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Intratumoral Vaccination with Vaccinia-Expressed Tumor Antigen and Granulocyte Macrophage Colony-Stimulating Factor Overcomes Immunological Ignorance to Tumor Antigen[1]

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ABSTRACT

Using a murine transitional cell carcinoma tumor model, MB49, which naturally expresses the male antigen HY, we evaluated whether tumor ignorance as determined by lack of a systemic immune response could be overcome by immunization with vaccinia expressed tumor antigen and granulocyte macrophage colony-stimulating factor. Systemic tumor ignorance of MB49 was demonstrated by the lack of a splenic HY-specific CTL response in MB49-bearing female mice. In contrast, we demonstrated HY-specific CTL priming in the draining lymph nodes. MB49-bearing female B6 mice were immunized with VVHY+VVGMCSF intratumorally or in the contralateral flank. Intratumoral VVHY, VVGMCSF, and keyhole limpet hemocyanin (to produce CD4 help) generated splenic HY-specific CD8 CTLs, whereas immunization with the combination in the contralateral flank or single agents given intratumorally failed to yield a splenic response. Purified male T cells injected intratumorally, as a source of HY antigen, also generated a HY-specific response, whereas contralateral immunizations did not. These finding expand the understanding of tumor immunological ignorance and support intratumoral vaccination as a strategy for immunotherapy of established tumors.

INTRODUCTION

Immunological ignorance to tumor is most often defined as the lack of systemic (splenic) immunity (1–4). Recently, increasing evidence that immunization at different sites (compartments) differentially led to the generation of tumor-specific immune responses has led to a reevaluation of the concept of ignorance (5). Because tumor ignorance in some systems has been accounted for by decreased tumor antigen expression or altered dendritic cell function (6, 7), we addressed the efficacy of immunization with vaccinia-expressed tumor antigen plus GM-CSF[3] in generating systemic antitumor immunity. Interestingly, we found that presentation of vaccinia virus encoded tumor antigen, and GM-CSF treatment at the tumor site elicited a significantly greater tumor-specific immune response than immunization in the tumor-free contralateral flank. The enhanced systemic (splenic) response after intratumoral vaccination followed a significant increase in tumor specific CTLs in the tumor draining lymph node. This phenomenon was not unique to the recombinant vaccinia-encoded antigen as intralesional immunization with male T cells as a source of antigen resulted in similar responses. Although the majority of vaccine strategies, to date, use immunization at nontumor sites to avoid the influence of tumor-associated cytokines, these data support the clinical evaluation of intratumoral vaccinations.

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[3] The abbreviations used are: GM-CSF, granulocyte macrophage colony-stimulating factor; VVbGal, vaccinia virus expressing the β-galactosidase gene; KLH, keyhole limpet hemocyanin; pfu, plaque-forming unit(s); APC, antigen presenting cell; DLN, draining lymph node.

MATERIALS AND METHODS

Animals and Tumor. Four to 6-week-old C57BL/6J (B6) mice were obtained from Jackson Labs (Bar Harbor, ME) and maintained in a HEPA-filtered cage system for at least 1 week before use. The MB49 tumor [7,12-dimethylbenz(*a*)anthracene-induced in male C57BL/6 bladder epithelial cells] was provided by Dr. Timothy Ratliff when at Washington University (St. Louis, MO). Tumors and splenic cell cultures were maintained in complete media (TCM) composed of RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM Na pyruvate, 50 IU/ml penicillin/streptomycin, 0.5× MEM amino acids solution, and 100 μM MEM nonessential amino acids solution (Life Technologies, Inc.).

Construction of VVHY and VVGMCSF. The complete 2.4-kb *Uty* cDNA that codes for the H-2D^b epitope of HY (8) was cloned into pBluescript. The construction first required cloning the *Uty* gene in three independent ~800-bp fragments obtained by PCR amplification of MB49 cDNA into pBluescript. The first fragment, which contained the initiation codon, was engineered to present an internal ribosomal entry site element and Sal/*Hin*dIII restriction sites to assist in incorporation into pBluescript and subsequent ligation with the additional two fragments. A naturally occurring vaccinia termination signal sequence was also located and eliminated by site directed mutagenesis. The *Uty* cDNA was removed from pBluescript and cloned into vaccinia recombination plasmid pSC65 (9). The murine GM-CSF coding sequence was PCR-amplified using the primers MGMP(+) (5′-CGCTAAG-CTTGCCACCATGTGGCTGCAGAATTTACTTTTCC-3′) and MGMM(−) (5′-GCGGGATCCTCATTTTTGGACTGGTTTTTTGC-3′); the 5′ primer contains a *Hin*dIII site and a Kozak consensus sequence proceeding the start codon, whereas the 3′ primer contains a *Bam*HI site after the termination codon. The GM-CSF cDNA was first directionally cloned into pBluescript (Stratagene, La Jolla, CA) using the *Hin*dIII and *Bam*HI restriction sites and then removed from pBluescript using *Sal*I and *Not*I and cloned into vaccinia recombination plasmid pSC65 (9). Recombination of the GM-CSF/pSC65 plasmid and *Uty*/pSC65 plasmid with the vaccinia virus genome (VVGMCSF) was performed in CV-1 cells followed by plaque purification in the presence of 5′-bromo-2′-deoxyuridine in 143B HuTK cells as described previously (10). VVGMCSF was found to produce a high level of murine GM-CSF in infected cells as measured by ELISA, and VVHY conferred sensitivity to HY-specific CTL when infected in the HY-negative EL4 cell line. As a negative control, a recombinant VVbGal gene was used. VVbGal was kindly provided by Dr. Laurence Eisenlohr (Thomas Jefferson University, Philadelphia, PA).

Vaccinia Immunizations. Mice received s.c. injections of 1×10^6 MB49 and, after 1 week, were vaccinated twice, 1 week apart, with 3×10^7 pfu of VVHY plus 7.5 μg of KLH (Sigma, St. Louis, MO) with or without 3×10^7 pfu of VVGMCSF. Two weeks after the second and final vaccination, splenocytes were harvested for *in vitro* restimulation. Control male-specific effector cells were generated by i.p. priming of female C57/BL6 mice with 5×10^7 male splenocytes. After 2 weeks, spleens were harvested and (7×10^6) splenocytes restimulated with (3×10^6) irradiated male splenocytes in 24-well plates at 37°C.

Flow Cytometry. Lymphocytes were harvested and suspended in PBS/5% FCS supplemented with 0.1% w/v sodium azide. Cells were double stained with FITC-CD8α (BD PharMingen, San Diego, CA) and the *Uty* (WMHHN-MDLI)-specific tetramer labeled with phycoerythrin provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, GA). Flow cytometry was conducted on a FACScalibur and analyzed using the WinMDI software package.

^{51}Cr Release Assay. Cr release assays were performed as described previously (11). Briefly, splenic or lymph node cells (7×10^6) were cultured with

(3×10^6) irradiated male splenocytes in a total of 2 ml of TCM + 50 μM β-mercaptoethanol 24-well plates for 5 days at 37°C, 5% CO_2 and harvested. MB49 tumor cells were labeled, washed, and plated (1×10^4) with effector cells were in a total of 200 μl of TCM to a 96-well round-bottomed plate. Plates were incubated for 4 h at 37°C, 5% CO_2 and then 100 μl of supernatant were removed and ^{51}Cr release measured with a gamma counter (Packard Bioscience, Meriden, CT). Percent specific lysis was calculated from the formula [(experimental release − spontaneous release) × 100/(maximal release in 5% TX-100 − spontaneous release)].

Statistical Analysis. Results were expressed as the mean ± SE with significance determined by Student's *t* test at the $P < 0.05$ level.

RESULTS

HY-Specific CTL Are Found in the Draining Lymph Nodes but not Spleens of MB49-Bearing Mice. We studied the generation of antitumor CTL generation in a murine transitional cell carcinoma, MB49, which expresses the male antigen (HY). The male antigen, a minor histocompatibility antigen, has been well characterized to mediate transplant rejection of male skin grafts on female hosts. Male-specific CD8 CTL against the gene encoding MHC class I epitopes of HY have been demonstrated to mediate this transplant rejection (12). In accordance with the current definition of ignorance, we found MB49 bearing-mice did not expand HY-specific CTL nor *Uty* tetramer-positive CD8 T cells in spleen (Fig. 1). However, when we examined the tumor draining lymph node, we detected HY-specific CTL present as determined by a ^{51}Cr release assay and *Uty* tetramer analysis after 5 days of *in vitro* HY restimulation. Tetramer results presented are after *in vitro* restimulation. Although precursor analysis also showed levels above control, the magnitude did not allow for statistical analysis.

HY-Specific CTL from Male or VVHY+KLH-Immunized Female Mice Lyse MB49 *in Vitro* and Protect against MB49 Challenge *in Vivo*. Although tumor-specific CTLs have demonstrated antitumor efficacy (13), we confirmed that MB49 was susceptible to HY-specific CTL both *in vitro* and *in vivo*. HY-specific CTL was generated by vaccinating naive female B6 mice with either one i.p. injection of 5×10^7 male splenocytes or by two s.c. injections, 2 weeks apart, of 3×10^7 pfu VVHY+KLH. Two weeks after the final vaccination, splenocytes were harvested and HY-specific CTL activity against MB49 was assessed after *in vitro* HY restimulation (Fig. 2, *A* and *B*). Vaccinated mice were also challenged with MB49 to assess the ability of an effective CTL response to HY to eliminate MB49 *in vivo* and thus validate our model (Fig. 2, *C* and *D*). HY-specific CTL lysed MB49 *in vitro* with HY-immune mice protected against challenge with MB49. Specifically, immunization against the MHC class I-restricted HY epitope, *Uty*, expressed by VVHY was also capable of generating HY-specific CTL with antitumor effects.

Intralesional but not Contralateral VVHY+GMCSF Vaccination Generates Systemic Tumor-Specific CTL. We have previously reported that the local tumor environment is inhibitory toward the generation of type 1 responses to MB49-HY (14). As numerous investigators have shown that immunization with i.m. or s.c. injections outside the tumor environment have resulted in antitumor CTL effects in a number of systems, we initially set to determine whether s.c. VVHY+VVGMCSF injections contralateral to the tumor would generate systemic tumor-specific immune effects in the MB49 system. One week after female B6 mice were s.c. injected with MB49, the tumor-bearing mice were vaccinated twice, 1 week apart, with VVHY+VVGMCSF plus KLH in the flank contralateral to the tumor. One week after the second and final vaccination, splenocytes were harvested and restimulated with irradiated male splenocytes for 5 days. The resultant cells were assayed in a ^{51}Cr release assay and stained for *Uty* tetramer-specific CD8 T cells (Fig. 3). Contralateral immunizations did not generate systemic *Uty*-specific CD8 CTLs, indicating an inability to break ignorance to the Uty tumor specific-antigen. Because of our determination of the presence of *Uty*-specific CD8 CTL in the DLN, we hypothesized that intratumoral vaccination might generate a systemic immune response. Intratumoral *versus* contralateral injections of VVHY+VVGMCSF plus KLH were compared head-to-head for efficacy in generating systemic HY-specific CTL and CD8 T-cell responses. Interestingly, the intratumoral injec-

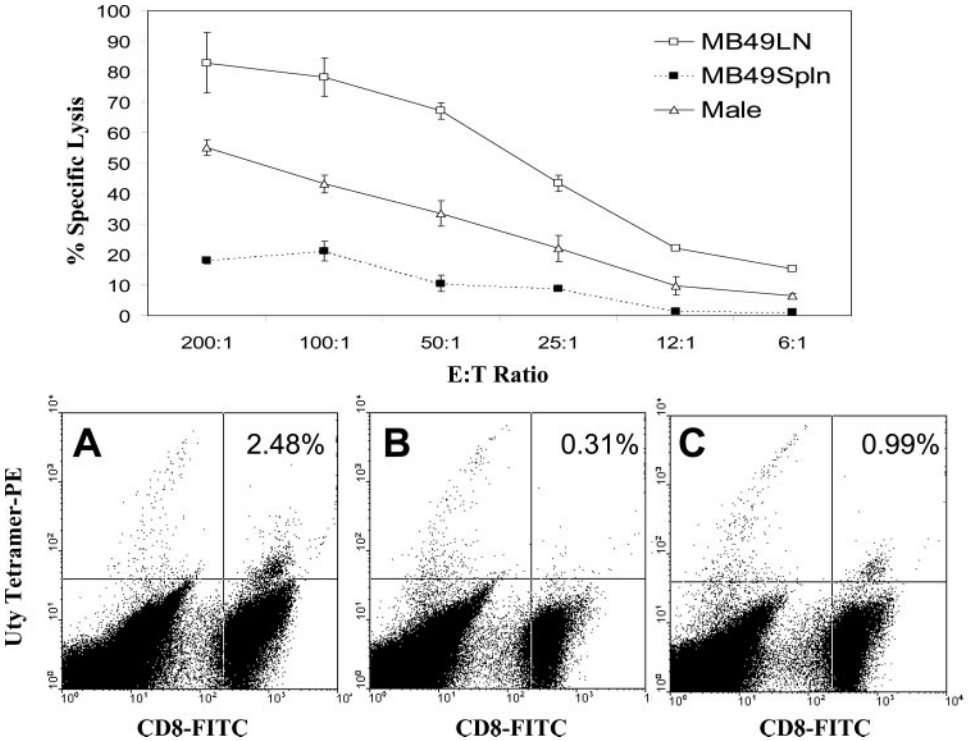

Fig. 1. HY-specific CTL are found in the draining lymph nodes but not spleens of MB49-bearing mice. Female B6 mice were s.c. injected with MB49 or injected i.p. with 5×10^7 male splenocytes as a male positive control. After 2 weeks, the draining lymph nodes (*A*) or splenocytes (*B*) from MB49-bearing female B6 mice or splenocytes from male-primed female B6 mice (*C*) were harvested and restimulated in culture with irradiated male splenocytes for 5 days. The cultures were then harvested as effector cells for use in either a ^{51}Cr release assay or stained for *Uty* tetramer-positive CD8 T cells.

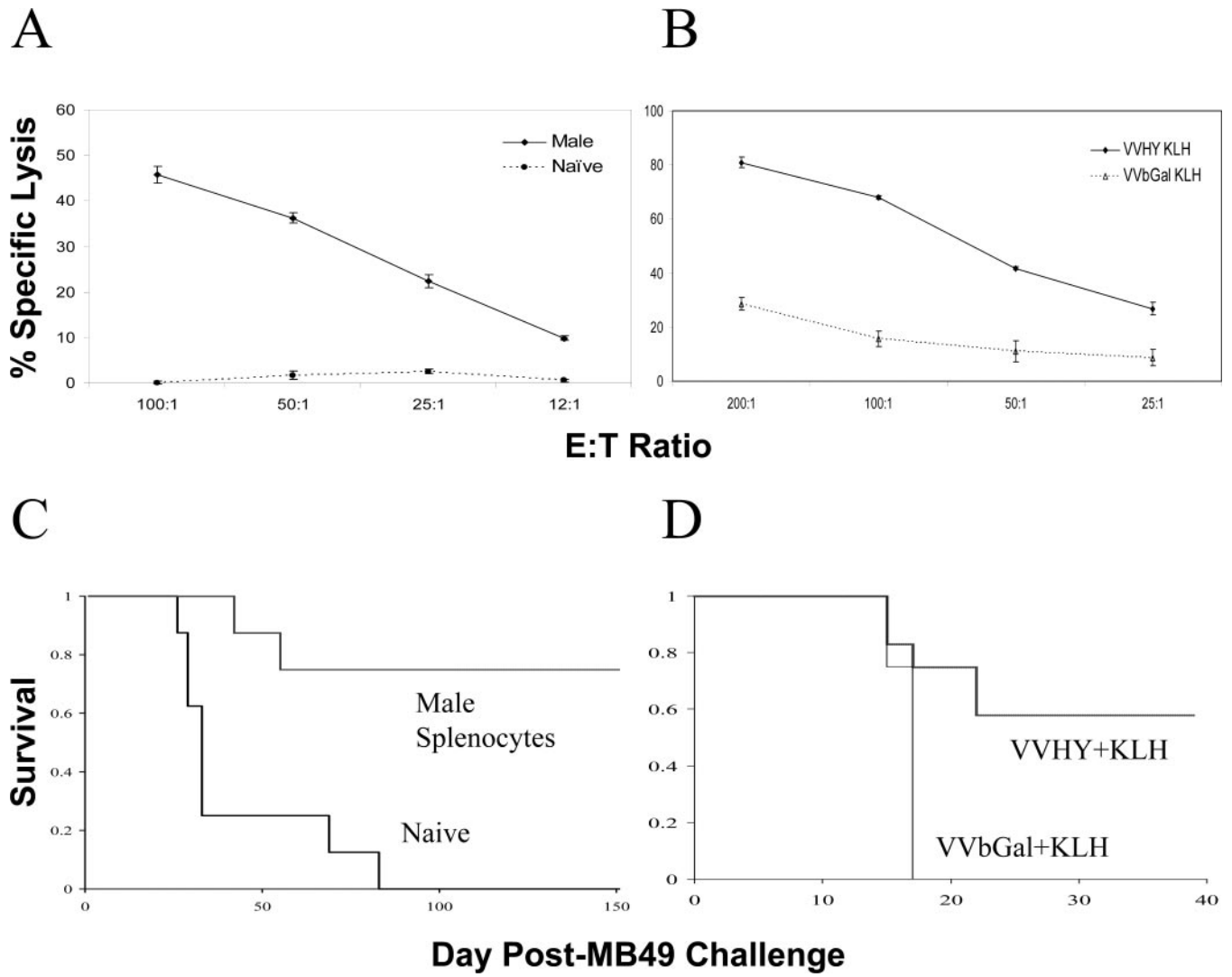

Fig. 2. HY-specific CTL from male or VVHY+KLH-immunized female mice lyse MB49 *in vitro* and protect against MB49 challenge *in vivo*. Female B6 mice were vaccinated with i.p. injection of 5×10^7 male splenocytes (*A* and *C*) or by two s.c. injections, 2 weeks apart, of 3×10^7 pfu VVHY+KLH (*B* and *D*). Two weeks after the final vaccination, splenocytes were harvested, and HY-specific CTL activity against MB49 was assessed after *in vitro* HY restimulation (*A* and *B*), or vaccinated female mice were s.c. challenged with 1×10^6 MB49 and survival was monitored (*C* and *D*).

tions of VVHY+VVGMCSF plus KLH were capable of generating dramatically higher systemic levels of male-specific CTLs and *Uty* tetramer-positive CD8 T cells compared with the contralateral injections (Fig. 3).

Combined Intratumoral VVHY and VVGMCSF Vaccination Generates Increased Systemic Antitumor Immune Responses Compared with the Individual Vaccines. GM-CSF has proven to be a powerful adjuvant in tumor immune therapies (15). Localized GM-CSF treatment for neuroblastomas in mice has also been shown to be efficacious (16). Previous experiments have focused on single agent administration of GM-CSF alone without additional provisions of tumor antigen, and we have studied intratumoral GM-CSF gene transfer in mouse models and clinical trials. To determine whether both VVHY and VVGMCSF were required to generate a systemic male-specific responses, MB49-bearing mice were injected intratumorally with VVHY, VVHY+VVGMCSF, VVbGal, or VVbGal+VVGMCSF twice, 1 week apart, and resultant splenocytes restimulated with male splenocytes as described above. Only the combination of VVHY+VVGMCSF was capable of generating systemic levels of *Uty*-specific CD8 CTLs (Fig. 4).

Intratumoral but not Contralateral Male T Cells Similarly Generates Systemic Male-Specific CTL. Finally, to additionally validate the immunocompetency of the tumor-bearing mice in generating tumor-specific CTL responses and to eliminate the possibility that the discrepancy in immunization response in each site of vaccination was purely because of antigen load, male splenocytes as a source of HY were injected either contralaterally or intratumorally. MB49-bearing female mice were vaccinated once either intratumorally or in the contralateral site, and resultant splenocytes harvested after 2 weeks and restimulated with male splenocytes as described above. Consistent with the VVHY data, male cells injected intratumorally were capable of generating systemic male-specific CD8 CTL responses and *Uty* tetramer-positive cells, whereas contralateral injections were not (Fig. 5). In addition, harvesting splenocytes 1–3 weeks after male splenocyte vaccination did not alter the frequency *Uty* tetramer-positive cells. Because the tumor environment has been demonstrated to suppress dendritic cell function, we then chose to determine whether only the male T-cell component (as antigen) of our male splenocyte mixture could also prime for male-specific CTL in tumor-bearing mice. This would clarify the role of host APC function at the tumor or immunization site. Male T cells but not female T cells injected intratumorally were capable of immunizing a male-specific CTL and *Uty* tetramer-positive CD8 T-cell response in tumor-bearing female mice (Fig. 6), demonstrating that resident host APC associated

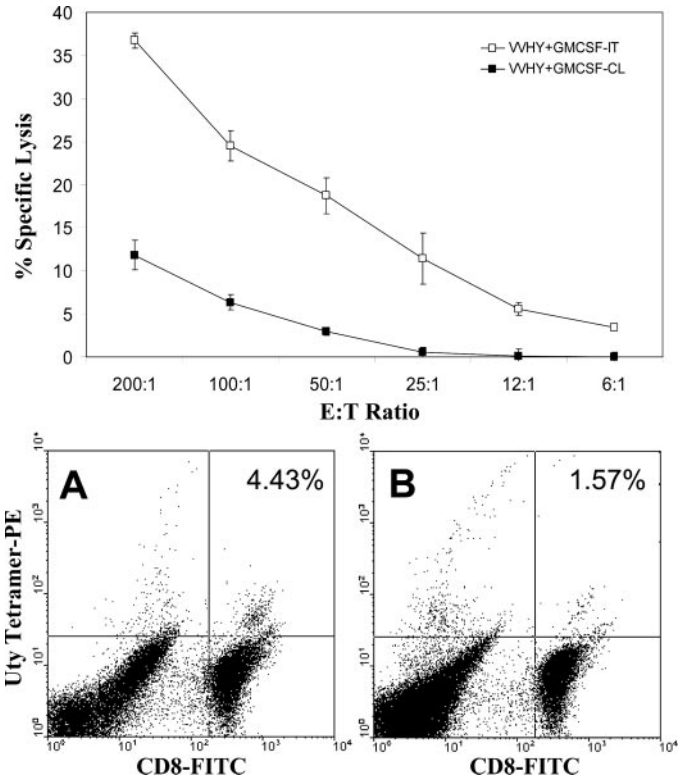

Fig. 3. Intralesional but not contralateral VVHY+GMCSF vaccination generates systemic tumor-specific CTL. One week after female B6 mice were s.c. injected with MB49, the tumor-bearing mice were vaccinated twice, 1 week apart, with either intratumoral (*A*) or contralateral (*B*) injections of VVHY+VVGMCSF. One week after the second and final vaccination, the splenocytes were harvested and restimulated in culture with irradiated male splenocytes for 5 days. The cultures were then harvested as effector cells for use in either a ^{51}Cr release assay or stained for *Uty* tetramer-positive CD8 T cells.

with tumor were capable of stimulating productive T-cell responses given antigen.

DISCUSSION

For the first time, we demonstrate that the intratumoral coinjection of recombinant vaccinia vectors expressing GM-CSF and a tumor-associated antigen generate an enhanced systemic tumor-specific immune response in a system where traditional immunization fails. The efficacy of active tumor vaccine strategies depends on the immunocompetence of the tumor-bearing host. Suppressive actions on the induction of antitumor immune responses through APC inhibition and ignorance are clearly mechanisms by which tumors avoid immune recognition. Here, we show that tumor growth leads to the presence of antigen-specific immune responses in the DLN of tumor-bearing mice but not spleen and that systemic HY-specific T cells can be preferentially generated with intratumoral immunization. This lymph node localization of antigen-specific cells is consistent with a Scott *et al.* (17) study of adoptively transferred tumor-specific T cells migrating only to the DLN. Taken together with other studies, our findings suggest that the local tumor environment may be a prime immunization location in generating effective responses because DLN may already contain tumor-specific T cells that can further be expanded or activated through intratumoral vaccinations. Furthermore, the concept of tumor ignorance previously encompassing the systemic and local lack of tumor antigen recognition may possess more sophisticated components, including limited local tumor immune responses without systemic manifestations. These data also call for a revisiting of the definition of immune ignorance, which, to date, has primarily based on a lack of systemic responses without evaluation of the tumor-draining lymph nodes.

The positive immunization effects of providing both exogenous antigen and GM-CSF at the tumor site is consistent with findings by others that tumors which present MHC class I and tumor antigens do not effectively maintain activated CTL responses (2) and that intratumoral vaccination may be an attractive strategy for recruitment of tumor-specific T cells to the tumor site. Mescher *et al.* (18) describes the loss of antitumor immune response attributable to CTL migration away from the tumor site, making it even more critical to maintain the recruitment and antigen presence at the tumor site. Finally, the Schreiber lab recently reported elegant experiments using an inducible transfectant that expressed either low or high tumor antigen and demonstrated that increased tumor antigen expression was capable of overcoming tumor ignorance via cross-presentation (6). Their data indirectly supports our conclusion that increased tumor antigen at the tumor site elicits an immune response while also supporting our direct

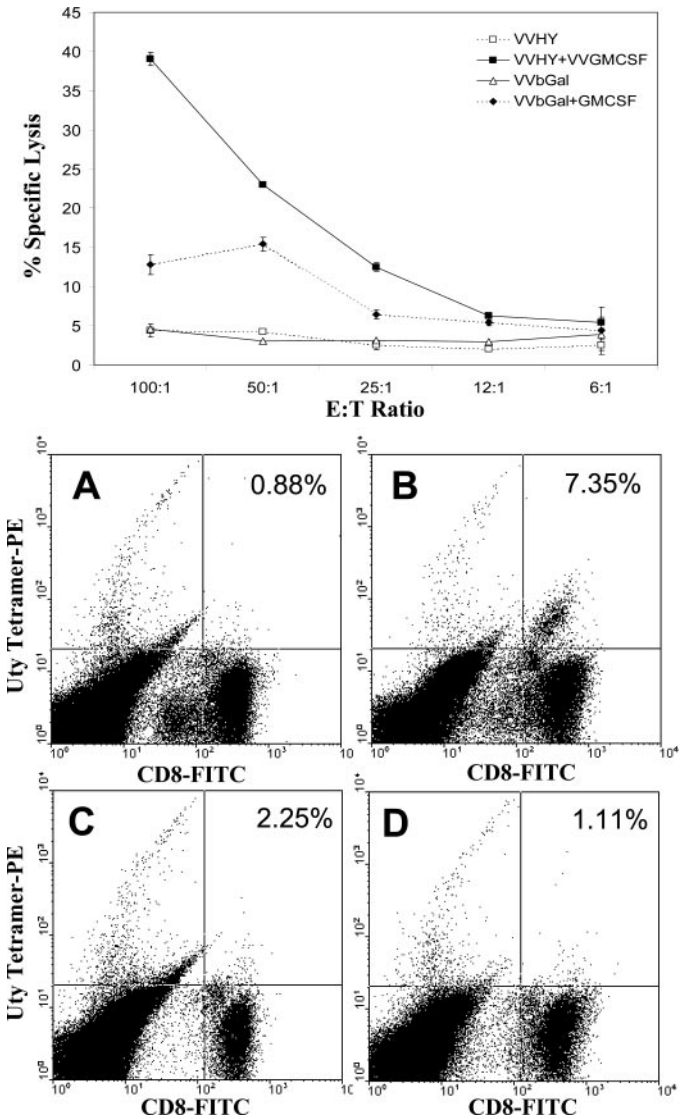

Fig. 4. Intratumoral delivery of VVHY and GM-CSF generate systemic antitumor immune responses. One week after vaccinating tumor-bearing mice intralesionally with either VVHY (*A*), VVHY+GMCSF (*B*), VVbGal (*C*), or VVbGal+GMCSF (*D*), twice, 1 week apart, the splenocytes were harvested and restimulated in culture with irradiated male splenocytes for 5 days. The cultures were then harvested as effector cells for use in either a ^{51}Cr release assay or stained for *Uty* tetramer-positive CD8 T cells.

evidence of cross-presentation of male antigen by female APCs (Fig. 6).

It should be noted that although our strategy of intervention enhances systemic immunity to the tumor, the tumor did not regress. The lack of tumor regression may be because of multiple effects, including the kinetics of tumor growth whereby the tumor volume overwhelms the antitumor response (19). Also, the generation of tumor-specific T cells may be insufficient or inactive at the tumor site because of immunosuppressive factors, including interleukin 10 as we have described previously (20–22). We are currently performing studies to determine the phenotype and migration characteristics of these tumor reactive CTLs in the draining lymph nodes. Because of potential inhibitory factors, additional adjuvants may be critical in activating or enhancing this response. In conclusion, our results demonstrate the presence of antigen-specific CTL in the tumor-draining lymph nodes in the absence of systemic (splenic) immunity. The presence of tumor-specific CTL at the DLN provide rationale for intratumoral vaccination with both a recombinant vaccinia vector-expressed tumor-specific antigen and cytokine such as GM-CSF, leading to the generation of enhanced levels of systemic antitumor immunity. Given the availability of virally encoded vaccines to known tumor antigens and GM-CSF, translation of these findings to clinical evaluation is currently planned.

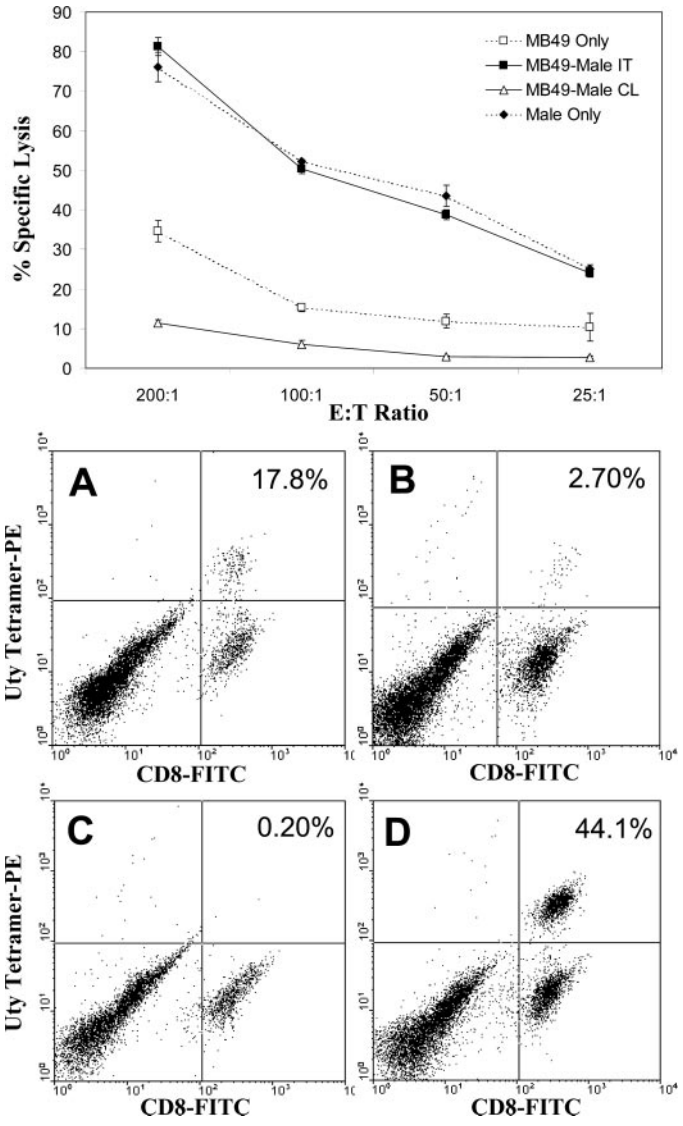

Fig. 5. Intratumoral but not contralateral male splenocytes were also capable of generating systemic male-specific CTL. MB49-bearing female mice were vaccinated either intratumorally (*A*) or in the contralateral site (*C*) with male splenocytes. A group of MB49-bearing mice were left untreated (*B*) as a negative control and naïve female B6 mice were injected i.p. with 5×10^7 male splenocytes as a male positive control (*D*). After 2 weeks, splenocytes were harvested and restimulated *in vitro* for 5 days and then assessed for male specific CTL generation by a ^{51}Cr release assay, and *Uty* tetramer-positive cells were enumerated.

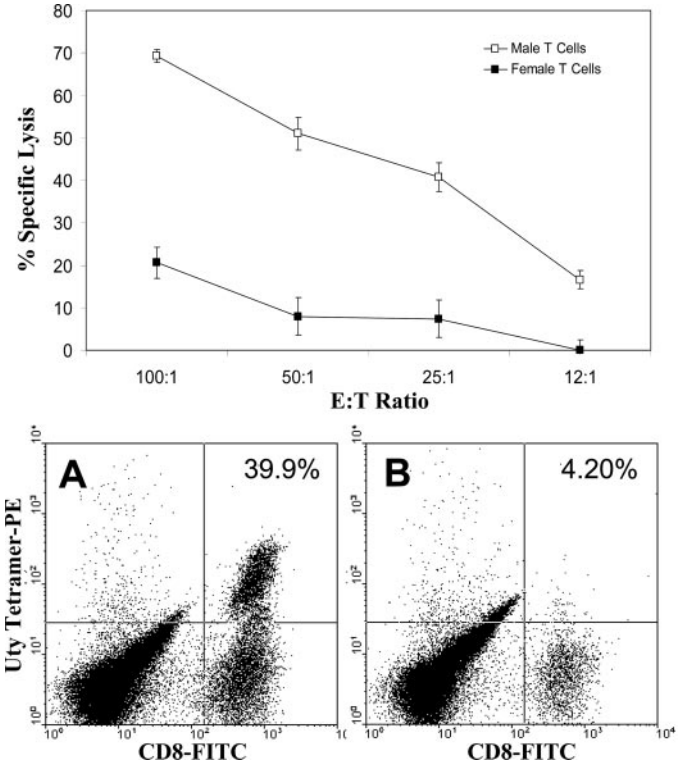

Fig. 6. Intratumoral male T splenocytes are capable of generating systemic male-specific CTL. MB49-bearing female mice were vaccinated intratumorally with male (*A*) or female (*B*) T cells. After 2 weeks, splenocytes were harvested and restimulated *in vitro* for 5 days and then assessed for male-specific CTL generation by a ^{51}Cr release assay, and *Uty* tetramer-positive cells were enumerated.

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