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Tumor Immunity against a Simian Virus 40 Oncoprotein Requires CD8$^+$ T Lymphocytes in the Effector Immune Phase$^\nabla$

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The required activities of CD4$^+$ T cells and antibody against the virally encoded oncoprotein simian virus 40 (SV40) Tag have previously been demonstrated by our laboratory to be mediators in achieving antitumor responses and tumor protection through antibody-dependent cell-mediated cytotoxicity (ADCC). In this study, we further characterize the necessary immune cell components that lead to systemic tumor immunity within an experimental pulmonary metastatic model as the result of SV40 Tag immunization and antibody production. Immunized animals depleted of CD8$^+$ T cells at the onset of experimental tumor cell challenge developed lung tumor foci and had an overall decreased survival due to lung tumor burden, suggesting a role for CD8$^+$ T cells in the effector phase of the immune response. Lymphocytes and splenocytes harvested from SV40 Tag-immunized mice experimentally inoculated with tumor cells synthesized increased *in vitro* levels of the Th1 cytokine gamma interferon (IFN-γ), as assessed by enzyme-linked immunosorbent assay (ELISA) and flow cytometry assays. CD8$^+$ T-cell activity was also heightened in SV40 Tag-immunized and tumor cell-challenged mice, based upon intracellular production of perforin, confirming the cytolytic properties of CD8$^+$ T cells against tumor cell challenge. Altogether, these data point to the role of recombinant SV40 Tag protein immunization in initiating a cytotoxic T-lymphocyte (CTL) response during tumor cell dissemination and growth. The downstream activity of CD8$^+$ T cells within this model is likely initiated from SV40 Tag-specific antibody mediating ADCC tumor cell destruction.

Determining the immunologic mechanisms involved in antitumor responses can provide valuable insight into developing and formulating appropriate immunotherapeutic strategies against a range of human cancers (25). Cell-mediated immunity involving CD8$^+$ T lymphocytes is generally regarded as the primary response to utilize due to its potent and efficient cytotoxicity against tumor cell targets *in vitro* and in animal models (10). Indeed, the proof of concept of this approach is best characterized by specialized conditioning protocols that involve autologous transfer of tumor infiltrating lymphocytes (TILs) in metastatic melanoma patients, with objective responses that approximate 70% (8). However, the efficacy of TILs harvested from additional cancer types have been less than effective, and additional strategies, such as genetic modification of peripheral blood mononuclear cells, are being explored to improve and extend the approach of cytotoxic T-lymphocyte (CTL) immunotherapy clinically (33, 46).

The roles of immune components such as CD4$^+$ T cells and antibody have been given less attention within the context of promoting tumor immunity against a range of tumor antigens. For example, the ability of CD4$^+$ T cells to activate humoral immunity can lead to antitumor responses that involve antibody-dependent cell-mediated cytotoxicity (ADCC) (17). In this scenario, antibody binds its targeted antigen and effectors such as natural killer (NK) cells lyse tumorigenic cells through interaction with the Fc region of the bound antibody. The efficacy of ADCC has been realized in scenarios involving breast cancer and non-Hodgkin's lymphoma, for example, and to date, the only FDA-approved immunologic treatments against these malignancies involve antibody-based therapies (5).

The concurrent roles of antibody—with specific emphasis on ADCC—and CD8$^+$ T-cell immunity within the context of tumor immunity have not been widely reported. Several recent studies have commented on the ability of antibody-bound tumor cells, particularly as a whole tumor cell-dendritic cell (DC) vaccination approach, to initiate CTL activity by engaging DCs through Fc receptors (9, 19, 34). However, to our knowledge, the mechanistic aspects of ADCC (e.g., NK-mediated lysis) promoting CD8$^+$ T-cell activity have been explored in relatively few studies (27, 41). From an immunotherapeutic standpoint, it may be preferable in certain settings to induce both the humoral and cell-mediated arms of the immune system to offset the progression of tumor cell growth and dissemination. Namely, these strategies could include active or passive approaches to first effectively induce ADCC in response to a tumor antigen, which would promote CTL activity against additional tumor targets through cross-presentation.

Our laboratory has been involved in determining the immunologic mechanisms of tumor immunity induced by the virally encoded tumor-specific antigen simian virus 40 (SV40) large tumor antigen (Tag). The mechanistic aspects of SV40 Tag-induced tumor immunity have been examined within an experimental murine model of pulmonary metastasis. To date, CD4$^+$ T cells and SV40 Tag-specific antibody have been

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implicated as required immune components within this murine system in order to achieve complete systemic tumor immunity (18). These studies demonstrated that during the course of immunization with SV40 Tag (i.e., the induction-phase response), $CD4^+$ T cells were required to induce an SV40 Tag humoral response. The specific role of the antibody response against an experimental tumor cell challenge was observed to involve ADCC-mediated clearance pathways (4, 23).

In the present study, we further characterize the immunologic response to SV40 Tag immunization by observing the necessary immune cell components following experimental challenge (i.e., the effector-phase response) with a tumor cell line expressing SV40 Tag. With the development of an SV40 Tag antibody response following SV40 Tag immunization *in vivo*, $CD8^+$ T-cell depletion during the effector phase resulted in the formation of lung tumor foci and decreased survival not observed with the abrogation of $CD4^+$ T cells. SV40 Tag-immunized mice also displayed a heightened Th1 response and $CD8^+$ CTL activity after experimental tumor cell challenge, as assessed by enzyme-linked immunosorbent assay (ELISA) and flow cytometry assays. In all, these data indicate that $CD8^+$ T cells mediate tumor immunity following antibody activation in response to the tumor-specific antigen SV40 Tag. We hypothesize that $CD8^+$ T-cell activity is initiated due to cross-presentation mechanisms as a result of ADCC activity against SV40 Tag. We are not aware of another published report that formulates a role for ADCC activity against a viral oncoprotein in this manner in order to engage $CD8^+$ T-cell activation.

SV40 Tag has been reported to be expressed in a number of human cancers, including malignant pleural mesothelioma and non-Hodgkin's lymphoma, although a causal link between SV40 infection and tumorigenesis is uncertain (11, 24, 35). The results of this study have direct implications for the construction of an appropriate immunotherapeutic strategy for patients suffering malignancies expressing the SV40 Tag tumor-specific antigen.

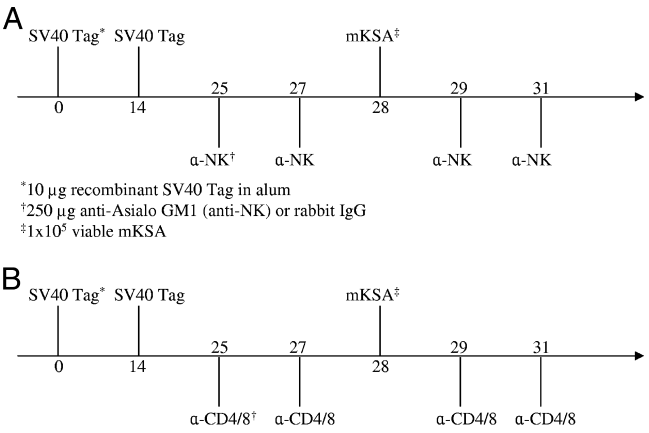

FIG. 1. Murine schedule for immunization, T-cell subset depletion, and tumor cell challenge. Mice were immunized i.p. with 10 μg recombinant SV40 Tag at 0 and 14 days and were challenged i.v. with 1×10^5 viable mKSA tumor cells on day 28. (A) One study assessed the role of NK cells in the effector phase by depleting animals with 250 μg anti-asialo GM1 (anti-NK) or a control rabbit IgG reagent, as indicated. Lungs were excised 18 days following tumor inoculation and were analyzed for the presence of tumor nodules. (B) To deplete specific T-cell populations in the effector immune phase, 100 μg of GK1.5 (anti-CD4), 2.43 (anti-CD8), GK1.5 and 2.43, or a control rat IgG preparation was administered i.p. during the course of tumor challenge, at 25, 27, 29, and 31 days. One set of experiments examined lungs obtained at 14 and 30 days post-mKSA cell challenge for the development of tumor foci. In a second set of experiments, the roles of T-cell subsets were determined by analyzing the survival of groups of mice, using an end-point cutoff of 60 days following tumor cell challenge.

MATERIALS AND METHODS

Cells. The SV40-transformed BALB/c mouse kidney fibroblast cell line mKSA (20) was used for experimental tumor cell challenge studies. mKSA cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (HyClone, Logan, UT), supplemented with 0.1 mM nonessential amino acids, 100 units/ml penicillin, 500 μg/ml streptomycin (Sigma, St. Louis, MO), and 10% heat-inactivated fetal bovine serum (HyClone). Cells were incubated at 37°C in a 5% CO_2 atmosphere. Prior to administration to mice, cells were detached from flasks by 1 mM EDTA–phosphate-buffered saline (PBS) (pH 7.5) treatment, washed, and adjusted to a 1×10^5 cell count in a total volume of 50 μl PBS.

***In vivo* depletion reagents against T lymphocytes and NK cells.** Murine $CD4^+$ and $CD8^+$ T lymphocytes were depleted *in vivo* using the rat IgG monoclonal antibodies (MAbs) GK1.5 and 2.43, respectively. These depletion reagents were purified using affinity chromatography, and total protein content was estimated by the absorbance at 280 nm. Both purified rat IgG MAbs and a control rat IgG preparation were administered intraperitoneally (i.p.) to mice at 100 μg in 0.1 ml PBS on days 25, 27, 29, and 31. Mice were experimentally challenged with tumor cells on day 28 (Fig. 1). To examine the efficiency of T-cell subset depletion, splenocytes and lymph nodes from MAb-treated mice were analyzed by flow cytometry, using rat MAbs specific for mouse CD4 (clone RM4-5) (BD Biosciences, Franklin Lakes, NJ) and mouse CD8 (clone YTS169.4) (BD Biosciences), as previously described (18). Compared to untreated and rat IgG-treated control mice, >95% of either T-cell population was depleted (data not shown).

In a separate study, NK cells were depleted by *in vivo* depletion using the rabbit IgG preparation anti-asialo GM1 (Cedarlane Laboratories, Burlington, NC), following the purification and delivery guidelines described above. Control groups of mice were treated with a nonimmune rabbit IgG preparation. The efficacy of NK cell depletion was first assessed through flow cytometry by staining splenocytes with a rat MAb specific for mouse CD49b (clone DX5) (BD Biosciences). Again, compared to the control treated mice, >95% of the NK cell population was depleted in the anti-asialo GM1-treated group.

Mice, immunization, and tumor cell challenge. Six- to 8-week-old BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained under standard conditions. Treatment and care of animals were performed in accordance with institutional guidelines and the Animal Welfare Assurance Act.

Groups of mice were immunized i.p. 2 weeks apart on days 0 and 14 with 10 μg of recombinant SV40 Tag in alum, while control groups received alum alone (Fig. 1). Sera were obtained from animals 7 days following the first and second immunizations to determine anti-SV40 Tag antibody reactivity by ELISA. On day 28, mice were experimentally challenged intravenously (i.v.) with 1×10^5 viable mKSA cells in a total volume of 50 μl PBS. In order to assess tumor burdens in mice, lungs were obtained 14 and 30 days following mKSA tumor cell challenge and were analyzed for the presence of lung tumor foci (43). To further assess the effects of tumor cell challenge, we evaluated the survival of mice following *in vivo* T-cell depletion and mKSA experimental challenge, where an end-point cutoff for survival of 60 days post-tumor challenge was employed (3, 4, 36).

Lung tumor focus determination. Following experimental tumor cell challenge, mice were euthanized at 14 and 30 days post-mKSA cell administration to determine the presence of lung tumor foci. After intratracheal injection using 10% India ink, left lungs were extracted from mice and stained in Fekete's destaining solution. Enumeration of tumor foci on the ventral surface of the lung was carried out using a FluorChem 8000 digital imaging system (Alpha Innotech Co., San Leandro, CA). Density threshold and focus diameter parameters were employed to consistently count lung tumor foci and to minimize subjectivity. These methods have been described previously by our laboratory (43).

SV40 Tag ELISA. To determine anti-SV40 Tag antibody reactivity, sera from mice were analyzed using indirect ELISA as previously described (3, 36, 37). A cutoff optical density value for positive reactivity and end-point titer determinations was established to be approximately three times the value obtained for a 1:20 dilution of preimmune serum. In similar assays, anti-SV40 Tag antibody failed to bind a control hepatitis B virus surface antigen (data not shown).

Intracellular flow cytometry. Groups of naïve mice were immunized with alum alone or with SV40 Tag on days 0 and 14 and either left unchallenged or experimentally challenged with 1×10^5 mKSA cells at day 21. Fourteen days following tumor cell challenge (day 35), lymph nodes were obtained from euthanized mice and homogenized in PBS. The bulk cell suspension was loaded onto a Histopaque-1077 gradient solution (Sigma), and the lymphocyte layer was harvested by centrifugation and washed in PBS according to the manufacturer's instructions. The final cell pellet was resuspended in 5 ml RPMI medium supplemented with phorbol myristate acetate (PMA; 50 ng/ml), ionomycin (1 μM), and brefeldin A (1 μg/ml) and then was stimulated for 5 h at 37°C.

All fluorescence-activated cell sorting (FACS) reagents were purchased from BD Biosciences, unless otherwise stated. For each analysis, 1×10^6 cells were pelleted, resuspended in 100 μl blocking buffer (1% bovine serum albumin [BSA]–0.5% sodium azide in PBS), and incubated at room temperature in the dark for 20 min. Cell surface molecules were targeted by staining CD3 with phycoerythrin (PE)-Cy5-conjugated anti-mouse CD3e (145-2C11) followed by the addition of 100 ng fluorescein isothiocyanate (FITC)-conjugated anti-mouse reagent against CD4 (RM4-5) or CD8a (53-6.7), with an incubation time of 20 min. After fixation, the cells were pelleted, permeabilized by resuspension in 100 μl BD Perm/Wash that contained 100 ng of PE-conjugated anti-mouse gamma interferon (IFN-γ) (XMG1.2) or perforin (OMAK-D) (eBioscience, San Diego, CA), and again incubated for 20 min at room temperature.

After intracellular cytokine staining, the cells were pelleted, resuspended in 1 ml FACS flow sheath fluid, and analyzed with a Becton Dickinson FACSVantage SE instrument using CellQuest software. Analysis of the samples consisted of first gating on CD3-positive cells, followed by the examination of additional cell markers and intracellular IFN-γ or perforin production. Data are presented as total percentages of the cell population, determined by first averaging values from three individual mice in each group and then subtracting background values obtained from lymphocytes harvested from alum- or SV40 Tag-immunized animals that were not experimentally challenged with tumor cells.

IFN-γ ELISA. Splenocyte secretion of IFN-γ was assessed by use of an OptEIA ELISA kit (BD Biosciences). Briefly, splenocytes were obtained and pooled from four alum- and four SV40 Tag-immunized mice 2 weeks following mKSA tumor cell challenge. Cells were incubated at 37°C for 7 days in DMEM in the presence of irradiated mKSA cells at a 20:1 effector-to-target (E:T) ratio, with interleukin-2 (IL-2) (20 U/ml) and IL-7 (5 pg/ml), in 24-well plates (14). Splenocytes were washed extensively with DMEM, transferred to 96-well plates in DMEM at E:T ratios of 5:1 to 40:1, based on 2×10^4 target cells, and incubated at 37°C for 24 h without additional stimuli. Supernatants were collected from wells and assayed for the presence of IFN-γ by ELISA. The plate was developed following the manufacturer's instructions, and optical density values were converted to pg/ml amounts of IFN-γ, using a recombinant IFN-γ standard curve.

Histology. Lung organs were obtained from alum- and SV40 Tag-immunized mice challenged with mKSA cells and were fixed in 10% formalin. Tissues were sectioned and stained by hematoxylin and eosin (H&E), and an institutional pathologist examined samples for tumor lesions in a blinded fashion.

Statistical analysis. Antibody serum titer values were statistically assessed by logarithmic transformation followed by one-way analysis of variance (ANOVA). An unpaired two-sample *t* test was considered for all other analyses. Statistical significance was indicated at *P* values of <0.05.

RESULTS

Anti-SV40 Tag antibody response develops normally prior to T-cell-subset depletion. Mice were immunized with alum alone or with 10 μg recombinant SV40 Tag in alum a total of 2 times at 0 and 14 days (Fig. 1). Seven days following the first and second immunizations, sera were obtained and analyzed for anti-SV40 Tag reactivity by indirect ELISA. Groups of mice were then depleted of specific T-cell subsets in the effector-phase immune response, prior to and after experimental tumor cell challenge.

TABLE 1. SV40 Tag-specific antibody titers in mice prior to treatment with anti-CD4 or anti-CD8 monoclonal antibodies[a]

Immunization/ treatment	Antibody titer (mean ± SEM [range]) at day 7	
	Post-1st immunization	Post-2nd immunization
Alum	<50	<50
SV40 Tag/rat IgG	<50	1,610 ± 663 (50–3,200)
SV40 Tag/anti-CD4	<50	1,783 ± 455 (<50–3,200)
SV40 Tag/anti-CD8	<50	1,167 ± 509 (50–3,200)

[a] Mouse sera were obtained 7 days following the first and second immunizations with SV40 Tag, and anti-SV40 Tag antibody reactivity was determined using indirect ELISA. Fourfold serial dilutions were performed on sera, beginning with a dilution of 1:50, and the absorbance at 410 nm was determined. A final dilution that resulted in an optical density above the cutoff value was the end-point titer. Values represent the mean (range) antibody end-point titers for animals in each immunized/treated group. The alum- and SV40 Tag/rat IgG-treated groups contained 5 mice, while the SV40 Tag/anti-CD4 and SV40 Tag/anti-CD8 groups consisted of 9 animals.

As shown in Table 1, all groups immunized with SV40 Tag generated detectable anti-SV40 Tag levels before treatment with depletion reagents targeting $CD4^+$ or $CD8^+$ T cells in the effector-phase immune response. The mean antibody titer for SV40 Tag/rat IgG control mice was 1,610, with a range of 50 to 3,200. Similarly, mean titers for anti-CD4- and anti-CD8-treated groups were 1,783 and 1,167, respectively. As expected, animals immunized with alum alone did not develop an SV40 Tag-specific humoral response. Based on end-point titers, the anti-SV40 Tag antibody responses were similar among the SV40 Tag-immunized experimental groups ($P > 0.05$; ANOVA).

These SV40 Tag antibody titer data were expected, given that $CD4^+$ T cells remain functional in the induction-phase response of SV40 Tag immunization and serve to prime and activate B cells, which differentiate into plasma cells to produce SV40 Tag-specific antibody. Our laboratory has previously reported on the requirement of $CD4^+$ T cells and anti-SV40 Tag antibody during the early course of immunization (i.e., induction phase) to achieve systemic tumor immunity (18). With the current depletion scheme, comparable anti-SV40 Tag antibody responses were attained in all SV40 Tag-immunized groups. Therefore, depletion with MAbs against T-cell subsets affected those T-cell populations at the time of tumor challenge (i.e., effector phase), allowing examination of the effector cell roles at the onset of tumor cell dissemination and growth.

NK cells are required for the effector-phase immune response. We previously published on the ability of SV40 Tag antibody to initiate ADCC reactions against mKSA tumor cells (4, 23). To further support the required role of ADCC and to define a necessary effector cell responsible for ADCC-mediated reactions within our tumor challenge system, we depleted NK cells in the effector phase, as outlined in Fig. 1A. Eighteen days following tumor inoculation, all animals (5/5) immunized with alum developed lung tumor foci (Table 2). As expected, those mice immunized with SV40 Tag and treated with a control rabbit IgG reagent were protected from tumor inoculation, as no tumor nodules were discovered. However, among SV40 Tag-immunized mice depleted of NK cells, all animals (5/5) succumbed to tumor challenge.

TABLE 2. Development of lung tumor foci in mice depleted of NK cells during the onset of tumor challenge[a]

Immunization/treatment	% Lung focus development (no. unprotected/no. challenged)
Alum	100 (5/5)
SV40 Tag/rabbit IgG	0 (0/5)
SV40 Tag/anti-NK	100 (5/5)

[a] SV40 Tag-immunized mice were depleted of NK cells during the effector phase response, as shown in Fig. 1A. Eighteen days following tumor challenge, lungs were excised from euthanized animals and stained for the presence of tumor growth. Values are reported as the percentage of animals succumbing to tumor inoculation, based on the total number of mice challenged. Percentages are based on 5 mice per treatment group.

These results demonstrate that NK cells are required in SV40 Tag-immunized animals at the onsite of mKSA cell challenge. We previously demonstrated *in vitro* ADCC activity by using monoclonal antibodies and serum reactive to SV40 Tag (4). The *in vivo* requirement for Fcγ receptors has also been described by our group, supporting a role for Fcγ receptor-expressing immune cells in SV40 Tag-immunized mice (23). Therefore, with support of these earlier studies and the data outlined in Table 2, NK cells help to function as mediators of ADCC in the presence of an SV40 Tag antibody response.

Depletion of $CD8^+$ T cells results in lung tumor focus development. Eleven days after immunization with SV40 Tag, groups of mice were first depleted of $CD4^+$ or $CD8^+$ T cells before tumor cell challenge with mKSA cells (Fig. 1B). To determine the immunologic role of these depleted T-cell subsets within an experimental pulmonary metastasis challenge model, animals were euthanized at least 2 weeks following tumor cell challenge, and lungs were obtained and evaluated for the presence of lung tumor foci, using a computer-assisted counting method.

In lungs obtained at 14 days post-mKSA cell inoculation, control mice immunized with alum alone developed 11.6 foci, on average, with a range of 9 to 14 (Fig. 2). Lung foci were undetectable in a control group immunized with SV40 Tag and depleted using a rat IgG preparation. In contrast, anti-CD8 treatment resulted in an average of 2 foci (range, 0 to 5), and 6 of 7 mice succumbed to tumor challenge. A double depletion scheme of anti-CD4 and anti-CD8 treatment yielded a similar mean focus value of 2.2 (range, 0 to 7). Interestingly, those mice receiving MAbs against $CD4^+$ T cells did not develop lung foci overall due to tumor challenge and had fewer foci than $CD8^+$ T-cell-depleted animals ($P < 0.05$; two-sample t test). Anti-CD4 treatment resulted in a mean focus value of 0.3 (range, 0 to 2), where 7 of 8 mice remained tumor free at the time of euthanasia. A comparison of the lung focus means between alum-treated control mice and anti-CD8-treated animals also indicated a statistical reduction in tumor load ($P < 0.05$; two-sample t test), suggesting that additional immunologic mechanisms are occurring in the effector-phase response in SV40 Tag-immunized/$CD8^+$ T-cell-depleted mice to mediate antitumor immunity.

Representative images of lungs obtained at 14 and 30 days

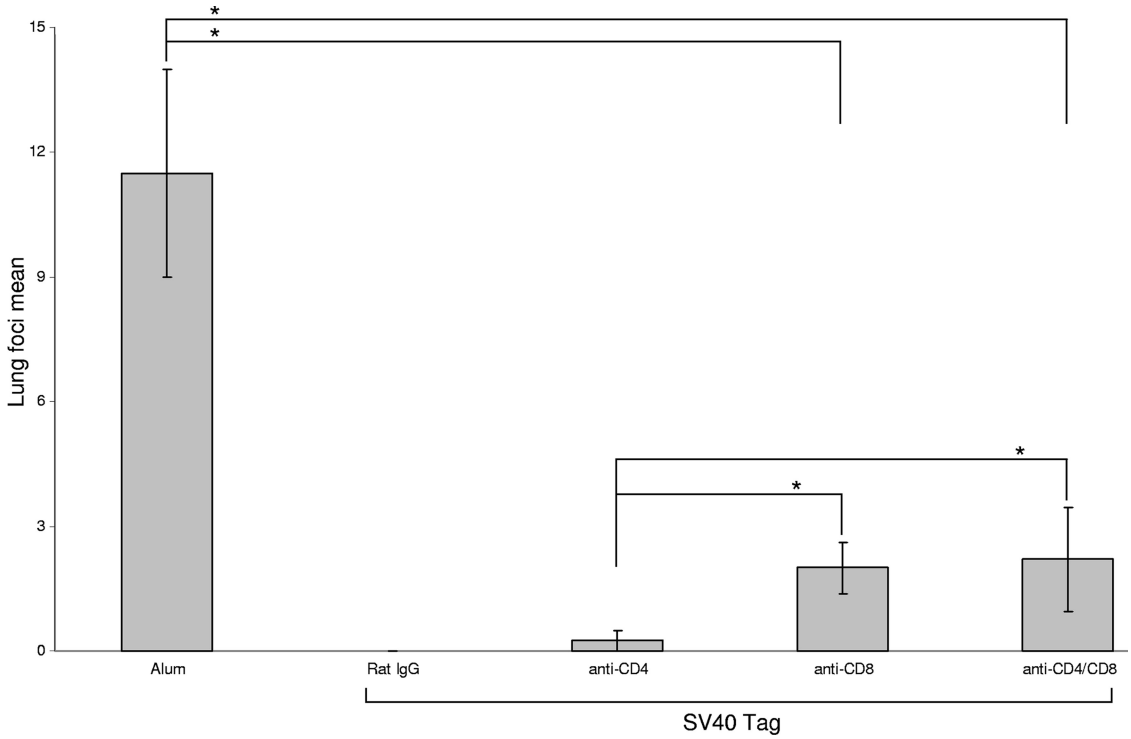

FIG. 2. Lung focus development in mice depleted of T-cell subsets during the effector-phase immune response to tumor challenge. Groups of mice were immunized with recombinant SV40 Tag protein and subsequently depleted of T-cell subsets during the course of experimental challenge with mKSA tumor cells. To assess tumor burden, lungs were obtained from euthanized animals at 14 days post-tumor cell challenge, and lung tumor focus numbers were analyzed using a computer-assisted method. All SV40 Tag-immunized groups consisted of 5 to 8 mice per experimental group, while data for the alum control group were the results for 3 animals. Error bars show standard errors of the means (SEM).

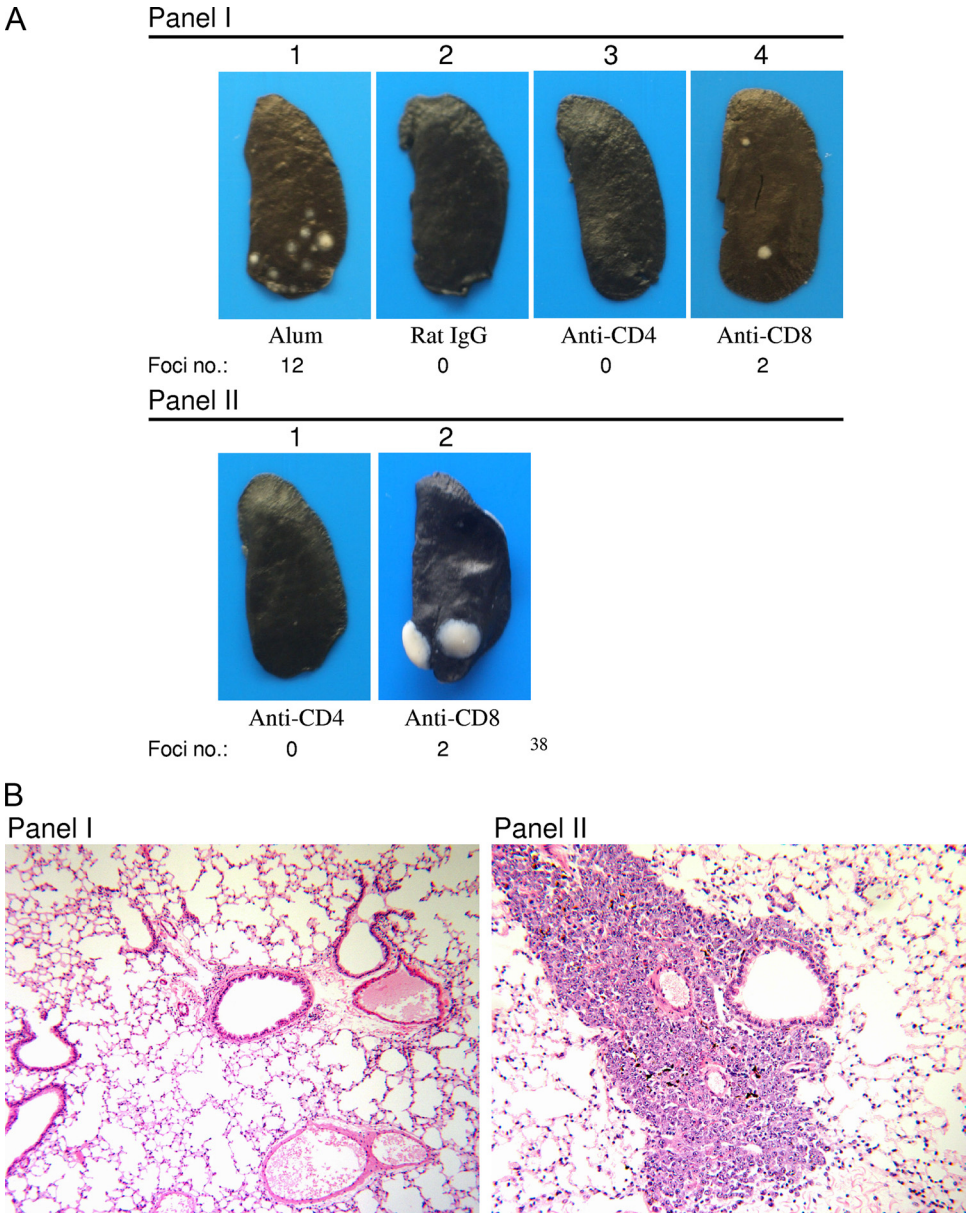

FIG. 3. Representative lung tumor nodules in SV40 Tag-immunized mice depleted of $CD4^+$ or $CD8^+$ T cells. (A) (I) Lungs obtained at 14 days post-mKSA cell tumor challenge, showing tumor focus formation in alum control (1) and anti-CD8-treated (4) mice. There was a lack of tumor foci in rat IgG control (2) and anti-CD4-treated (3) mice. (II) Lungs obtained 30 days following mKSA cell challenge, showing tumor focus formation in CD8-depleted mice (2), with a lack of tumor foci in anti-CD4-treated animals (1). The lung focus number for each respective lung is notated. (B) Representative H&E stains of lungs obtained from tumor-challenged mice immunized with either SV40 Tag (I) or alum (II).

post-mKSA cell challenge are depicted in Fig. 3A. As detailed in panels I and II, tumor foci were observed in SV40 Tag control-immunized mice depleted of $CD8^+$ T cells in the effector-phase immune response to mKSA tumor cell inoculation, and these lung nodules were confirmed to be tumors by H&E analysis (Fig. 3B). It is interesting that although the overall focus number remained unchanged, the focus diameter increased rapidly in unprotected animals from 14 to 30 days post-tumor challenge. Although we have not directly studied the mechanisms governing outgrowth of foci in CD8-depleted animals at 30 days postchallenge, it remains possible that tumor growth proceeds as a result of immunosuppressive effects of established tumor growth and/or the absence of a specific population of activated $CD8^+$ T cells. The *in vivo* growth kinetics of mKSA cells is the cause of death in unprotected animals within our experimental metastasis challenge model (45).

Since the CD8 and doubly depleted CD4/8 groups did not result in statistical differences in the degree of lung tumor burden and since anti-CD4 treatment did not lead to overall focus formation, the role of $CD4^+$ T cells as required mediators of tumor immunity upon the administration of tumor cell challenge can be ruled out. Taken together, these data demonstrate the necessary *in vivo* role of $CD8^+$ T cells to achieve

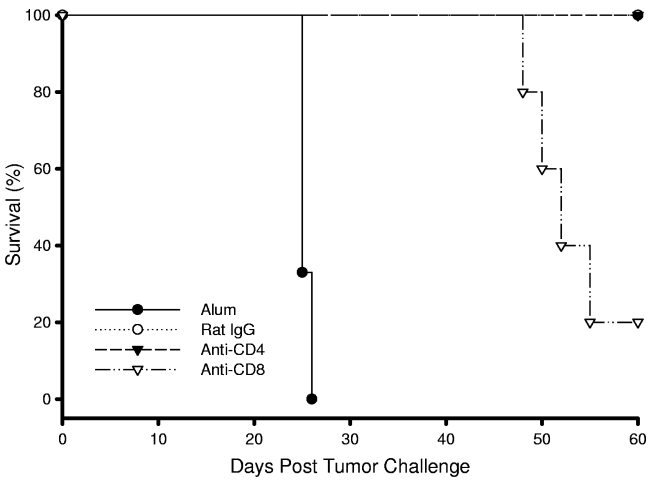

FIG. 4. Survival of mice following T-cell subset depletion and tumor challenge. Tumor burdens in treated groups were determined based on survival of mice following mKSA cell inoculation and $CD4^+$ T-cell or $CD8^+$ T-cell depletion. A 60-day end point following tumor cell challenge was established as a cutoff for survival determination. Immunized/treated groups consisted of 3 alum-, SV40 Tag/rat IgG-, or SV40 Tag/anti-CD4-treated mice, while 5 mice comprised the SV40 Tag/anti-CD8 group.

systemic tumor immunity during the effector-phase immune response within an experimental pulmonary metastasis challenge system.

Survival against tumor challenge is impaired in $CD8^+$ T-cell-depleted mice. In a second set of experiments, the role of T-cell subsets in depleted animals was determined based on survival after mKSA tumor cell challenge. Following the same immunization, depletion, and experimental challenge schedule (Fig. 1B), a 60 day post-tumor cell challenge end point was utilized in order to observe tumor immunity, as previously described by our laboratory (3, 4, 36). Figure 4 shows that unprotected animals immunized with alum alone survived for 25 days post-mKSA tumor cell challenge, on average (range, 25 to 26 days), due to lung tumor burden. In contrast, control animals immunized with SV40 Tag and injected i.p. using a rat IgG preparation did not develop lung tumor foci and survived for more than 60 days post-mKSA tumor cell challenge.

The data in Fig. 4 are depicted as percentages of survival versus days post-tumor cell challenge. Survival of mice depleted of $CD4^+$ T cells *in vivo* was similar to that of control SV40 Tag-immunized mice, as animals survived for more than 60 days post-tumor challenge and no animals developed lung tumor foci, as assessed by postmortem analysis. In contrast, $CD8^+$ T-cell depletion resulted in a mean survival of 53 days, with tumor focus formation evident in all mice euthanized at the 60-day end point. More specifically, by 60 days postchallenge, 4 of 5 animals (80%) did not survive as a result of the occurrence and burden of lung tumor foci (Fig. 4). The survival characteristics of alum-immunized mice and $CD8^+$ T-cell-depleted animals were also consistent with the lung focus mean values of the two groups (Fig. 2). Although $CD8^+$ T-cell depletion resulted in overall decreased survival of tumor inoculation, these treated mice had longer survival times than those of alum-immunized mice. These results, again, suggest that additional immune effector cells mediate complete systemic tumor immunity in SV40 Tag-immunized mice at the onset of tumor cell challenge. However, based on the mortality of SV40 Tag-immunized and T-cell-depleted mice, $CD8^+$ T cells were clearly required in preventing the occurrence of lung tumor foci in the effector-phase immune response and in initiating long-term survival of animals following tumor cell challenge.

IFN-γ is upregulated in tumor-challenged mice immunized with SV40 Tag. We next determined the extent to which SV40 Tag immunization promoted a Th1-driven response against tumor challenge within the effector phase of the immune response. Briefly, groups of naïve mice were immunized with alum alone or with SV40 Tag on days 0 and 14, and at day 21, animals were either left unchallenged or experimentally challenged with 1×10^5 mKSA cells. Fourteen days following tumor challenge (day 35), lymphocytes were harvested from mice, stimulated *in vitro* with PMA-ionomycin as detailed in Materials and Methods, and analyzed by flow cytometry for $CD4^+$ and $CD8^+$ T-cell intracellular synthesis of IFN-γ.

Alum immunization induced minimums of 3.7% and 4.4% stained $CD4^+$ or $CD8^+$ T cells, respectively, producing IFN-γ (data not shown). When mice were immunized with alum and subsequently challenged with mKSA tumor cells, the $CD4^+$ and $CD8^+$ T-cell populations demonstrated 3.1- and 1.8-fold increases, respectively, in their IFN-γ cytokine profiles, although these increases were not statistically significant ($P > 0.05$; two-sample t test). On the other hand, unchallenged SV40 Tag-immunized groups displayed <2% IFN-γ-stained cells among both CD4 and CD8 cells (data not shown). Inoculation of mKSA cells into SV40 Tag-immunized animals resulted in approximately 14-fold increases in $CD4^+$ and $CD8^+$ T-cell intracellular synthesis of IFN-γ ($P < 0.05$; two-sample t test).

Additionally, lymphocytes obtained from SV40 Tag-immunized and challenged animals were observed to have significant increases in the percentages of $CD4^+$ and $CD8^+$ T cells producing intracellular IFN-γ compared to those from mKSA cell-challenged mice immunized with alum (Fig. 5A). Flow cytometry data for challenged mice were first corrected for background values obtained from lymphocyte production of IFN-γ in unchallenged animals. For the alum-challenged group, means of 7.7% $CD4^+$ T cells and 3.4% $CD8^+$ T cells were observed. SV40 Tag-immunized animals challenged with mKSA cells developed IFN-γ production in 22.1% of $CD4^+$ T cells and 20.9% of $CD8^+$ T cells. Compared to the alum group values, SV40 Tag immunization resulted in 2.9- and 6.1-fold increases in the intracellular level of IFN-γ in $CD4^+$ and $CD8^+$ T cells, respectively ($P < 0.05$; two-sample t test). Representative flow cytometry dot plots are provided in Fig. 5B.

We next determined the IFN-γ secretion profiles of splenocytes harvested from alum-treated and SV40 Tag-immunized mice challenged with mKSA cells. Two weeks following tumor cell inoculation, splenocytes were obtained and pooled from animals and then specifically stimulated *in vitro* with irradiated mKSA tumor cells in the presence of IL-2 and IL-7 for 7 days. Cells were washed, reconstituted in DMEM at various cell densities (ranging from 5:1 to 40:1 E:T, based on a 2×10^4 cell target), and incubated for 24 h without further stimuli. Cell-free supernatants were then analyzed for the presence and amount (pg/ml) of IFN-γ through indirect ELISA. At all cell concentrations tested, splenocytes obtained from SV40 Tag-

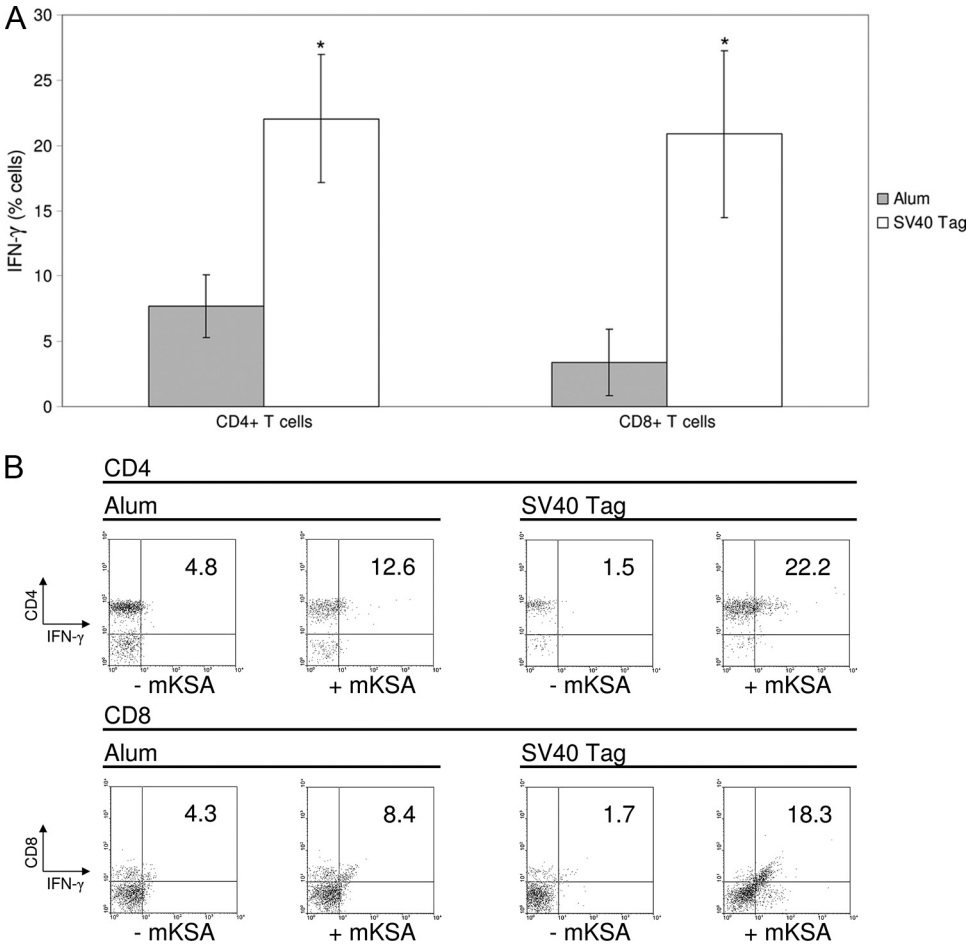

FIG. 5. IFN-γ level and cell distribution in lymphocytes in SV40 Tag-immunized and tumor-challenged animals. (A) Mice were immunized with alum alone or with SV40 Tag a total of 2 times and challenged with 1×10^5 viable mKSA tumor cells. Fourteen days following tumor inoculation, mice were euthanized and lymphocytes were isolated, stimulated *in vitro* with PMA-ionomycin, and analyzed by flow cytometry for intracellular synthesis of IFN-γ. The bars show the total percentages of stained cells, determined by averaging results for 3 mice in each group; error bars show the SEM. (B) Representative flow cytometry profiles of IFN-γ-producing T cells in alum- and SV40 Tag-immunized mice challenged with mKSA cells.

immunized mice secreted greater levels of IFN-γ than did splenocytes harvested from alum-immunized animals ($P < 0.05$; two-sample t test) (Fig. 6). For example, at both 5:1 and 10:1 E:T splenocyte ratios, expression of IFN-γ was >2-fold higher in SV40 Tag-immunized animals than in alum control mice.

Taken together, these results demonstrate the ability of immune effector cells from SV40 Tag-immunized mice to synthesize and secrete upregulated levels of the Th1 cytokine IFN-γ after the onset of tumor cell growth and spread.

CD8+ T cells from SV40 Tag-immunized and challenged mice exhibit increased perforin production. Due to the required role of CD8+ T cells to mediate *in vivo* tumor protection and the increases in CD4+ and CD8+ T-cell synthesis of IFN-γ *in vitro*, we next determined whether SV40 Tag immunization results in heightened CTL activity after mKSA tumor cell challenge. To evaluate this possibility, we analyzed CD8+ T-cell production of the perforin enzyme, which is expressed by activated CTLs and induces cytotoxicity in targeted cells. Groups of animals were again immunized with either alum or SV40 Tag and inoculated with mKSA tumor cells. Two weeks

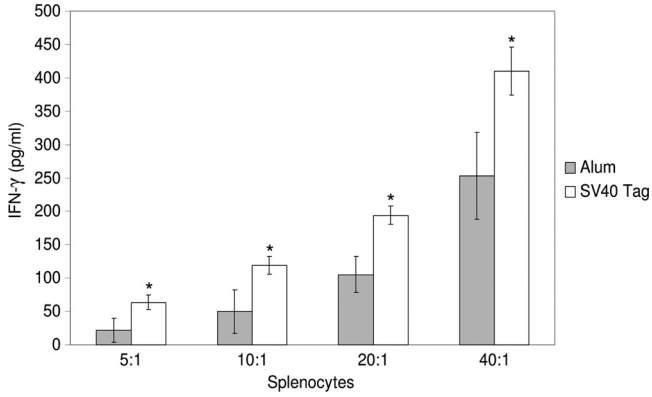

FIG. 6. Splenocyte *in vitro* secretion of IFN-γ in response to mKSA tumor cells. Two weeks after mKSA tumor cell challenge, splenocytes were harvested and pooled from 4 alum- and 4 SV40 Tag-immunized mice and stimulated for 7 days in the presence of irradiated mKSA cells with IL-2 and IL-7. Splenocytes were washed, reconstituted at various E:T ratios, based on a 2×10^4 cell target, and incubated in culture for 24 h at 37°C without additional stimuli. Supernatants were assayed by ELISA for the presence of IFN-γ, and optical density values were converted to pg/ml values by use of a recombinant IFN-γ standard curve. Error bars show standard deviations (SD).

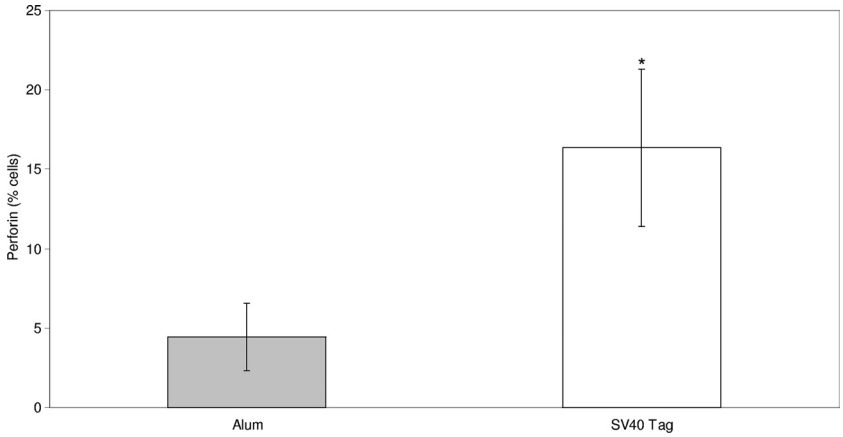

FIG. 7. $CD8^+$ T-cell intracellular synthesis of perforin in SV40 Tag-immunized and tumor-challenged mice. Fourteen days following tumor inoculation with 1×10^5 viable mKSA cells, alum- or SV40 Tag-immunized mice were euthanized and lymphocytes were isolated, stimulated *in vitro* with PMA-ionomycin, and analyzed by flow cytometry for intracellular synthesis of perforin. The bars show the total percentages of stained cells, determined by averaging results for 3 mice in each group. Error bars show the SEM.

following mKSA cell challenge, lymphocytes were harvested, stimulated *in vitro* with PMA-ionomycin, and stained with antibodies specific to the surface molecules CD3 and CD8 and to intracellular perforin. Flow cytometry was utilized to analyze the intracellular levels of perforin in challenged groups of immunized mice.

The unchallenged SV40 Tag-immunized group showed <2% of cells exhibiting a $CD8^+$ phenotype and producing perforin (data not shown). On the other hand, the SV40 Tag-immunized and mKSA tumor cell-challenged group displayed an approximately 12-fold increase in $CD8^+$ T cells producing perforin ($P < 0.05$; two-sample *t* test), an increase similar to that observed for $CD8^+$ T cells producing IFN-γ for this group. A comparison between alum-immunized groups left unchallenged or challenged showed $CD8^+$ T cells producing perforin, but differences between the levels of perforin were not statistically significant ($P > 0.05$; two-sample *t* test) (data not shown). Once levels of perforin production were corrected for background values of unchallenged animals immunized with either alum or SV40 Tag, the alum challenge group displayed a mean of 4.4% $CD8^+$ T cells positive for perforin synthesis (Fig. 7). On the other hand, SV40 Tag immunization and tumor inoculation resulted in a mean of 16.4% perforin-producing $CD8^+$ T cells. Compared to the alum values, SV40 Tag immunization resulted in a 3.7-fold increase of intracellular synthesis of perforin in $CD8^+$ T cells ($P < 0.05$; two-sample *t* test).

In all, these results suggest that SV40 Tag recombinant protein immunization and mKSA tumor cell challenge induced a heightened Th1 response (assessed by IFN-γ synthesis) during the effector-phase response to mKSA tumorigenic cells. The ability to heighten the response to such a degree likely allows SV40 Tag-immunized animals to mount CTL reactions *in vivo*, as typified by heightened levels of perforin production in $CD8^+$ T cells stimulated *in vitro*.

DISCUSSION

Previous studies from our laboratory have demonstrated a required role for $CD4^+$ T cells and SV40 Tag-specific antibody in the induction-phase response to the SV40-transformed mKSA tumor cell line in an experimental murine model of pulmonary metastasis (18). In these experiments, mice were treated with antibodies against $CD4^+$ and $CD8^+$ T-cell subsets during the course of immunization with recombinant SV40 Tag protein, and only those animals depleted of $CD4^+$ T cells developed lung tumor foci. We hypothesized that $CD4^+$ T cells were functioning as helper cells to promote B-cell differentiation into antibody-secreting plasma cells, since the lack of an SV40 Tag antibody response in $CD4^+$ T-cell-depleted mice correlated with reduced tumor immunity. The role of antibody within this animal model was ascribed to promoting ADCC in order to achieve tumor immunity. Our past studies using *in vitro*-stimulated peritoneal exudate cells from BALB/c mice indicated a role for ADCC tumor clearance of mKSA cells, using either reactive serum or monoclonal antibodies specific to SV40 Tag antibody (4). The requirement of SV40 Tag antibody and an ADCC induction-phase mechanism for tumor immunity was further supported by *in vivo* studies using immunologically deficient Fcγ receptor knockout mice (23). Briefly, animals deficient in the activating Fcγ I/III receptors (expressed by macrophages and NK cells) succumbed to tumor cell challenge, while mice deficient in the inhibitory Fcγ receptor IIB mounted protective antitumor responses.

In contrast to the induction-phase response, the current study provides evidence that $CD4^+$ T cells do not play a predominant role in antitumor immune responses in the effector phase. Those animals abrogated of functioning $CD8^+$ T cells succumbed to mKSA cell challenge and had an overall decreased survival following tumor cell inoculation, indicating the required *in vivo* effects of $CD8^+$ T cells in this experimental tumor challenge system. Yet there also appeared to be some protective effects in SV40 Tag-immunized mice depleted of $CD8^+$ T cells, based upon reduced lung foci and increased survival post-tumor challenge compared to unprotected control-immunized mice. Since all mice mounted an SV40 Tag antibody response due to immunization prior to the depletion of T-cell populations in the effector phase, the latter results would support our previous reports and the data outlined in

Table 2 in the current study showing that SV40 Tag antibody mediates ADCC at the onset of tumorigenic growth and dissemination, particularly as it relates to NK cell function.

The Th1 response was heightened for at least 14 days following the i.v. inoculation of mKSA tumor cells into mice immunized with SV40 Tag. Both $CD4^+$ and $CD8^+$ T cells harvested from lymph nodes synthesized increased intracellular levels of IFN-γ after *in vitro* stimulation with PMA-ionomycin. We extended these studies to include splenocytes from SV40 Tag-immunized/tumor cell-challenged animals and specifically activated the cells in culture with irradiated mKSA cells to observe the similar effect of increased IFN-γ expression. Although $CD4^+$ T cells were capable of displaying an elevated Th1 phenotype, their direct role *in vivo* was not required in the effector-phase response, particularly as it related to the interaction with $CD8^+$ T cells. Based on the Th1 profile of SV40 Tag-immunized and tumor-challenged mice, $CD8^+$ T cells were likely mediating cytotoxic reactions against mKSA tumor cells *in vivo*. To confirm this CTL activity, $CD8^+$ T cells were harvested from lymph nodes of SV40 Tag-immunized animals at 14 days post-mKSA tumor cell inoculation. When stimulated for 5 h *in vitro* with PMA-ionomycin, $CD8^+$ T cells synthesized increased intracellular levels of the cytotoxicity-specific enzyme perforin. This enzyme is expressed in functional CTLs.

Our characterization of the antitumor response to i.v. challenge with mKSA cells in SV40 Tag-immunized mice is as follows. SV40 Tag immunization induces a $CD4^+$ T-cell immune response that activates B-cell production of SV40 Tag-specific antibody in the induction phase of the immune response (18). With the introduction of tumorigenic cells and the progression of experimental metastasis, a Th1 response is heightened and the secreted antibody binds to surface-expressed SV40 Tag on mKSA cells, initiating Fc receptor-mediated ADCC by innate effector cell populations such as NK cells and macrophages (4, 23). The cytokine environment, as well as tumor-killing events by components of the innate system, activate DCs (6, 15). These mature DCs are then capable of promoting a $CD8^+$ T-cell response to tumor cells without the direct need for $CD4^+$ T cells (12, 30, 42). This characterization of the antitumor response not only is distinct in terms of ADCC promoting CTL activity but also is unique in that we are not aware of a published study that has implicated a viral oncoprotein to engage tumor-specific $CD8^+$ T cells through ADCC.

Within our own model, the late-stage $CD8^+$ T-cell response is unlikely to be solely SV40 Tag specific. Rather, with ADCC occurring, DCs process and present a range of exogenously released antigens and necrotic tissue from lysed mKSA cells via major histocompatibility complex (MHC) class I pathways through cross-priming mechanisms. Cross-priming was recently described by our group, using a similar tumor challenge system employing a murine mammary carcinoma cell line, designated 4T1, and the 4T1 cell line transfected with plasmid DNA carrying the SV40 Tag gene, designated 4T1.Tag (22). In this study, BALB/c mice were first immunized with SV40 Tag and challenged subcutaneously (s.c.) with 4T1.Tag, followed by s.c. challenge with 4T1 2 weeks later. All animals immunized and challenged in this fashion were protected from the development of lung metastasis from both tumorigenic cell lines. Specificity within this system for tumor-associated antigen cross-priming and tumor cell destruction was also demonstrated, as groups failed to be protected from a secondary 4T1 s.c. injection after first being immunized with SV40 Tag and challenged with mKSA cells.

In the present study, our results differ slightly with respect to a previous report by Utermöhlen and colleagues, where BALB/c mice were immunized with SV40 Tag and challenged i.p. with mKSA tumor cells (40). During the course of immunization and tumor cell challenge (i.e., the induction and effector phases), both $CD4^+$ and $CD8^+$ T-cell subsets were found to be required immunologic components to achieve protection from mKSA cell tumorigenic growth *in vivo*. The cytokine profile of $CD4^+$ T cells obtained from the tumor microenvironment was Th1 driven, indicating that $CD4^+$ T cells were functioning in a capacity to help prime a $CD8^+$ T-cell response, though weak CTL activity was detected only at the tumor site. Our analysis, on the other hand, points to the importance of a $CD8^+$ T-cell response without the aid of $CD4^+$ T-cell help during the effector-phase response, although there was an overall increase in $CD4^+$ T-cell production of IFN-γ following tumor challenge. The discrepancies of our results reported here and those contributed by Utermöhlen and colleagues (40) may simply be a result of the route of tumor inoculation promoting differences in the ensuing immune response. In our experimental pulmonary challenge model, we have attempted to simulate advanced disease, which is commonly diagnosed in the clinic and remains an impediment to immunotherapeutic intervention. In our nonmetastatic tumor challenge model where BALB/c mice are injected i.p. with mKSA cells and tumors grow locally, $CD8^+$ T cells served to induce protective tumor immunity in mice immunized with a plasmid DNA vector carrying the SV40 Tag transgene, which was unable to elicit an SV40 Tag-specific antibody response *in vivo* (2). However, humoral and cellular immunity is required to achieve protective effects in an experimental metastatic setting when either recombinant SV40 Tag protein or plasmid DNA vaccines are utilized (26, 44).

The requirement for $CD8^+$ T-cell activity against SV40 Tag-expressing mKSA tumor cells after recombinant SV40 Tag protein immunization has been reported previously (1, 40, 47). However, this is the first report detailing the requirement of CTL activity after the onset of mKSA tumor cell challenge (i.e., the effector-phase response) in an experimental pulmonary metastasis model. The requirement of $CD8^+$ T cells in both the nonmalignant and experimental metastasis models is interesting given the historical evidence that BALB/c mice are low- or nonresponders with regard to generating CTL activity against SV40 Tag (21, 28, 31). To date, only one subdominant SV40 Tag-specific $H\text{-}2K^d$ CTL epitope has been characterized as leading to antitumor effects against mKSA cells, but the CTL clones were generated in SV40-immunized mice only after repeated *in vitro* stimulation with irradiated mKSA cells (28). In contrast, C57BL/6 mice readily respond to SV40 Tag with a potent CTL response, and many immunodominant CTL epitopes have been characterized (39). Yet in our model using BALB/c mice, we were able to readily detect CTL activity 2 weeks following tumor cell challenge. We hypothesize that the heightened CTL response was a result of cross-presentation of

neo-antigens expressed by the tumor cell, as previously explained.

Given the reduced degree to which BALB/c mice can mount a CTL response to SV40 Tag, our experimental pulmonary metastasis model relates to the constraints commonly observed in the clinical setting. Patients are typically immunosuppressed as a result of widespread cancer progression and establishment and treatment with standard protocols, which may include surgery, chemotherapy, and radiation therapies. CTL activity against targeted tumor antigens in these individuals is generally low, whether this is due to the above immunosuppressive effects and/or to central tolerance mechanisms that do not allow the widespread distribution of CTLs with highly reactive receptors specific to tumor-associated antigens (25, 29). By understanding the immunologic pathways involved in antitumor responses and tumor immunity, more efficacious immunotherapeutic strategies can be implemented to halt the progression of tumorigenic growth. Relative to our study with the tumor-specific antigen SV40 Tag, a variety of cancers, including non-Hodgkin's lymphoma and malignant pleural mesothelioma, have demonstrated amplifiable SV40 Tag gene sequences (11, 24). Since our murine system typifies the requirement for a primary antibody response followed by activation of the cell-mediated arm of the immune system, it may be advantageous for development of cancer vaccine strategies to elicit both arms of the immune system in SV40 Tag-expressing human tumors. This vaccination strategy may be most useful in a setting where, for example, an individual is at high risk of developing malignant pleural mesothelioma due to asbestos exposure. Immunization with recombinant SV40 Tag protein would generate antibodies that would target Tag-expressing tumor cells, resulting in their lysis via ADCC. The destroyed tumor cells would express neo-antigens that would function to cross-prime $CD8^+$ T cells and to target other tumor cells that express these tumor-associated neo-antigens. Additionally, our results have relevance to other virally related human chronic infections that can result in cancer, such as hepatitis B virus and human papillomavirus virus (13), where the roles of antibody and $CD8^+$ T cells have been indicated to provide enhanced protection from tumorigenesis (7, 16, 32, 38).

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