the percentage (A, B) or absolute number (C, D) of IFN- γ -producing cells were measured by intracellular staining. ** denotes $p < 0.01$. (E) Representative FACS plots from mice immunized with either wild type vaccinia virus (VV) or VV-p79, four days after tumor inoculation. Plots are gated on CD8+ cells and the numbers denote the percentage CD8+ cells staining positive for IFN- γ .

Figure 4. Enhanced granzyme B production elicited by FS73R vaccine vector. Mice were treated as in Fig. 3, and spleens or lymph node cells stained for intracellular granzyme B. Mean fluorescence intensity of granzyme B staining from draining lymph node (A) or spleen (B). (C) shows the proportion of cells staining for high levels of granzyme B in the lymph node. (D) shows representative granzyme B staining from lymph node cells, shaded isotype control, thick line FS73R, thin line FS73. Bar indicates gate placement.