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Comparison of simian virus 40 large T antigen recombinant protein and DNA immunization in the induction of protective immunity from experimental pulmonary metastasis

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Abstract In this report we examine the ability of a recombinant tumor antigen preparation to prevent the establishment of experimental pulmonary metastasis. Baculovirus-derived recombinant simian virus 40 (SV40) large tumor antigen (T-Ag) was injected into BALB/c mice followed by challenge with an intravenous injection of syngeneic SV40-transformed tumorigenic cells. The experimental murine pulmonary metastasis model allows for the accurate measurement of metastatic lessions in the lungs at various times after the challenge, using computer-assisted video image analysis. Following challenge, lung metastasis and survival data for the groups of mice were obtained. Animals immunized with recombinant SV40 T-Ag showed no detectable sign of lung metastasis and survived for more than 120 days after challenge. Antibodies specific for SV40 T-Ag were detected in the serum of immunized mice by enzyme-linked immunosorbent assay. Splenocytes obtained from mice immunized with recombinant SV40 T-Ag did not lyse syngeneic tumor cells, indicating that no cytotoxic T lymphocyte response was induced. Control mice developed extensive lung metastasis and succumbed to lethal tumor within 4 weeks after challenge. These data indicate that immunization with the recombinant SV40 T-Ag induces protective, T-Ag-specific immunity in an experimental pulmonary tumor metastasis model.

Key words SV40 T-Ag · Tumor immunity · Pulmonary metastasis · Vaccine

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Introduction

The primary tools used in cancer treatment include surgery, chemotherapy, and radiation therapy or combinations thereof. These modalities represent highly invasive and relatively non-specific treatments, thus alternative methods to treat or prevent cancer are under active investigation. Included in these alternative methods are prophylactic and therapeutic vaccination strategies that target tumor-specific or tumor-associated antigens expressed on the cancer cell. Although a number of investigations have described the utilization of cancer vaccines as a means to treat cancer (reviewed in [9]), several problems have been identified that have limited the progress in this field of investigation. Progress in the identification of tumor antigens that can be employed as tumor vaccines has been slow. Tumor-associated antigens are expressed on both normal cells and tumor cells, hence the induction of immune responses to self antigens is problematic. There may also be difficulty in obtaining large quantities of the highly purified tumor antigen because its complex nature does not allow one to implement the newer technologies presently employed in the development of vaccines against infectious agents (reviewed in [3]). Finally, the lack of experimental animal models where tumor formation can be evaluated and accurately quantified has hampered efforts to examine the effectiveness of certain tumor vaccines in vivo.

Surgical removal of the primary tumor can be curative in many cancer patients. However, surgery may expose tumor cells to the circulation, which can lead to metastasis and tumor establishment at distal sites. Chemotherapy and/or radiation therapy are normally used to eliminate metastatic cells; however, this may only control disease temporarily. Immunotherapeutic strategies may have different effects on a localized primary tumor and on metastatic lesions (e.g., [2, 23, 42]). In these studies, models of experimental metastasis appeared to be more difficult for inducing evidence of

tumor immunity than were primary solid tumor models [23, 42].

Previous studies employing murine models for simian virus 40 (SV40) tumors have used the intraperitoneal route for introduction of SV40 tumorigenic cells into a syngeneic murine system, with the induction of primary solid tumor and lethal tumor burden serving as a reference point [5, 54]. Alternatively, intradermal inoculation of SV40 tumorigenic cells results in a variable tumor mass that can be measured for quantitative purposes [25]. Neither of these two routes of inoculation represents a model for tumor metastasis. Other animal models of SV40-induced tumors have employed newborn hamsters challenged with SV40 or hamsters inoculated with SV40 tumorigenic cells (reviewed in [38]). However, the lack of availability of immunological reagents specific for the hamster has hampered the development of this animal model.

We have recently described an experimental murine pulmonary metastasis model employing syngeneic SV40 tumor cells [66]. In this model, the intravenous (i.v.) route of SV40 tumorigenic cell administration results in tumorigenic cell metastasis to the lungs and eventual pulmonary tumor formation. Additional reference points for use in monitoring tumor induction and progressive tumor development in organs and tissues other than the lung have also been defined within this model. Quantification of the tumors induced by SV40 tumorigenic cells based on the number and size of foci observed in the lungs of inoculated BALB/c mice, is accomplished by video-assisted computer imaging [65]. This in vivo tumor model is useful for the evaluation of immunologically based therapies with the goal of preventing the development of metastatic foci in the lungs and subsequent metastatic events. In the present report, we use this newly developed SV40 murine tumor metastasis model to examine the ability of immunization with recombinant SV40 large tumor antigen (T-Ag) to produce protective immunity against SV40-tumorigenic-cell-induced pulmonary metastasis. Our results indicate that recombinant SV40 T-Ag represents an effective prophylactic cancer vaccine that completely prevents pulmonary tumor metastasis within this model.

Materials and methods

Mice, cells and media

Female BALB/c mice, 6–8 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, Me.) and maintained under standard conditions. Treatment and care of the animals were in accordance with institutional guidelines and the Animal Welfare Assurance Act. The SV40-transformed BALB/c mouse kidney fibroblast cell line, designated mKSA [27], is tumorigenic in BALB/c mice and was used for in vivo tumor induction. mKSA cells were cultured in Dulbecco's modified Eagle medium with L-glutamine (Gibco BRL) supplemented with 0.1 mM non-essential amino acids, 500 units/ml penicillin, 500 μg/ml streptomycin (Mediatech, Washington D.C.) and 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, Md.). Flasks were incubated in a humidified, 5% CO_2 atmosphere at 37 °C. Prior to injection, cells were detached from flasks by 5 min exposure to phosphate-buffered saline (PBS) and 1 mM EDTA (pH 7.5). The tumor cells were washed once, resuspended in PBS, counted, and adjusted to the appropriate density with additional PBS prior to injection.

Antigen production and purification

Recombinant SV40 T-Ag was generated in the Sf9 insect cell line, using a baculovirus expression vector system [31], and immunoaffinity-purified by methods previously described [53, 55]. The purity of recombinant SV40 T-Ag was assessed by methods described elsewhere [54]. The concentration was determined, using an absorption coefficient of 1.14 for a 1% solution at 280 nm prior to alum precipitation by methods described in detail previously [24].

Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to recombinant SV40 T-Ag

SV40 T-Ag specificity and the titer of mouse sera were examined by a previously described indirect ELISA [4, 5, 54]. An absorbance at 410 nm, determined to be approximately three times that obtained for a 1:10 dilution of the preimmune sera, was established as the cutoff for positive antibody reactivity and determination of the antibody endpoint titer [64]. Anti-(SV40 T-Ag) antibodies failed to bind a control baculovirus recombinant viral antigen (hepatitis B surface antigen; HBsAg) in similar assays (data not shown).

Cytotoxic T lymphocyte assay

To measure SV40 T-Ag cytotoxic T lymphocyte (CTL) activity, a 4-h ^{51}Cr-release assay was performed as previously described [7]. Briefly, splenocytes were obtained from immunized mice prior to tumor challenge, and employed as effector cells. A secondary in vitro stimulation of effector cells was performed using irradiated mKSA cells. Viable mKSA were labeled with 100 μCi ^{51}Cr and employed as the target cells. Target cells were used at a concentration of 5×10^4 cells, and various effector to target cell ratios were examined. The percentage specific lysis was calculated as previously described [7].

Immunization and tumor challenge

Groups of ten BALB/c mice were immunized via intraperitoneal injection with either an alum precipitate of immunoaffinity-purified recombinant SV40 T-Ag, or alum alone. In previous studies, we demonstrated that injection of alum alone gave comparable results to injection with alum-precipitated control antigens with regard to the lack of protective tumor immunity against SV40 tumorigenic cells [5, 54]. Recombinant SV40-T-Ag-immunized mice were primed with 5 μg recombinant SV40 T-Ag as an alum precipitate. Three subsequent injections of 2 μg recombinant SV40 T-Ag as an alum precipitate were given at approximately 14-day intervals.

Approximately 30 days after the final immunizations, groups of mice were injected with a 50-μl volume of either 5×10^5 (initial experiments) or 1×10^6 (confirmatory experiments) mKSA cells in PBS into the dorsal tail vein. For the lower dose, the majority of the animals were used for tumor challenge survival experiments. Four of the ten animals from each group given the higher dose (10^6 mKSA cells) were euthanized 10 days after challenge, at which time a post-mortem examination was conducted. The remaining six animals were used in tumor challenge survival experiments. To evaluate the effects of genetic immunization, groups of mice were immunized intramuscularly with plasmid DNA containing the SV40 T-Ag gene or with a control plasmid not containing the T-Ag gene. These plasmids have been described in detail elsewhere [7]. Mice immunized with plasmid DNA were primed with 100 μg DNA in saline and subsequently received three additional injections of 100 μg DNA per injection at approximately 14-day inter-

vals. Mice were challenged 30 days following the fourth injection with 5×10^5 mKSA cells and experiments were performed as described above.

Pulmonary tumor foci quantification

The left lung of each animal was removed after euthanasia and stained by injecting lobes with 10% India ink. Lungs were then suspended in Fekete's destaining solution [67], using a technique described previously [66]. After destaining for 15 min, the number of foci visible on the ventral surface of the lung was quantified using an IS-1000 digital imaging system (Alpha Innotech Corporation, San Leandro, Calif.). Density threshold parameters were defined to ensure that the foci counted consistently fell above a gray scale value of 25 (compared to black lung background). Size threshold parameters were set to count only those foci that were more than 4 pixels in diameter on the computer image [65]. A range for the number of foci and an estimate of their diameter were defined and employed as indicators of disease progression. In instances where tumor foci were deemed too numerous to count and the individual foci became indistinguishable because of overlap (tumor foci number >60), visible foci from an individual lung are shown and not quantified.

Survival experiments

In order to asses the long-term outcome of the various doses of mKSA cells in inoculated mice, animals from each group were allowed to progress in illness and observed daily for up to 120 days after challenge to evaluate mean survival time.

Statistical analysis

Data were analyzed using the mean collected for animals in each group and the standard error of the mean to demonstrate the amount of variability within each group.

Results

Components of the immune response induced by recombinant SV40 T-Ag compared to DNA immunization

To examine the humoral immune responses to SV40 T-Ag, anti-SV40 T-Ag responses were measured by ELISA following each immunization with recombinant SV40 T-Ag (Table 1). The antibody responses were specific for SV40 T-Ag. The preimmune sera as well as sera specific for HBsAg failed to recognize SV40 T-Ag (data not shown). The reciprocal anti-SV40 T-Ag antibody endpoint titers in BALB/c mice ranged from 50 to 3200 and from 12,800 to 1,638,400 following two and four injections with recombinant SV40 T-Ag respectively. To evaluate the CTL responses, splenocytes were obtained from mice immunized with recombinant SV40 T-Ag after the fourth injection. At various effector to target cell ratios, the immune splenocytes fail to lyse syngeneic tumor cells in the CTL assay (data not shown). Less than 5% specific lysis was observed with effector cells obtained from SV40-T-Ag-immunized mice (E:T cell ratios ranged from 6:1 to 200:1). This was comparable to the levels of specific lysis obtained with effector cells from control immunized mice. These results indicate that recombinant SV40 T-Ag immunization does not induce a specific CTL response in the splenocytes of immune mice; however, it does induce SV40-T-Ag-specific antibodies. Mice immunized with plasmid DNA containing the SV40 T-Ag gene did not generate detectable antibodies to SV40 T-Ag following the fourth injection (titers <50). However, in vitro CTL responses were observed in splenocytes from SV40-T-Ag-DNA-immunized mice when compared to mice immunized with recombinant SV40 T-Ag and control plasmid ($>30\%$ specific lysis at an E:T cell ratio of 100:1; data not shown). These results confirm our previous studies on the dichotomy of the anti-(SV40 T-Ag) immune response to SV40 T-Ag protein compared to genetic immunization [5, 7].

Table 1 Anti-(SV40 T-Ag) endpoint titers in sera from immunized mice. Titers were determined by an indirect enzyme-linked immunosorbent assay. The groups consisted of 20 female BALB/c mice, immunized and bled biweekly. The titers represent the reciprocal of the dilution of sera that had three times the absorbance value obtained with a 1:10 dilution of the preimmune sera. The mean of each group is shown with the range of individual values in parenthesis. *SV40* simian virus 40, *T-Ag* large tumor antigen

Group	Number of injections	Anti-(SV40 T-Ag) titer
Alum	1	<50
	2	<50
	3	<50
	4	<50
SV40 T-Ag/alum	1	<50
	2	615 (50–3,200)
	3	123,093 (1,600–819,200)
	4	311,885 (12,800–1,638,400)

Protection from lethal tumor challenge

In initial experiments, we examined whether immunization with recombinant SV40 T-Ag protects the mice from lethal tumor challenge and the establishment of experimental pulmonary metastasis. In these experiments, we evaluated two parameters indicative of protective tumor immunity: survival after tumor cell challenge and no development of lung tumor foci. Table 2 shows that the SV40-T-Ag-immunized mice were protected from a lethal tumor challenge of 5×10^5 mKSA cells. Specifically, control mice survived a mean of 24 days after challenge (a range of 22–31 days). Mice immunized with SV40 T-Ag survived longer than 120 days, at which time no tumors were evident. It is clear that SV40 T-Ag immunization also prevented establishment of experimental pulmonary metastasis (Fig. 1A). One mouse from each group was euthanized 25 days after tumor cell inoculation and the lungs were evaluated for tumor foci. The lung obtained from the SV40-T-Ag-immunized mouse exhibited no visible tumor foci, whereas a lung obtained from a control mouse contained more than 60 tumor foci.

To confirm our initial observations, we selected a higher tumor challenge dose and added an additional parameter to evaluate tumor immunity, namely quantification of tumor foci in the lungs. We examined mortality among control and immunized mice after challenge with an i.v. injection of mKSA cells (Table 2). The mean survival time for six control mice inoculated with 1×10^6 mKSA cells was 21 days (range 19–23 days). Recombinant SV40-T-Ag-immunized mice survived for more than 120 days, during which time no sign of illness or tumors was apparent.

Plasmid-DNA-immunized mice that had been previously shown to be completely immune to tumor in a primary solid tumor model [7] demonstrated partial to no protection in the experimental pulmonary metastasis model with regard to survival. Mice immunized with plasmid DNA containing the SV40 T-Ag gene were challenge with 5×10^5 mKSA cells and their mean survival time was 31 days (range 27–41 days). This compared to 23.5 days (range 23–25 days) for mice immunized with control plasmid DNA (Table 2).

Table 2 Effect of SV40 T-Ag immunization on survival after metastatic challenge. Each group contained BALB/c mice immunized four times and challenged once with mKSA cells. Survival time values represent the means of the individual survival times. Values in parenthesis represent the range

Group	Dose	No. of survivors	Mean survival time (days)
Alum	5×10^5	0/9	24 (22–31)
	1×10^6	0/6	21 (19–23)
SV40 T-Ag/alum	5×10^5	12/12	>120
	1×10^6	6/6	>120
SV40 T-Ag DNA	5×10^5	0/5	31 (27–41)
Control DNA	5×10^5	0/6	23.5 (23–25)

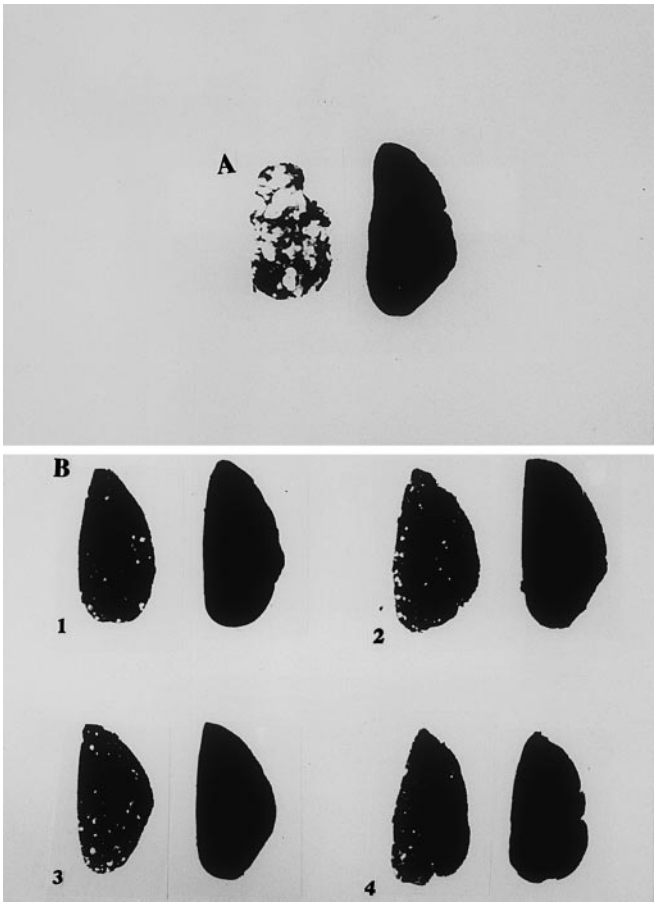

Fig. 1 A Representative video image prints of lungs taken from (*left*) an alum-injected BALB/c mouse and (*right*) a BALB/c mouse immunized with simian virus 40 large tumor antigen 25 days after i.v. inoculation with 5×10^5 mKSA cells. **B** Photographs of lungs obtained from BALB/c mice taken 10 days after inoculation with 1×10^6 mKSA cells. Lungs on the *right* of each panel (1–4) are from immunized mice. The *left* of each panel shows lungs from the control group of mice

Protection from development of lung tumor foci

The presence of foci in the lungs is used as a main indicator of disease progression in this SV40 murine tumor metastasis model. Lungs were obtained from mice in each group and bleached with Fekete's solution, which results in a clear distinction between foci (white) and normal lung tissue (black) after staining. Both the size and number of foci were measured 10 days after inoculation. To quantify the lung foci, computer-assisted image analysis of the lung ventral surface was employed. Density and size threshold parameters were defined in order to keep counts consistent from experiment to experiment, as previously described [65]. Table 3 shows data for individual mice and mean values for the groups. Upon examination of lungs taken from control mice 10 days after inoculation, a mean of 33 foci were counted (Table 3). Foci diameters fell between 5 and 8 pixels

Table 3 Tumor formation measured as the number of visible foci on lungs of BALB/c mice after metastatic challenge. The mean foci count ± SD for control mice is 33 ± 9.9. *ND* not determined since no visible foci were detected

Group	Dose	Day	No. mice	Foci count	Foci size (pixels)
Alum	1×10^6	10	1	42	5–8
	1×10^6	10	1	26	5–8
	1×10^6	10	1	23	5–8
	1×10^6	10	1	41	5–8
SV40 T-Ag/alum	1×10^6	10	1	0	ND
	1×10^6	10	1	0	ND
	1×10^6	10	1	0	ND
	1×10^6	10	1	0	ND
SV40 T-Ag DNA	5×10^5	10	1	1	11
	5×10^5	10	1	31	10
	5×10^5	10	1	2	14
	5×10^5	10	1	1	23
Control DNA	5×10^5	10	1	35	8
	5×10^5	10	1	27	11
	5×10^5	10	1	32	8
	5×10^5	10	1	30	14

when the computer image was analyzed. No foci were detected on lungs taken from immunized mice. Images of representative lungs taken from both control immunized mice and recombinant SV40-T-Ag-immunized mice are shown in Fig. 1B. Together, these data clearly indicate that SV40 T-Ag immunization can prevent the establishment of pulmonary tumors in this experimental animal model of pulmonary metastasis. Alternatively, mice immunized with SV40 T-Ag DNA contained lung foci following challenge with 5×10^5 mKSA cells. All four mice examined exhibited lung foci, and one of the four had lung foci comparable in number to those in the group of mice immunized with control DNA (Table 3). The other three mice in the group immunized with SV40 T-Ag DNA had fewer lung foci than the controls; however, the size of the foci, based on pixel units, was comparable or larger. These data indicate that SV40 T-Ag DNA immunization fails to provide complete tumor immunity in this experimental pulmonary metastasis model.

Discussion

A number of immunotherapeutic strategies to treat a variety of cancers have been investigated both in animal models and in human clinical trials (reviewed in [11, 37]). These strategies include in vitro expansion and adoptive transfer of tumor-specific cytotoxic T cells in the presence or absence of recombinant cytokines [45], the direct use of cytokines that inhibit tumor growth [58], tumor-reactive monoclonal antibodies conjugated to toxin [61, 63], monoclonal antibodies specific for tumor antigens [17–19, 33, 43], genetic introduction of immunological molecules into the tumor cells [8, 41, 60], and tumor-antigen-based vaccines (reviewed in [3, 9, 12, 20, 29, 30, 52, 57]). Examination of the potential of the latter approach, namely tumor antigen vaccines, was the basis for the investigation described here.

A number of investigators have previously described the induction of protective tumor immunity via prophylactic vaccination. For the most part, these studies have indicated a role cell-mediated immune (CMI) responses, in particular CTL for the induction of tumor immunity [10, 12–14, 42, 46, 51, 56]. Yet when these strategies to induce tumor immunity are applied to therapeutic vaccination, often only partial responses are obtained and tumor immunity is rarely complete [10, 13, 14, 23, 42, 56, 71]. The induction of tumor immunity by various immunotherapeutic strategies has utilized a variety of murine models; however, the outcome of vaccination is not predictable [10, 13, 23, 70] and is reminiscent of the situation with infectious disease vaccines. Indeed, murine models were poor predictors of the clinical efficacy in humans of the herpes simplex virus vaccine, vaccines against protozoan infections, such as schistosomiasis, and the formulation of combined bacterial and viral pediatric vaccines. In contrast, mouse potency models are still employed to predict the human immunogenicity of hepatitis B virus surface antigen vaccines [1]. These observations suggest that murine models provide evidence to support studies in humans, but may not represent true predictors of the human situation (reviewed in [26]). Nevertheless, success in a prophylactic modality provides justification for attempting therapeutic regimens, and murine models represent the cost-effective choice for such studies. Murine models may also not predict the ability of a tumor cell to undergo mutation in vivo, which results in a tumor antigen variant that is not recognized by the pre-existing induced immune response, as has been reported in human B cell lymphoma (reviewed in [52]).

Previously, we demonstrated that recombinant SV40 T-Ag is an effective protein-based vaccine for the prevention of primary solid tumors induced by inoculation with syngeneic SV40-transformed cells into BALB/c mice [5, 54]. In additional studies, we demonstrated that plasmid DNA encoding the SV40 T-Ag gene was also an effective genetic vaccine for the prevention of primary solid tumors [7]. A dichotomy in the induction of anti-(SV40 T-Ag) immune responses was observed when protein was used for immunization instead of DNA. Recombinant SV40 T-Ag induced antibodies that mediated in vitro tumor cell lysis by antibody-dependent cell-mediated cytotoxicity, T cell proliferative responses, and the secretion of Th1-type cytokines, namely interferon γ and interleukin-2 [5, 6]. This was supported by the induction of both IgG1 and IgG2a anti-(SV40 T-Ag) subclasses of antibodies and the IgG2a:IgG1 ratio [5]. However, major-histocompatibility-complex(MHC)-class-I-restricted CTL responses were not induced by this immunization scheme. Plasmid DNA immunization resulted in the induction of MHC-class-I-restricted CTL responses in the splenocytes of immunized BALB/c mice with little to no anti-(SV40 T-Ag)-specific antibodies being generated [7]. These data indicate that both humoral and CMI mechanism(s) played a role in tumor immunity in this non-metastatic solid-tumor model. We have now extended our studies to examine the ability of recombinant SV40 T-Ag and plasmid DNA containing the SV40 T-Ag gene to prevent experimental murine pulmonary metastasis. BALB/c mice were immunized with a baculovirus-derived recombinant SV40 T-Ag preparation and subsequently challenged i.v. with syngeneic SV40 tumorigenic cells. All mice immunized with SV40 T-Ag produced SV40-T-Ag specific antibodies, as detected by ELISA. Again, no specific CTL activity was apparent when immune splenocytes were used as effector cells along with secondary in vitro stimulation in an attempt to expand the CTL population. Following immunization and challenge, the survival rates of mice were evaluated and revealed that immunization with SV40 T-Ag is completely protective against a lethal tumor challenge in this model. Conversely, immunization with plasmid DNA encoding the SV40 T-Ag gene resulted in partial or no protection in the SV40 T-Ag experimental pulmonary metastasis model. Together, these data suggest the possibility of an antibody-based

mechanism in the prevention of the experimental pulmonary metastasis. Studies by other investigators have suggested a role for antibodies in tumor immunity [70]. Synthetic human mucin, MUCI peptides conjugated to a carrier protein, generated antibodies but no T cell responses, including delayed-type hypersensitivity, lymphocyte proliferation, and CTL responses, when used to immunize mice. In a prophylactic vaccination format, significant protection was observed from a challenge with MUCI-expressing tumor cells. Lung foci were reduced by approximately 50%–70% when compared to control challenge group of mice, yet protection from tumors was not complete. In addition, this study demonstrated that one could not a priori select which MUCI peptide conjugate formulation would induce protection from tumor formation in mice without immunization and experimental challenge. Conjugation of MUCI to the carrier protein, involving a particular heterobifunctional cross-linking agent and the experimental adjuvant QS-21, was required for the induction of optimal tumor immunity. A potential role for antibody in tumor immunity and the prevention of metastasis in vivo has been recently described. Antibodies specific for the GD2 ganglioside prevented syngeneic micrometastasis in a murine lymphoma model [71]. In our system, additional studies are warranted to determine the exact role of antibodies and T cell populations in the induction of immunity in vivo within this SV40 T-Ag system.

A number of investigations have characterized the immune response to SV40 T-Ag in murine systems. It has been well established that BALB/c mice ($H\text{-}2^d$) do not respond to SV40 T-Ag, as far as induction of CTL responses are concerned, following challenge or immunization with SV40-transformed cells [15, 28, 39, 40, 62]. This has also been extended to immunization with recombinant SV40 T-Ag in alum adjuvants [5]. These data are not surprising, in that immunization with soluble proteins without modification or appropriate adjuvants only rarely induces $CD8^+$ CTL responses [16, 34, 35, 44, 47–49, 72]. Investigators have also demonstrated that BALB/c mice can reject subcutaneously administered primary tumors if they have been previously immunized with irradiated tumor cells, SV40-transformed allogeneic and xenogeneic cells, SV40-T-Ag-expressing adenovirus, and purified SV40 T-Ag protein [5, 21, 22, 32, 54, 68, 69] (reviewed in [59]). We and others have demonstrated that genetic immunization with DNA encoding SV40 T-Ag will provide complete immunity against subcutaneous tumors in BALB/c mice [7, 50]. In these studies, DNA immunization induced MHC-class-I-restricted $CD8^+$ CTL responses that were associated with tumor immunity [7, 50]. These investigations also confirmed the inability of immunization with SV40 T-Ag protein to induce CTL responses in BALB/c mice [7, 50], with data similar to those described herein. More recently, CTL responses have been induced in BALB/c mice by immunization with vaccinia virus expressing SV40 T-Ag [36]. These studies highlight the role of antigen processing and presentation pathways in the recruitment of distinct T cell subsets into the immune response. In addition, these data support the notion that both humoral an CMI responses play a role in immunity against primary SV40-T-Ag-expressing solid tumors.

To characterize the ability of immunization with SV40 T-Ag to protect from pulmonary metastasis, lung tumor foci were measured and enumerated following challenge. In our previous studies to develop the pulmonary metastasis model, digital image analysis of the lungs indicated that, 10 days after challenge, the lungs possess tumor foci that are small and few enough for an accurate measurement of number and size to be obtained [65, 66]. Therefore, this assay time and dose were chosen to analyze size and number of lung tumor foci as an indicator of tumor burden in the lung. No lung tumor foci were detected in mice immunized with recombinant SV40 T-Ag. These data indicate that immunization with recombinant SV40 T-Ag can prevent the establishment of pulmonary metastasis within this model.

In the development and characterization of this murine tumor model, the presence of SV40 tumorigenic cells in a number of organs and tissues was evaluated and characterized by morphological identification and by immunofluorescene directed to SV40 T-Ag expression [66]. The tumorigenic cells expressing SV40 T-Ag are detectable in organs and tissues distal to the site of inoculation and are indicative of tumor metastasis. In previous studies where SV40 T-Ag was shown to induce tumor immunity in murine systems, the animal models employed were non-metastatic. Tumor immunity was determined on the basis of either survival [5, 54] or measurement of a solid tumor via intradermal inoculation with tumor cells [25]. Therefore, the animal model employed in this present study represents a useful tool for studies associated with tissue-specific tropism in metastasis in addition to potential in studies of the mechanism(s) of tumor-specific immune responses. The in vivo tumor model we have employed is useful for the evaluation of immunologically based therapies with the goal of preventing the development of metastatic foci in the lungs of inoculated syngeneic mice by directing the immune response towards a tumor-specific antigen expressed on the tumor cells. Our results indicate that immunization with recombinant SV40 T-Ag, but not plasmid DNA containing the SV40 T-Ag gene, protects from lethal tumor challenge within this model and prevents the establishment of pulmonary tumors with the capability for subsequent metastasis. Based on our previous studies using a primary solid-tumor model, the experimental pulmonary metastasis model utilized here appears to be a more stringent system for evaluating SV40-T-Ag-based vaccination and immunotherapeutic strategies. A recent study has described a vaccinia human papillomavirus type 16 E7 prophylactic vaccination strategy that prevents the establishment of an experiemental pulmonary metastasis in eight out of ten mice challenged intravenously with E6/E7-expressing tumor cells [23]. To our knowledge, this is the second example

where immunization with a virally encoded tumor-specific antigen alone prevented the establishment of tumors within an experimental pulmonary metastasis, animal model system. However, in our studies, recombinant SV40 T-Ag induced complete tumor immunity within this tumor model system. These data imply a role for antibodies in SV40 T-Ag immunity against an experimental metastasis. Additional studies are required to evaluate the role of T cells in the induction of this observed immunity and whether therapeutic vaccination strategies will have any in vivo effect on the tumor burden within this system. Other investigations that utilized vaccines based on virally encoded tumor-specific antigens have demonstrated some antitumor effects in therapeutic modalities: however, these antitumor effects were not as pronounced as the tumor immunity observed in prophylactic regimens [23]. In certain situations, where genetics, environment, and exposure to a carcinogenic agent may present a high risk for metastatic cancers, prophylactic vaccination strategies may be warranted.

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References

1. Attanasio R, Lanford RE, Dilley D, Stunz GW, Notvall L, Henderson AB, Kennedy RC (1991) Immunogenicity of hepatitis B surface antigen derived from the baculovirus expression vector system: a mouse potency study. Biologicals 19:347–353
2. Baskar S, Glimcher L, Nabavi N, Jones RT, Ostrand-Rosenberg S (1995) Major histocompatibility complex class II^+ $B7\text{-}1^+$ tumor cells are potent vaccines for stimulating tumor rejection in tumor-bearing mice. J Exp Med 181:619–629
3. Benton PA, Kennedy RC (1997) DNA strategies for the treatment of cancer. Curr Top Microbiol Immunol 226:1–20
4. Bright RK, Shearer MH, Kennedy RC (1993) Comparison of the murine humoral immune response to recombinant simian virus 40 large tumor antigen: epitope specificity and idiotype expression. Cancer Immunol Immunother 37:31–39
5. Bright RK, Shearer MH, Kennedy RC (1994) Immunization of BALB/c mice with recombinant simian virus 40 large tumor antigen induces antibody-dependent cell-medicated cytotoxicity against simian virus 40-transformed cells. An antibody-based mechanism for tumor immunity. J Immunol 153:2064–2071
6. Bright RK, Shearer MH, Kennedy RC (1995) Examination of lymphokines induced in mice following immunization with recombinant simian virus 40 large tumor antigen. Cancer Immunol Immunother 40:206–211
7. Bright RK, Beames B, Shearer MH, Kennedy RC (1996) Protection against a lethal tumor challenge with SV40-transformed cells by direct injection of DNA encoding SV40 large tumor antigen. Cancer Res 56:1126–1130
8. Chen L, Ashe S, Brady WA, Hellenstrom I, Hellenstrom KE, Ledbetter JA, McGowan P, Linsley PS (1992) Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell 71:1093–1102
9. Cohen J (1993) Cancer vaccines get a shot in the arm. Science 262:841–843
10. De Bruijn MLH, Schuurhuis DH, Vierboom MPM, Vermeulen H, de Cock KAJ, Ooms ME, Ressing ME, Toebes M, Franken KLMC, Drijfhout JW, Ottenhoff THM, Offringa R, Melief CJM (1998) Immunization with human papillomavirus type 16 (HPV16) oncoprotein-loaded dendritic cells as well as protein in adjuvant induces MHC class I-restricted protection to HPV 16-induced tumor cells. Cancer Res 58:724–731
11. Dranoff G, Mulligan RC (1995) Gene transfer as cancer therapy. Adv Immunol 58:417–454
12. Fernandez N, Duffour MT, Perricaudet M, Lotze MT, Tursz T, Zitvogel L (1998) Active specific T-cell-based immunotherapy for cancer: nucleic acids, peptides, whole native proteins, recombinant viruses, with dendritic cell adjuvants or whole tumor cell-based vaccines. Principles and future prospects. Cytokines Cell Mol Ther 4:53–65
13. Fernando GJ, Stewart TJ, Tindle RW, Frazer IH (1998) Th2-type $CD4^+$ cells neither enhance nor suppress antitumor CTL activity in a mouse tumor model. J Immunol 161:421–2427
14. Fields RC, Shimizu K, Mule JJ (1998) Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. Proc Natl Acad Sci USA 95:9482–9487
15. Gooding LR (1979) Specificities of killing by T lymphocytes generated against syngeneic SV40 transformants: studies employing recombinants within the H-2 complex. J Immunol 122:1002–1008
16. Heeg K, Kuon W, Wagner H (1991) Vaccination of class I major histocompatibility complex (MHC)-restricted murine $CD8^+$ cytotoxic T lymphocytes towards soluable antigens: immunostimulating-ovalbumin complexes enter the class I MHC-restricted antigen pathway and allow sensitization against the immunodominant peptide. Eur J Immunol 21:1523–1527
17. Herlyn D, Wettendorff M, Koprowski H (1989) Modulation of cancer patients' immune responses by anti-idiotypic antibodies. Int Rev Immunol 4:347–357
18. Herlyn D, Sears HF, Ernst CS, Iliopoules D, Steplewski Z, Koprowski H (1991) Initial clinical evaluation of two murine IgG2a monoclonal antibodies for immunotherapy of gastrointestinal carcinoma. Am J Clin Oncol 14:371–378
19. Herlyn D, Harris D, Zaloudi KJ, Sperlagh M, Maruyama H, Jacob L, Kieny MP, Scheck S, Somasundaram R, Hart E, Ertl H, Mastrangelo MJ (1994) Immunomodulatory activity of monoclonal anti-idiotypic to anti-colorectal carcinoma antibody CO17-1A in animals and patients. J Immunother 15:303–311
20. Hsu FJ, Carpar CB, Czerwinski D, Kwak LW, Liles TM, Syrengelas A, Taidi-Laskouski, Levy R (1997) Tumor-specific idiotype vaccines in the treatment of patients with B-cell lymphoma-long-term results of a clinical trial. Blood 89:3129–3135
21. Jay G, Jay FT, Chang C, Friedman RM, Levine AS (1978) Tumor-specific transplantation antigen: use of the $Ad2^+ND_1$ hybrid virus to identify the protein responsible for simian virus 40 tumor rejection and its genetic origin. Proc Natl Acad Sci USA 75:3055–3059
22. Jay G, Jay FT, Chang C, Levine AS, Friedman RM (1979) Induction of simian virus 40-specific tumor rejection by the $Ad2^+ND_2$ hybrid virus. J Gen Virol 44:287–296
23. Ji H, Chang EY, Lin KY, Kurman RJ, Pardoll DM, Wu TC (1998) Antigen-specific immunotherapy for murine lung metastatic tumors expressing human papillomavirus type 16 E7 oncoprotein. Int J Cancer 78:41–45
24. Kennedy RC, Dreesman GR (1984) Enhancement of the immune response to hepatitis B surface antigen. In vivo administration of antiidiotype induces anti-HBs that express a similar idiotype. J Exp Med 159:655–665
25. Kennedy RC, Dreesman GR, Butel JS, Lanford RE (1985) Suppression of in vivo tumor formation induced by simian virus 40-transformed cells in mice receiving antiidiotypic antibodies. J Exp Med 161:1432–1449
26. Kennedy RC, Shearer MH, Hildebrand WH, Simmonds RS (1997) Nonhuman primates and their potential use in immunologically based investigations. Immunologist 5:150–156
27. Kit S, Kurimura T, Dubs DR (1969) Transplantable mouse tumor line induced by injection of SV40-transformed mouse kidney cells. Int J Cancer 4:384–392
28. Knolwes BB, Koncar M, Pfizenmaier K, Solter D, Aden DP, Trinchieri G (1979) Genetic control of the cytotoxic T cell re-

sponse to SV40 tumor-associated specific antigen. J Immunol 122:1798–1806
29. Kohler H, Raychaudhuri S, Chen JJ, Saeki Y (1989) Idiotypic responses induced by tumor: an autocrine network. Int Rev Immunol 4:311–320
30. Kwak LW, Campbell MJ, Czerwinski DS, Hart S, Miller RA, Levy R (1992) Induction of immune responses in patients with B-cell lymphoma against the surface immunoglobulin idiotype expressed by their tumors. N Engl J Med 327:1209–1215
31. Lanford RE (1988) Expression of simian virus 40 T antigen in insect cells using a baculovirus expression vector. Virology 167:72–81
32. Law LW, Takemoto KK, Rogers MJ, Ting Rc (1977) Induction of simian virus 40 (SV40) transplantation immunity in mice by SV40 transformed cells of various species. J Natl Cancer Inst 59:1523–1526
33. Miller RA, Lowder J, Meeker TC, Brown S, Levy R (1987) Anti-idiotypes in B-cell tumor therapy. NCI Monogr 3:131–134
34. Nair S, Zhou X, Huang L, Rouse BT (1992) Class I restricted CTL recognition of a soluble protein delivered by liposomes containing lipophilic polylysines. J Immunol Methods 152:237–243
35. Newman MJ, Wu JY, Gardner BH, Munroe KJ, Leombruno D, Recchia J, Kensil CR, Coughlin RT (1992) Saponin adjuvant induction of ovalbumin-specific $CD8^+$ cytotoxic T lymphocyte responses. J Immunol 148:2357–2362
36. Newmaster RS, Mylin LM, Fu TM, Tevethia SS (1998) Role of a subdominant H-$2K^d$-restricted SV40 tumor antigen cytotoxic T lymphocyte epitope in tumor rejection. Virology 244:427–441
37. Pardoll DM (1993) New strategies for enhancing the immunogenicity of tumors. Curr Opin Immunol 5:719–725
38. Pass HI, Kennedy RC, Carbone M (1996) Evidence for and implications of SV40-like sequences in human mesotheliomas. In: DeVita VT Jr, Hellman S, Rosenberg SA (eds) Advances in oncology. Lippincott, Philadelphia, Pa., pp 89–108
39. Pfizenmaier K, Trinchieri G, Solter D, Knowles BB (1978) Mapping of H-2 genes associated with T cell-mediated cytotoxic responses to SV40-tumor-associated specific antigens. Nature 274:691–693
40. Pfizenmaier K, Pan SH, Knowles BB (1980) Preferential H-2 association in cytotoxic T cell responses to SV40 tumor-associated specific antigens. J Immunol 124:1888–1891
41. Plautz GE, Yang ZY, Gao X, Huang L, Nabel GJ (1993) Immunotherapy of malignancy by in vivo gene transfer into tumors. Proc Natl Acad Sci USA 90:4645–4650
42. Pulaski BA, Ostrand-Rosenberg S (1998) Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. Cancer Res 58:1486–1493
43. Raychaudhuri S, Saeki Y, Chen JJ, Iribe H, Fuji H, Kohler H (1987) Tumor specific idiotype vaccines. II. Analysis of the tumor-related network response induced by the tumor and by internal image antigens (Aβ2 beta). J Immunol 139:271–278
44. Reddy R, Zhou F, Nair S, Huang L, Rouse BT (1992) In vivo cytotoxic T lymphocyte induction with soluble proteins administered in liposomes. J Immunol 148:1585–1589
45. Rosenberg SA, Lotze MT, Yang JC, Topalian SL, Chang AE, Schwartzentruber DJ, Aebersold P, Leitman SA, Linehan WM, Seipp CA (1993) Prospective randomized trial of high-dose interleukin-2 alone or in conjuction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. J Natl Cancer Inst 85:622–632
46. Samuel J, Budzynski WA, Reddish MA, Ding L, Zimmermann GL, Krantz MJ, Koganty RR, Longenecker BM (1998) Immunogenicity and antitumor activity of a liposomal MUC1 peptide-based vaccine. Int J Cancer 75:295–302
47. Schirmbeck R, Zerrahn J, Kuhrîber A, Deppert W, Reimann J (1993) Immunization of mice with the N-terminal (I-272) fragment of simian virus 40 large T-antigen (without adjuvants) specifically primes cytotoxic T lymphocytes. Eur J Immunol 23:1528–1534
48. Schirmbeck R, Bohm W, Reimann J (1994) Injection of detergent-denatured ovalbumin primes murine class I-restricted cytotoxic T cells in vivo. Eur J Immunol 24:2068–2072
49. Schirmbeck R, Melber K, Kuhrîber A, Janowicz ZA, Reimann J (1994) Immunization with soluble hepatitis B virus surface protein elicits murine H-2 class I-restricted $CD8^+$ cytotoxic T lymphocyte responses in vivo. J Immunol 152:1110–1119
50. Schirmbeck R, Bohm W, Reimann J (1996) DNA vaccination prime MHC class I-restricted, simian virus 40 large tumor antigen-specific CTL in H-2^d mice that reject syngeneic tumors. J Immunol 157:3550–3558
51. Schreurs MWJ, Annemiek J, Figdor CG, Adema GJ (1998) Genetic vaccination against the melanocyte lineage-specific antigen gp 100 induces cytotoxic T lymphocyte-mediated tumor protection. Cancer Res 58:2509–2514
52. Schultze JL (1997) Vaccination as immunotherapy for B cell lymphoma. Hematol Oncol 15:129–139
53. Shearer MH, Lanford RE, Kennedy RC (1990) Monoclonal anti-idiotypic antibodies induce humoral immune responses specific for simian virus 40 large tumor antigen in mice. J Immunol 145:932–939
54. Shearer MH, Bright RK, Kennedy RC (1993) Comparison of humoral immune responses and tumor immunity in mice immunized with recombinant SV40 large tumor antigen and a monoclonal anti-idiotype. Cancer Res 53:5734–5739
55. Simanis V, Lane DC (1985) An immunoaffinity purification procedure for SV40 large T antigen. Virology 144:88–100
56. Sivanandham M, Shaw P, Bernik SF, Paoletti E, Wallack MK (1998) Colon cancer cell vaccine prepared with replication-deficient vaccinia viruses encoding B7.1 and interleukin-2 induce antitumor response in syngeneic mice. Cancer Immunol Immunother 46:261–267
57. Strassburg CP, Kasai Y, Seng BA, Miniou P, Zaloudik J, Herlyn D, Koprowski H, Linnenbach AJ (1992) Baculovirus recombinant expressing a secreted form of a transmembrane carcinoma-associated antigen. Cancer Res 52:815–821
58. Teng MN, Park BH, Koeppen HK, Tracey KJ, Fendly BM, Sohreiber H (1991) Long-term inhibition of tumor growth by tumor necrosis factor in the absence of cachexia or T-cell immunity. Proc Natl Acad Sci USA 88:3535–3539
59. Tevethia SS (1980) Immunology of simian virus 40. In: Klein G (ed) Viral oncology. Raven, New York, pp 581–601
60. Townsend SE, Allison JP (1993) Tumor rejection after direct costimulation of $CD8^+$ T cells by B7-transfected melanoma cells. Science 259:368–370
61. Trail P, Wilner D, Lasch SJ, Henderson AJ, Hofstead S, Casazza AM, Firestone RA, Hellstrom I, Hellstrom KA (1993) Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. Science 261:212–215
62. Trinchieri G, Aden DP, Knowles BB (1976) Cell-mediated cytotoxicity to SV40-specific tumor-associated antigens. Nature 261:312–314
63. Vitetta ES, Thorpe PE, Uhr JW (1993) Immunotoxins: magic bullets or misguided missiles? Immunol Today 14:252–259
64. Warren RQ, Wolf H, Zajac RA, Boswell RN, Kanda P, Kennedy RC (1991) Patterns of antibody reactivity to selected human immunodeficiency virus type 1 (HIV-1) gp 160 epitopes infected individuals grouped according to $CD4^+$ cell levels. J Clin Immunol 11:13–21
65. Watts AM, Kennedy RC (1998) Quantitation of tumor foci in an experimental murine tumor model using computer-assisted video imaging. Anal Biochem 256:217–219
66. Watts AM, Shearer MH, Pass HI, Kennedy RC (1997) Development of an experimental murine pulmonary metastasis model incorporating a viral encoded tumor specific antigen. J Virol Mehtods 69:93–102
67. Wexler H (1996) Accurate identification of experimental pulmonary metastases. J Natl Cancer Inst 36:641–645
68. Zarling JM, Tevethis SS (1973) Transplantation immunity to simian virus 40-transformed cells in tumor-bearing mice. I. Development of cellular immunity to simian virus 40 tumor-

specific transplantation antigens during tumorigenesis by transplanted cells. J Natl Cancer Inst 50:137–147
69. Zarling JM, Tevethis SS (1973) Transplantation immunity to simian virus 40-transformed cells in tumor-bearing mice. II. Evidence for macrophage participation at the effector level of tumor cell rejection. J Natl Cancer Inst 50:149–157
70. Zhang S, Graeber LA, Helling G, Ragupathi G, Adluri S, Loyd KO, Livingston PO (1996) Augmenting the immunogenicity of synthetic MUC1 peptide vaccines in mice. Cancer Res 56:3315–3319
71. Zhang H, Zhang S, Cheung NV, Ragupathi G, Livingston PO (1998) Antibodies against GD2 ganglioside can eradicate syngeneic cancer micrometastases. Cancer Res 58:2844–2849
72. Zhou F, Rouse BT, Huang L (1992) Induction of cytotoxic T lymphocytes in vivo with protein antigen entrapped in membranous vehicles. J Immunol 149: 1599–1604