Allogeneic Tumor Cells Expressing Fusogenic Membrane Glycoproteins as a Platform for Clinical Cancer Immunotherapy

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Abstract

Purpose: Fusogenic membrane glycoproteins (FMG), such as the vesicular stomatitis virus G glycoprotein (VSV-G), represent a new class of gene therapy for cancer that cause cytotoxic fusion on expression in tumor cells. In addition, FMG-mediated tumor cell death stimulates antitumor immunity, suggesting potential applications for FMG-expressing cellular vaccines. This study addresses the promise of FMG-expressing allogeneic tumor cells, which are most practical for clinical use, as a novel platform for *ex vivo* and *in situ* vaccination.

Experimental Design: Murine B16 melanoma–derived cell lines expressing autologous or allogeneic MHC class I, expressing fusogenic or nonfusogenic VSV-G, were used to vaccinate mice *in vivo* against a live tumor challenge. Exosome-like vesicles released by fusing allogeneic cells (syncitiosomes) and intratumoral injection of fusing vaccines were also tested as novel therapeutic strategies for their antitumor effects.

Results: Expression of fusogenic VSV-G enhanced the immunogenicity of an allogeneic cellular vaccine, which was more effective than a fusing autologous vaccine. Allogeneic syncitiosomes were only as effective as cellular vaccines when administered with adjuvant, demonstrating that syncitiosomes cannot account entirely for the mechanism of immune priming. Intratumoral injection of FMG-expressing allogeneic cells led to significant tumor regression using both fusogenic or nonfusogenic VSV-G. However, specific priming against tumor-associated antigenic epitopes and protection against secondary rechallenge only occurred if the initial vaccine was competent for cell fusion.

Conclusions: FMG-expressing allogeneic tumor cells are a potent source of antitumor vaccines. Syncitiosomes given with adjuvant and intratumoral injection of fusing cells represent novel strategies well-suited to clinical translation.

Fusogenic membrane glycoproteins (FMG) are a family of proteins involved in viral entry into target cells. They have potential applications in the gene therapy of cancer because, on expression in tumor cells, they trigger extensive cell-to-cell fusion and eventual death (1). This cytotoxicity is not immediate, occurs at variable rates in different tumor cell lines, and is associated with metabolic depletion and mitochondrial failure (2). As syncitia expand and recruit surrounding cells not expressing the FMG, a significant bystander effect develops, characterized by nuclear fusion, features of autophagy, and premature chromosome condensation. Syncitial growth requires loss of membrane, and vesicles reminiscent of exosomes, termed syncitiosomes, are released by dying cells (3).

Delivery of FMG expression to target tumor cells is directly cytotoxic, with a bystander effect superior to that of suicide genes such as herpes simplex virus thymidine kinase. Such cytotoxicity has been shown in human tumor xenografts *in vivo*, transfected with FMG genes using plasmid DNA, adenoviral or lentiviral vectors (1, 4). These studies used a truncated gibbon ape leukemia retrovirus envelope gene (*GALV*; ref. 5), or a combination of measles F and H. Due to species differences in the receptors to which *GALV* and F/H bind (Pit-1 and CD46, respectively), these proteins do not mediate fusion between murine cells. Hence, *in vivo* studies with *GALV* or F/H have been restricted to human xenografts in immunodeficient mice.

As well as a direct cytotoxic bystander effect, FMG gene therapy can initiate antitumor immunity which leads to additional distant bystander killing. Various mechanisms may account for the immunogenicity of FMG-mediated tumor cell death. Cells die predominantly by nonapoptotic pathways with, in some models, induction of heat shock proteins (3). Heat shock proteins could act as innate "danger signals" for

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immune activation and chaperone tumor-associated antigens (TAA) into antigen-presenting cells (APC) such as dendritic cells for cross-priming to T cells (6). Experiments *in vitro* have confirmed the ability of fusing tumor cells, or the syncitiosomes they secrete, to deliver TAA to dendritic cells for cross-presentation and antigen-specific T cell activation (3, 7). The foreign, viral origin of FMG may also act as a nonspecific adjuvant to facilitate the generation of an antitumor immune response (8).

In vivo evidence for the immunogenicity of FMG-mediated tumor cell death has been shown in a murine melanoma model (7). These experiments used the vesicular stomatitis virus G glycoprotein (VSV-G) as the FMG, which triggers cell fusion in a pH-dependent manner. Transfection of tumor cells *ex vivo*, followed by a transient pH drop, allowed the generation of a T cell–dependent fusing vaccine, which was therapeutic in both vaccine/challenge protocols and treatment of early established disease. Importantly, in this system, the vaccine was most effective when it included an allogeneic melanoma line (K1735, derived from H-2^k C3H mice), in addition to the syngeneic B16 line (H-2^b, C57BL mice) against which protection was tested. This suggested that allogeneic MHC might act as an additional "danger" signal in this system (9); however, in these experiments, it was not possible to isolate and specifically characterize the role of allogeneic MHC as an immunogenic component of the fusogenic vaccine.

FMG may have further applications for tumor therapy. Recent data has shown that FMG-mediated cell fusion enhances the efficacy of oncolytic adenoviral, herpes simplex virus, or VSV-based tumor therapy (10–12), and that FMG can mediate fusion between tumor cells and dendritic cells, to generate potent, short-lived immunogenic hybrids (13).

Previous data had suggested that expression of allogeneic MHC may enhance the immunogenicity of FMG-expressing tumor cell vaccines, and that vesicles released by tumor cells (syncitiosomes) are a potential source of TAA for immune priming. In this study, we have addressed the specific role of allogeneic MHC expression in the immune response to FMG-mediated tumor cell death, and developed novel therapeutic strategies based on allogeneic tumor cell lines, designed for straightforward application in the clinical setting. Allogeneic cell vaccines can be prepared to clinical grade in bulk and are readily manipulated, making them a more practical source of TAA than individual, autologous preparations. There is also evidence that expression of allogeneic MHC can, of itself, enhance immune priming against TAA shared between the vaccine and the tumor of the patient to be treated (14, 15). Expression of FMG in allogeneic tumor cells may therefore provide a highly immunogenic vaccine which can be applied in the broadest clinical setting.

The data reported here shows that expression of allogeneic MHC may, of itself, enhance the immunogenicity of murine melanoma, but that cell fusion significantly increases antitumor immunity on allogeneic vaccination, which is more effective than autologous vaccination. Syncitiosomes released by fusing allogeneic tumor cells are immunogenic *in vivo* when given with adjuvant, and represent a potential vaccine preparation which can readily be prepared and stored for clinical administration. The importance of cross-priming of endogenous APC following FMG vaccination is shown by the ability of fusing class I-negative tumor cells to protect against a live melanoma challenge, in the process, successfully cross-priming a response against a defined, MHC class I–restricted TAA epitope. Finally, we have explored direct intratumoral injection of FMG-expressing allogeneic melanoma cells as a novel therapeutic strategy, designed to initiate fusion *in situ* between established autologous tumor and vaccine cells. We show that intratumoral injection of even nonfusing allogeneic tumor cells can result in the regression of established B16.OVA tumors. However, specific priming against TAA expressed by the autologous tumor and rejection of a secondary rechallenge, only occurs if the injected cells are fusing. These results show that FMG expression within allogeneic tumor cells provides a promising platform for developing novel strategies for patient vaccination.

Materials and Methods

Cell culture and transfection with VSV-G. The parental B16.F1, K1735, and B16.OVA cells used in this study have been described previously (7). The B78H1, B78H1.K^bD^b, and B78H1.L^d lines (a kind gift from L. Jaffee, Johns Hopkins University School of Medicine, Baltimore, MD) are B16-derived lines which express no, syngeneic, or allogeneic MHC class I, respectively (16). H2-K^b and H2-L^d expression in these lines was confirmed by fluorescence-activated cell sorting analysis using antibodies specific for each class I allele (BD PharMingen, San Diego, CA and Cedarlane Laboratories, Ontario, Canada, respectively). The B16-β-gal cell line had previously been generated by retroviral transfection of parental B16 with the β-gal gene. Cell lines were grown in DMEM, supplemented with 10% (v/v) FCS, and L-glutamine (all from Life Technologies, Inc., Rockville, MD). All cell lines were routinely tested for *Mycoplasma* and found to be free of infection.

FMG were expressed in tumor cells by transfection with 1 to 2 μg of pCMV-VSV-G (VSV-G) or pCMV-VSV G-E124 (VSV-G*), a point mutant of VSV-G which abolishes fusion of the protein by >90%, as previously described (7). All transfections were done using Effectene lipid reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Twenty-four hours later, cells were incubated in medium (pH 5.7) for 2 minutes, washed thrice in PBS, and then returned to normal medium. Twenty-four to 48 hours later, cells expressing VSV-G, but not VSV-G*, were extensively fused with up to 80% of tumor nuclei involved in syncytia. Fusing cell vaccines were prepared from such cultures 48 hours following pH drop.

In vivo *studies.* All procedures were approved by the Mayo Foundation Institutional Animal Care and Use Committee. C57BL/6 mice (n = 10 per group) were age- and sex-matched for individual experiments. To establish s.c. tumors, 2×10^5 B16 or B16.OVA cells were injected s.c. (100 μL) into the flank region. Animals were examined daily until the tumor became palpable; thereafter, the diameter, in two dimensions, was measured thrice weekly using calipers. Animals were killed when tumor size was $\sim 1.0 \times 1.0$ cm in two perpendicular directions. Animals were considered to have a tumor (to distinguish from a swelling or inflammation in reaction to injection) when a tumor measurement was in excess of 0.2 cm in the longest diameter; data is presented as the percentage of tumor-free mice in individual groups over time. All groups of mice in any one individual experiment were challenged on the same occasion using the same preparation of cells; a naïve group of mice was also injected with these cells at the same time.

Ex vivo *fusing vaccines.* Mice received two s.c. vaccinations (100 μL in PBS into the flank region) of 2×10^5 irradiated (100 Gy) B78H1, B78H1.K^bD^b, B78H1.L^d or a 1:1 mix of parental B16/K1735 cells 7 days apart as previously described (7). Mice were then rechallenged 7 days after the last vaccination with a s.c. injection of 2×10^5 parental B16 cells on the opposite flank and tumor growth was

monitored. Alternatively, as a model of early established disease, parental B16 was seeded on day 1, and contralateral vaccination was given on day 3.

Syncitiosome vaccines. Syncitiosomes were prepared from fusing B78H1.L^d cells as previously described (3). Briefly, supernatants from cultures of previously transfected cells were centrifuged sequentially at 300 × *g* for 10 minutes, twice at 800 × *g* for 15 minutes, and at 10,000 × *g* for 30 minutes. Syncitiosomes were pelleted from the supernatants at 100,000 × *g* for 1 hour and resuspended in 100 μL of PBS. Mice were given two syncitiosome vaccines, 50 μg protein in 100 μL into the flank region 7 days apart s.c., with or without admixed lipopolysaccharide (1 μg per injection), and rechallenged as described above.

Intratumoral injection of VSV-G expressing B78H1.L^d cells. On day 1, 2×10^5 B16.OVA cells were injected s.c. into the right flank of syngeneic C57BL/6 mice (primary tumor). When the primary tumor became palpable (typically 7 days following injection), 2×10^5 irradiated, B78H1.L^d cells (in 100 μL PBS) transfected with fusogenic VSV-G or nonfusogenic VSV-G* were injected intratumorally (or on the opposite flank), and primary growth was monitored. Those mice in which the primary tumor completely regressed following intratumoral injection were rechallenged on day 60, 50% with 2×10^5 B16.OVA cells and 50% with 2×10^5 parental B16, at the same time as a separate group of naïve animals for each challenge.

Measurement of antigen-specific responses following vaccination with VSV-G–expressing tumor cells. Mice were vaccinated with VSV-G/VSV-G*–expressing B78H1, or primary B16.OVA tumors were established and injected with VSV-G/VSV-G*–expressing B78H1.L^d, as described above. Ten to 14 days later, splenocytes were harvested and stimulated in the presence of the synthetic H-2K^b restricted OVA peptide SIINFEKL, H-2D^b restricted TRP-$2_{180\text{-}188}$ (SVYDFFVWL), and/or H-2D^b restricted hGP$100_{25\text{-}33}$ (KVPRNQDWL) as previously described (17); specific peptide recognition was determined by ELISA for IFN-γ.

Statistics. Data from the animal studies were analyzed by the log-rank test (18).

Results

FMG-mediated fusion of tumor cells expressing allogeneic MHC class I generates a potent* ex vivo *vaccine against murine melanoma. Previous work on the immunogenicity of an *ex vivo* vaccine fusing following transfection with the FMG VSV-G has shown protection and therapy in a murine melanoma model (7). When challenging with the poorly immunogenic parental B16 cell line, an autologous fusing B16 vaccine gave minimal protection. This was significantly enhanced by vaccinating with a 1:1 mixture of fusing B16 cells and K1735, an allogeneic melanoma line sharing limited antigens and expressing a different MHC phenotype. This data suggested that the presence of allogeneic MHC can enhance the efficacy of fusing vaccines, but could not exclude the effect of K1735 unrelated to its MHC status.

To study the role of MHC class I expression in this context specifically, we used the B78H1 panel of cell lines. B78H1 originally derives from B16 melanoma, but expresses no MHC class I on the cell surface due to the absence of RNA transcripts (16). Parental B16, used in our previous experiments, expresses low, but not absent, levels of class I. B78H1 has previously been genetically modified to express K^bD^b, which is syngeneic in C57BL/6 mice, or allogeneic L^d (19). Hence, these lines allow comparisons between immune responses generated by B16 lines which differ only in the MHC class I allele they express. Other factors, such as differing antigen expression profiles, are eliminated, so that any differences observed depend only on the cells' autologous/allogeneic status. Figure 1A shows the MHC status of B78H1, B78H1.K^bD^b, and B78H1.L^d by fluorescence-activated cell sorting analysis using antibodies specific for their differing class I alleles.

We then tested the immunogenicity of B78H1.K^bD^b and B78H1.L^d with or without FMG-mediated cell fusion. To control for FMG expression without fusion, control cells were transfected with pCMV-VSV-G G-E124 (VSV-G*), a nonfusogenic mutant of VSV-G which has been nonimmunogenic in previous vaccine/challenge or therapy B16 protocols (7).

As previously described, cell fusion following transfection with VSV-G (but not with VSV-G*) was triggered by a transient drop in medium pH *ex vivo*. As shown in Fig. 1B, vaccination with nonfusing irradiated B78H1.K^bD^b or B78H1.L^d generated some protection against parental challenge, with allogeneic B78H1.L^d showing a nonstatistically significant trend towards higher immunogenicity than syngeneic B78H1.K^bD^b (50% protection versus 10%). This is consistent with previous data showing that the presence of an allogeneic MHC class I allele of itself can facilitate the antitumor immune response generated after vaccination (14, 15). The inherent immunogenicity of B78H1.K^bD^b, expressing higher levels of syngeneic class I than parental B16, remained low (10% protection). When vaccinating tumor cells were transfected with fusogenic VSV-G, increased protection was seen in both the B78H1.K^bD^b and B78H1.L^d models, with tumor rejection rising from 10% to 50% ($P = 0.093$), and from 50% to 90% ($P = 0.047$), respectively; hence, statistical significance was reached only for allogeneic B78H1.L^d. When the fusing B78H1.K^bD^b and B78H1.L^d vaccines were compared directly, the allogeneic cells were more effective (90% versus 50% protection; $P = 0.049$). As previously published, a combined fusing vaccine of parental B16 and H-2^k K1735 was also effective, with 80% of mice protected ($P < 0.01$ compared with PBS control; ref. 7).

We then tested the most immunogenic vaccine (B78H1.L^d, which is also most suited to clinical application), in a more challenging model of early established disease. B16 tumors were seeded on day 1, and treated on day 3 with a single injection of fusing or nonfusing B78H1.L^d on the contralateral flank. In this more challenging model, as shown in Fig. 1C, growth of established B16 was effectively controlled by the fusing ($P < 0.01$), but not the nonfusing, B78H1.L^d vaccine.

This data shows, that whereas expression of an allogeneic class I allele may of itself increase the inherent immunogenicity of this melanoma model, expression of a fusogenic FMG significantly enhances the efficacy of an allogeneic vaccine. FMG-mediated cell fusion also shows a trend towards the improvement of a syngeneic prophylactic vaccine, although without reaching statistical significance. A fusing allogeneic vaccine is more effective than fusing autologous cells differing only in their MHC class I expression, and is also effective in controlling early established disease. This data supports the use of allogeneic tumor cells as a source of antigen for FMG-based immunotherapy.

Syncitiosomes prepared from allogeneic cells are an effective* in vivo *vaccine against murine melanoma when coadministered with adjuvant. Having shown the high immunogenicity of allogeneic tumor vaccines expressing FMG, we wished to further exploit potential mechanisms underlying this effect relevant to clinical application. During FMG-mediated cell

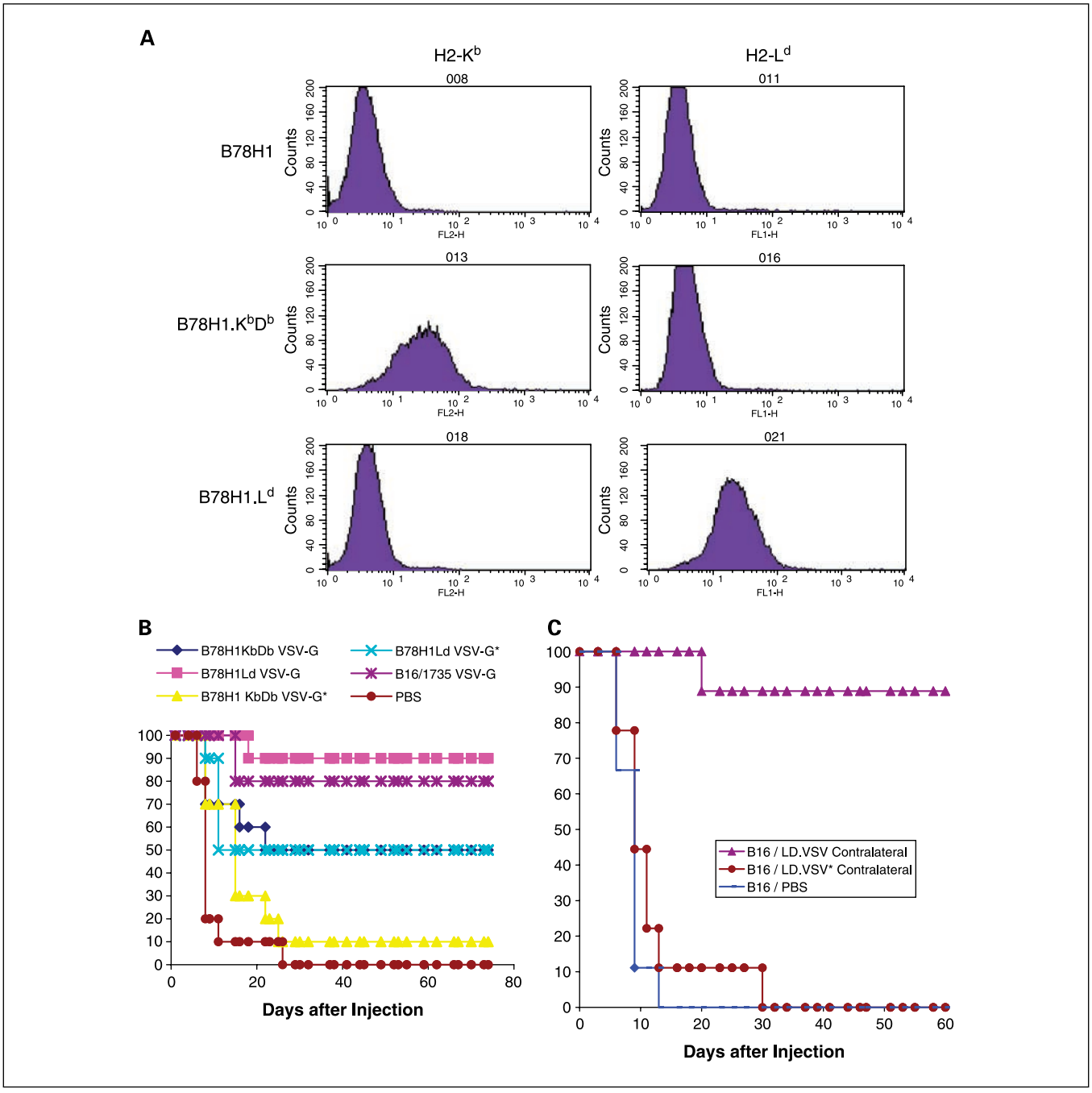

Fig. 1. Vaccination with VSV-G – transfected B16-derived cellular vaccines expressing syngeneic or allogeneic MHC class I. *A,* class I MHC expression on B78H1, B78H1.K^bD^b, and B78H1.L^d was confirmed by fluorescence-activated cell sorting analysis with antibodies specific for the different alleles. *B,* mice were vaccinated twice with irradiated B78H1.K^bD^b or B78H1.L^d cells expressing fusogenic VSV-G or nonfusogenic VSV-G*, and challenged with parental B16. A 1:1 mix of VSV-G – transfected B16/K1735 cells was also included as a positive control. *C,* as a model of early established disease, B16 tumors were seeded on day 1, and contralateral vaccines comprising VSV-G or VSV-G* – expressing B78H1.L^d administered on day 3. Although nonfusing allogeneic B78H1.L^d showed a trend to higher inherent immunogenicity than autologous B78H1.K^bD^b, the presence of fusion increased protection following vaccination in both models (although statistical significance was reached only for B78H1.L^d). Vaccination with fusing, but not nonfusing, allogeneic cells was also effective in early established disease.

fusion, tumor cells release vesicles similar to exosomes, previously termed "syncitiosomes" (3). Syncitiosomes are released in greater quantities than exosomes from intact cells, express immunologically relevant proteins such as TAA and heat shock proteins, and are an effective source of antigen for uptake and cross-presentation by dendritic cells. We therefore tested whether syncitiosomes prepared from fusing allogeneic tumor cells could generate *in vivo* tumor protection, potentially providing a convenient source of antigen for clinical administration, as currently proposed for exosomes (20). We focused on an allogeneic vaccine in this protocol, because established tumor lines represent the only practical source of the large number of cells needed for syncitiosome/exosome preparation; individualized autologous vaccines are difficult to establish and

maintain. Figure 2 shows that vaccination with syncitiosomes prepared from the B78H1.L^d line can provide some protection against a live tumor challenge (20% protection; P = 0.0004 compared with naïve control), but that this is greater when an adjuvant is added to the vaccine, in this case, lipopolysaccharide (100% protection, P = 0.0003 compared with syncitiosomes alone; ref. 21). This requirement for additional immunostimulatory signals with syncitiosome vaccines has also been described for standard exosomes (22), and suggests that syncitiosomes form only part of the mechanism by which the immune system may respond to fusing cellular vaccines. Syncitiosomes prepared from autologous B78H1.K^{b}D^b cells also had some *in vivo* activity, protecting 20% of animals against challenge when given with lipopolysaccharide, and none when given alone (data not shown); this lower potency compared with allogeneic syncitiosomes is consistent with the cellular vaccine data shown in Fig. 1B. Taken together, this data supports the use of vaccines comprising allogeneic syncitiosomes delivered with adjuvant as a novel approach for clinical application.

FMG vaccination leads to cross-priming of endogenous APC. Having established that FMG-expressing allogeneic tumor cells can be an effective source of TAA for vaccination, we next explored further potential *in vivo* mechanisms for this effect. In general terms, vaccinating tumor cells or their products can present antigen directly to reactive T cells, or cross-prime APC for T cell stimulation. The relative contribution of such direct and indirect antigen presentation *in vivo* remains controversial (23). We have previously shown *in vitro* that both fusing tumor cells and the syncitiosomes they secrete can cross-prime dendritic cells for presentation to antigen-specific CD8 T cells in both murine and human systems (3, 7). To address the role of cross-priming of endogenous APC *in vivo* following cellular vaccination, we used the B78H1 line. Although B78H1.L^d cells express only an allogeneic class I allele in this model, they could still potentially present tumor-associated or allogeneic antigens directly to the T cell receptors of host CD8 T cells (24), whereas class I-negative B78H1 cannot mediate any direct presentation at all. Hence, any antigen-specific priming

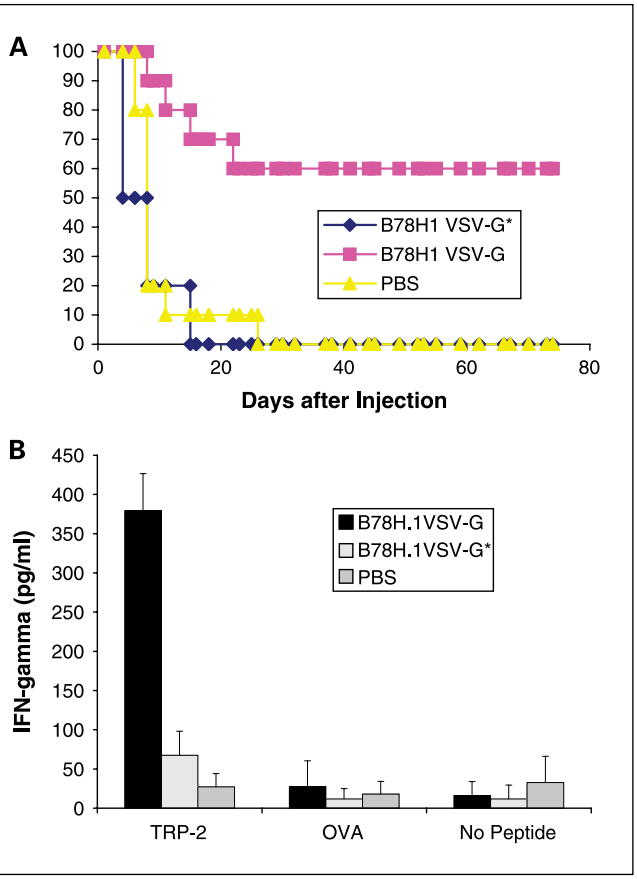

Fig. 3. Role of *in vivo* cross-priming of endogenous APCs following vaccination with fusing tumor cells. *A,* class I-negative B78H1 cells were transfected with fusogenic VSV-G or nonfusogenic VSV-G*, and used as a vaccine (2×10^5 cells on each of two occasions) against challenge with parental B16. PBS (100 μL) was also used as a control vaccine. Significant protection was seen with the fusing, but not nonfusing vaccine, implicating endogenous APC in priming against FMG-expressing fusing tumor cells. *B,* splenocytes were harvested from mice following VSV-G or VSV-G* – expressing B78H1 vaccination, and challenged *in vitro* with H-2^b-restricted epitopes from TRP-2 and SIINFEKL as a negative control. A significant IFN-γ response to TRP-2 was only seen in mice treated with fusing cells, correlating with the protection shown in (*A*).

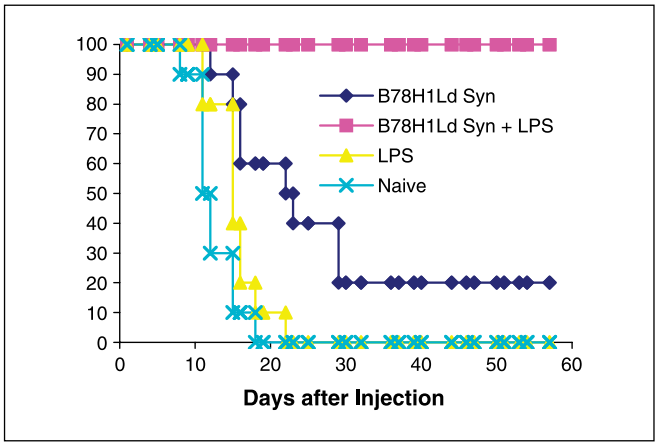

Fig. 2. Vaccination with syncitiosomes prepared from fusing allogeneic tumor cells. Mice were vaccinated twice with B78H1.L^d-derived syncitiosomes, with or without lipopolysaccharide as an adjuvant, and rechallenged with parental B16. Syncitiosomes alone afforded some protection against challenge, which was significantly greater on coadministration of lipopolysaccharide.

and associated protection generated following vaccination with B78H1 must rely on cross-priming of endogenous APC. As shown in Fig. 3A, B78H1 cells transfected with nonfusogenic VSV-G* were ineffective at protecting against a live tumor cell challenge, whereas vaccination with fusing B78H1 cells protected 60% of mice (P = 0.0003). Moreover, this protection was associated with *in vivo* generation of a splenocyte response against TRP-2, a defined MHC class I–restricted melanoma TAA expressed by B16, thus confirming antigen cross-priming from a class I-negative fusing vaccine *in vivo* (Fig. 3B). Consistent with our previous data showing the immunogenicity of fusing vaccines to be T cell–mediated (7), this supports a model in which fusing but not intact cells are an effective source of antigen for cross-priming of endogenous APC *in vivo*.

Intratumoral injection of VSV or VSV* expressing allogeneic cells can induce primary tumor regression, but only fusing cells generate immune memory. An alternative, novel application for allogeneic tumor cells expressing FMG is intratumoral injection of fusing cells into an established tumor *in vivo*. The hypothesis

is that injected cells will initiate immunogenic fusion *in situ* within the tumor, potentially incorporating both autologous and allogeneic cells and mimicking the successful combination vaccine previously generated *ex vivo* (7). Autologous TAA are potentially accessed without having to establish or manipulate individual tumors *ex vivo*.

First, to provide proof of principle that intratumorally injected transfected cells can act as a delivery vehicle for gene therapy, we tested *in vitro* whether one population of FMG-expressing tumor cells could form syncitia which will recruit and incorporate a second population of untransfected cells. Although previous data had shown a significant *in vitro* bystander effect for FMG cytotoxicity in a single population of transfected cells (1), this intratumoral strategy requires "delivery" of fusion from injected cells to established, unmodified tumor. To visualize this approach *in vitro*, we used two cell lines which can readily be distinguished by nuclear staining, B78H1.L^d and B16-β-gal. B16-β-gal cells transfected with VSV-G were plated at a 1:1 ratio with untransfected B78H1.L^d and allowed to adhere. Twenty-four hours following transient pH drop to initiate fusion, cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Figure 4A illustrates syncitia containing both blue nuclei derived from transfected B16-β-gal, and unstained nuclei from B78H1.L^d, establishing that FMG-expressing tumor cells can deliver fusion to a target untransfected tumor population, at least *in vitro*.

One concern in using allogeneic tumor cells as vaccines is that they will induce such a powerful immune response directed at alloantigens, that no effective priming against TAA expressed by the autologous tumor will emerge. In the next experiments, we therefore used B16.OVA as an established tumor, into which B78H1.L^d cells expressing VSV-G or VSV-G* were injected. This system allows tracking of immune priming against OVA as a TAA expressed by the tumor but *not* by vaccinating cells, in addition to monitoring endogenous melanoma TAA potentially expressed by both. As shown in Fig. 4B, injection of B16.OVA with irradiated B78H1.L^d cells expressing VSV-G, or its nonfusogenic mutant, VSV-G*, resulted in the regression of a significant number of primary tumors. Regression rates were higher for fusogenic vaccinating cells (100% versus 62%, $P < 0.0001$ and $P = 0.0013$, respectively, for each versus PBS control; $P = 0.0073$ for VSV-G versus VSV-G*), although clearly even nonfusing injected allogeneic cells can have activity in this setting. Those mice which had successfully rejected a primary B16.OVA tumor were then challenged a second time with live tumor cells, half with B16.OVA again, and half with parental B16. In this test of systemic immunity, clear differences emerged between animals which had initially been treated intratumorally with fusing or nonfusing cells. No mice in the VSV-G*–treated group rejected a second tumor, whereas all mice which had received VSV-G–expressing fusogenic B78H1.L^d into primary tumors were protected, against either B16 or B16.OVA ($P = 0.0001$ for both rechallenges; Fig. 4C).

To test for antigen-specific responses generated by this approach, spleens from mice treated with the intratumoral vaccines were harvested, and splenocytes challenged *in vitro* with defined MHC class 1-restricted peptides from OVA (SIINFEKL), TRP-2 and, in this experiment, gp100 (7). Figure 4D shows that specific responses, in parallel with protection against secondary *in vivo* challenge, were restricted to mice initially treated with fusing cells. The strongest antigenic response was against SIINFEKL, with gp100 inducing low, but detectable levels of IFN-γ from splenocytes. Surprisingly, no detectable response against TRP-2 was detected in this protocol (in contrast to Fig. 3B). Nevertheless, this data shows that whereas expression of allogeneic MHC by a vaccinating cell delivered intratumorally may be sufficient to induce significant primary regression, only cells fusing following VSV-G transfection induce a memory response sufficient to protect against rechallenge. This systemic immunity is directed against a powerful TAA expressed exclusively by the established tumor, but also potentially targets other tumor-associated melanoma antigens.

Ongoing experiments are now addressing whether intratumoral delivery of VSV-G–expressing vaccines is superior to vaccination at a distant site, both for control of established primary disease and generation of a memory T cell response. Preliminary data has suggested the superiority of intratumoral injections, with ~40% of mice in a single experiment rejecting secondary rechallenge following intratumoral vaccination–induced primary regression, compared with 15% when the initial treatment was contralateral to the established tumor (data not shown). However, the number of mice available for rechallenge in this experiment was small, and this difference did not reach statistical significance; further work is still required to compare different delivery routes for FMG-expressing vaccines.

Discussion

FMG-mediated tumor cell killing is a promising novel approach to genetic immunotherapy because it could stimulate antitumor immunity as well as cause direct cytotoxicity to malignant cells. Many features of FMG expression in tumor cells are potentially immunogenic, including the induction of necrotic cell death and release of exosome-like vesicles, previously termed syncitiosomes (3).

This study has focused on facets of FMG genetic immunotherapy related to novel and practical applications in the clinical setting. Previously published data had shown that an antitumor vaccine, generated *ex vivo* following transfection with VSV-G, could protect against B16 melanoma, but only with incorporation into the vaccine of an allogeneic melanoma line, K1735 (7). This suggested that additional immunostimulatory signals, such as allogeneic MHC, may enhance the success of a fusing vaccine, although the precise role of MHC expression (as opposed to other potential immunomodulatory properties of K1735) could not be defined. For clinical application, allogeneic cell vaccines are particularly attractive because they can be characterized (e.g., for antigen expression) and expanded to provide an "off the shelf" preparation. Cell lines can potentially be transduced to stably express FMG (for example, under the control of an inducible promoter), so that fusion can be triggered when required before injection into the patient. To define the precise role of allogeneic MHC in the immunogenicity of FMG vaccines, we used the B78H1 panel of cell lines, which differ only in the class I allele they express (19). We initially found, consistent with previous reports (14), that allogeneic MHC may of itself enhance the efficacy of a nonfusing, irradiated vaccine (Fig. 1B). However, transfection with fusogenic VSV-G enhanced the

immunogenicity of both autologous B78H1.K^bD^b and allogeneic B78H1.L^d prophylactic vaccines, although statistical significance was only reached for the allogeneic line. Fusing allogeneic cells were also effective at controlling early established disease (Fig. 1C). Hence, the combined approach of fusing vaccines in the context of allogeneic MHC expression provides a potent immune therapy ideally placed for clinical translation.

One potential mechanism underlying the immune response to fusing vaccines is the release of syncitiosomes. These vesicles, reminiscent of exosomes, are released by fusing cells following transfection with FMG (3). Like exosomes, they can deliver antigens to APC for cross-priming, and can readily be frozen and stored for clinical use. We therefore tested the *in vivo* efficacy of syncitiosomes, prepared from the allogeneic B78H1.L^d line. In this context, allogeneic lines are again the most realistic source of clinical syncitiosomes because individual expansion of autologous tumor for FMG transfection and syncitiosome preparation is impractical. We found that vaccination with B78H1.L^d syncitiosomes could protect against live tumor challenge, most effectively in the presence of an additional adjuvant (lipopolysaccharide; Fig. 2). This is consistent with recently published data showing that standard exosomes similarly require adjuvant for immunogenicity *in vivo* (22), and shows that syncitiosomes alone cannot account for the

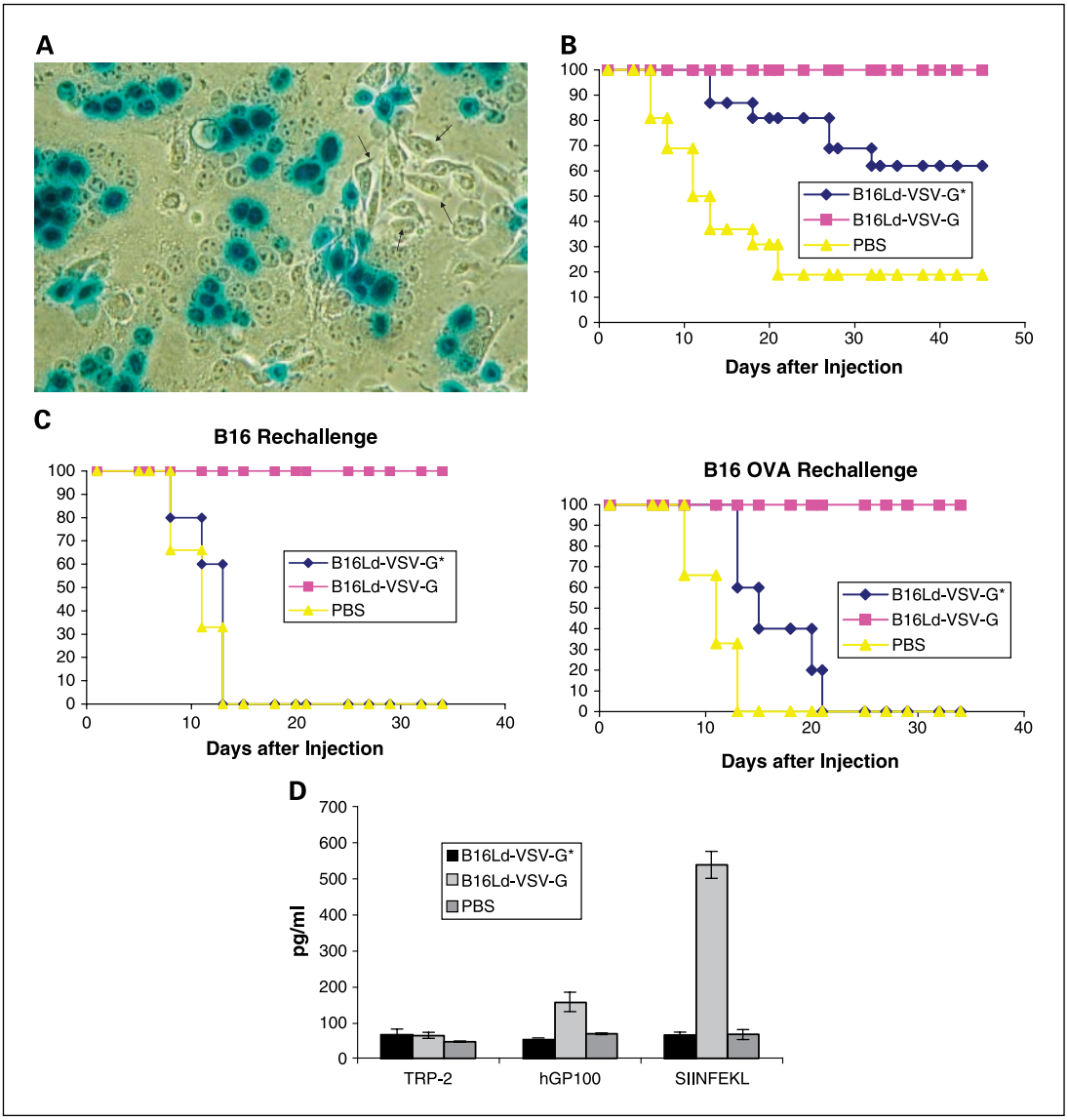

Fig. 4. Intratumoral injection of FMG-expressing allogeneic tumor cells. *A,* B78H1.Ld cells were transfected with fusogenic VSV-G, subjected to transient pH drop, and plated onto B16-β-gal cells *in vitro*; the coculture was stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside 16 hours later. The presence of syncitia containing both blue (B16-β-gal) and clear (B78H1.Ld) nuclei confirms the ability of VSV-G – expressing tumors to "deliver" fusion to an untransfected target cell population. The arrows outline an area of intact cells to help delineation from the syncitium, which makes up the rest of the plate. *B,* VSV-G or VSV-G* – transfected B78H1.Ld cells were injected into palpable, established B16.OVA tumors and primary growth was monitored. Significant primary regression was seen in both treatment groups, although tumor control was greater (100%) after injection of fusing cells. *C,* mice achieving complete regression of their primary tumor in (*B*) were further rechallenged on day 60 with either B16.OVA or parental B16. Mice treated initially with intratumoral injection of fusing, but not nonfusing B78H1.Ld, rejected both challenges. *D,* splenocytes were harvested from animals treated with intratumoral fusing or nonfusing B78H1.Ld cells, and challenged *in vitro* with the H-2b-restricted epitopes SIINFEKL, TRP-2, and gp100. IFN-γ was only seen in mice treated with fusing cells, correlating with the secondary protection shown in (*C*). The greatest specific response was to SIINFEKL, with lower IFN-γ detected on stimulation with gp100; no response was apparent against TRP-2.

effective immune response to fusing cell vaccines. Nevertheless, syncitiosomes prepared from an immortal allogeneic line, bulked and frozen prior to *in vivo* administration with adjuvant, may provide an effective novel vaccine for patient use. We are now exploring the potency of allogeneic syncitiosomes *in vivo* combined with other adjuvants, and directly comparing the immunogenicity of syncitiosomes and standard exosomes.

In vitro data has shown that both fusing tumor cells and the syncitiosomes they secrete can provide antigen to APC such as dendritic cells for cross-presentation to specific T cells (3, 7). To explore the *in vivo* role of APC cross-priming in FMG immunogenicity, we took advantage of the B78H1 line, which expresses no MHC class I, and therefore cannot directly present antigen to T cells (16). Figure 3 shows that vaccination with fusing, but not intact B78H1 cells, protected mice against B16 challenge, and that this protection was associated with the generation of a specific response against a melanoma-associated MHC class I–restricted antigenic epitope, TRP-2. This response to class I-negative fusing cells must have been initiated by cross-priming of endogenous APC, and confirms the *in vivo* relevance of our previous *in vitro* cross-presentation data (7); it also supports the development of combined strategies to deliver antigen from fusing cells *in vivo* in the context of maximal activation of endogenous APC.

Delivery of therapeutic genes remains a major challenge for gene therapy, including those designed to elicit an immune response. We tested a novel approach, using intratumoral injection of allogeneic tumor cells transfected with VSV-G to initiate fusion and potentially prime immunity within an established tumor. This strategy again uses fusion of allogeneic cells for potent immune priming, whereas potentially also recruiting autologous tumor cells and their associated antigens into developing syncitia. In addition, direct injection of fusing allogeneic cells into tumor, either prior to resection or in the palliative setting, is clinically straightforward. To track priming against a specific autologous TAA, B16 expressing OVA was used as a model of established disease.

Somewhat surprisingly, both nonfusing and fusing B78H1.L^d led to a significant number of regressions following intratumoral injection, although fusing cells were more potent (Fig. 4B). This suggests the initiation of a rapid innate response by expression of allogeneic MHC and/or VSV-G within the injected tumor. However, only the fusing vaccine primed responses directed at specific TAAs, i.e., OVA and to a lesser extent, gp100 (although here not TRP-2), and protected mice from a second rechallenge with either B16.OVA or parental B16 (Fig. 4C and D). Hence, the presence of fusion is required for specific T cell priming, and this response extends beyond the dominant model OVA antigen. We are currently investigating the CD4, CD8, and natural killer cell dependence of this immunity, to better understand the mechanisms underlying the differential nonspecific response elicited by nonfusing allogeneic tumor cells, and the TAA-specific response to fusion.

It is interesting that the repertoire of TAA-specific responses was different in the two different FMG-based vaccination strategies following which they were tracked. After vaccination with fusing MHC class I-negative B78H1, we found a significant TRP-2-specific response (confirming cross-priming; Fig. 1C), with no response against OVA (here used as a negative control which was not expressed by the vaccine cells). When priming against TAA was tracked following intratumoral injection of fusing B78H1.L^d into B16.OVA tumors, we detected strong anti-OVA splenocyte reactivity, but no response against TRP-2. In this experiment, gp100 was also tested and a relatively weak, although measurable, response was detected (Fig. 4D). Potential explanations for this discrepancy in anti-TRP-2 priming between the two protocols (both in the context of effective antitumor protection/regression), are (*a*) the different disease burdens of the two protocols (prophylactic vaccination versus primary therapy), (*b*) differing expression levels of specific melanoma TAA between these different B16 models, and (*c*) the effect of a dominant model antigen such as OVA (present in Fig. 4D but not Fig. 1C) on the repertoire of responses primed against other endogenous TAA following cellular vaccination. Nevertheless, this data supports a model in which appropriate cell-based vaccination strategies prime responses directed against a range of expressed TAA, leading to protection against live tumor challenge and potentially reducing the risk of tumor escape by antigen-loss tumor variants. The range of defined TAA we can track in these model systems probably represents only a small fraction of the TAA potentially involved in antitumor immunity. This is illustrated by the complete rejection of the secondary B16 challenge shown in Fig. 4C, in the context of *in vitro* reactivity restricted to only a low level response against gp100 (Fig. 4D). However, priming against a large number of TAA, which remain for the most part uncharacterized, represents one of the major attractions of allogeneic cell-based vaccination.

It is also noteworthy that a vaccine/challenge protocol using nonfusing B78H1.L^d cells offers some protection against live tumor challenge, suggesting memory (Fig. 1B), whereas intratumoral injection of nonfusing B78H1.L^d cannot protect against rechallenge, following partial primary control/regression (Fig. 4C). Moreover, in the more challenging setting of early established disease, contralateral treatment with nonfusing cells is ineffective (Fig. 1C). Differences in disease burdens between these various protocols using nonfusing cells, the site into which the vaccine is delivered, may also be critical, with normal skin differing significantly from the environment of an established tumor. Both may permit or even differentially facilitate some early innate immunity, whereas intratumoral FMG expression without fusion clearly prevents the generation of subsequent TAA-specific responses; further work is required to characterize these effects in more detail.

We are currently directly comparing FMG-expressing allogeneic cell therapy of established disease delivered either intratumorally (allowing fusion with autologous tumor cells *in situ*) or injected contralaterally into normal skin. Preliminary data is consistent with intratumoral injection as a more effective route of delivery for FMG-expressing allogeneic vaccines, although not conclusive. The access to autologous TAA, which is unique to the intratumoral route, is likely to become more significant in the clinical setting, where the extent of TAA shared between vaccine and patient will be more limited than in this model system in which all cell lines are originally B16-derived. We are therefore testing alternative models in which the vaccinating cells are not so closely matched in their antigenic profile with the injected tumor (e.g., injection of K1735 into B16.OVA). In addition, intratumoral injection of fusing cells could support and amplify delivery into tumors of other immunostimulatory genes such as cytokines, which can readily be coexpressed by the allogeneic line.

This study shows that FMG provides a cytotoxic and immunogenic gene therapy approach suitable for a range of

clinical applications. Expression of allogeneic MHC class I within cells undergoing FMG-mediated fusion generates a potent antitumor vaccine, supporting the practical use of established allogeneic lines expressing FMG in the clinic. Fusing cells, or the syncitiosomes they secrete given with adjuvant, are both effective vaccines, and intratumoral injection of transfected cells provides a further novel application for *in situ* antigen-specific priming. This data supports early testing of strategies based on allogeneic tumor cells expressing FMG in cancer patients.

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