Combined Administration with DNA Encoding Vesicular Stomatitis Virus G Protein Enhances DNA Vaccine Potency

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Received 15 September 2009/Accepted 7 December 2009

DNA vaccines have recently emerged at the forefront of approaches to harness the immune system in the prevention and treatment of viral infections, as well as the prevention and treatment of cancers. However, these vaccines suffer from limited efficacy since they often fail to produce significant antigen-specific CD8+ T-cell responses. We report here a novel concept for DNA vaccine design that exploits the unique and powerful ability of viral fusogenic membrane glycoproteins (FMGs) to couple concentrated antigen transfer to dendritic cells (DCs) with local induction of the acute inflammatory response. Intramuscular administration into mice by electroporation technology of a plasmid containing the FMG gene from vesicular stomatitis virus (VSV-G)—together with DNA encoding the E7 protein of human papillomavirus type 16, a model cervical cancer antigen—elicited robust E7-specific CD8+ T-cell responses, as well as therapeutic control of E7-expressing tumors. This effect could potentially be mediated through the immunogenic form of cellular fusion and necrosis induced by VSV-G, which in a concerted fashion provokes leukocyte infiltration into the inoculation site, enhances cross-presentation of antigen to DCs, and stimulates them to mature efficiently. Thus, the incorporation of FMGs into DNA vaccines holds promise for the successful control of viral infections and cancers in the clinic.

Due to their safety, low manufacturing cost, and ease of production, DNA vaccines have emerged as one of the most attractive approaches to prevent and treat viral infections, as well as cancers with defined tumor-associated antigens. Several studies have demonstrated that DNA vaccines can produce significant CD8+ T-cell-mediated immunity—which is often essential in the elimination of pathogens and malignancies—together with therapeutic benefit in various animal models of disease (4, 21–23, 29). However, DNA vaccines have achieved limited success in the clinic, since they generally elicit limited CD8+ T-cell responses in humans despite repeated high-dose administration (15, 28). Therefore, there is a critical need to develop DNA vaccines which generate these types of responses.

DNA vaccines are commonly delivered by inoculation into skeletal muscle, where myocytes uptake them and express their encoded antigen. Myocytes, however, lack major histocompatibility complex (MHC) class II and costimulatory molecules and, as a result, have poor ability to prime naïve T cells. Thus, the potency of DNA vaccines depends on sequential phases of antigen transfer from myocytes to sentinel dendritic cells (DCs), followed in close succession by the maturation of these DCs. The DCs then migrate to lymphoid organs, where they can present the antigen to and activate cognate naïve CD8+ T cells (11). In the absence of either of these phases, either a null or a tolerogenic immune response is generated.

One of the major challenges to the generation of CD8+ T-cell responses is therefore the design of vaccines that efficiently target antigen to and stimulate the maturation of DCs in a concerted manner. We hypothesized that this could be accomplished through the induction of an inflammatory form of myocyte death with concentrated antigen release to DCs upon DNA immunization. Viral fusogenic membrane glycoproteins (FMGs) represent ideal agents for eliciting such an effect. It has been shown that FMGs induce the fusion of cells into large multinucleated syncytia, which are lysed rapidly by nonapoptotic mechanisms (1) and in the process shed exosomes from the membrane (2). We reasoned that, since nonapoptotic death has been linked to stimulation of the innate immune system (25) and since exosomes have been reported to contain antigen that can be readily taken up by DCs (30), FMGs may bolster the release of antigens encoded by the DNA vaccine, leading to enhanced antigen-specific CD8+ T-cell responses.

We have previously created and characterized a panel of DNA vaccines targeting the E7 oncoprotein of human papillomavirus (HPV) type 16, a model cervical cancer antigen. Because E7 is required for the maintenance of the transformed cellular phenotype and is expressed in virtually all cases of cervical cancers, it provides an ideal molecular target against which to apply our vaccination approach. In head-to-head comparisons of these vaccines, we showed that one particular construct, CRT/E7, could generate the most potent immunological and therapeutic effect against E7-expressing tumors (14). However, although CRT/E7 can cure mice with small tumors, it invariably fails to control more advanced forms of disease. In light of this obstacle, we sought to determine whether VSV-G could render CRT/E7 therapeutically effective against otherwise refractory E7-expressing tumors. We
have previously characterized the growth kinetics of one such aggressive tumor model, TC-1, in mice and observed consistently that 1 week after subcutaneous challenge with $5 \times 10^5$ cancerous cells, the mass becomes clearly detectable by palpation and visual inspection, and vaccination with CRT/E7—even with repeated high dose administration—has negligible beneficial effect on disease progression or animal survival. At this point, we considered the cancer to be sufficiently advanced, and this timeline was therefore adopted for the tumor treatment experiments in the present study.

In the present study, we explored whether combined administration with VSV-G could successfully control the growth of aggressive TC-1 tumors refractory to treatment with CRT/E7 alone, and potential mechanisms by which this might be achieved. Collectively, our data indicate that incorporation of FMGs into DNA vaccines represents a therapeutically promising strategy for the generation of antigen-specific T-cell-mediated immunity against viral infections and cancer.

**MATERIALS AND METHODS**

**DNA constructs.** The VSV-G gene, cloned into the pCMV mammalian expression vector (27), was generously provided by Robert Weinberg (Massachusetts Institute of Technology, Cambridge, MA). The CRT/E7 (10) and green fluorescent protein (GFP) (12) plasmid constructs [contained in the pCDNA3.1(−) vector (Invitrogen, Carlsbad, CA)] have been previously described.

**Cell lines.** The human embryonic kidney 293 cell line (American Type Culture Collection, Manassas, VA) was grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 2 mM-L-glutamine, 100 U of penicillin/mL, and 100 g of streptomycin/mL (Invitrogen). By continuous passage of an immortalized DC line (26) kindly provided by Kenneth Rock (University of Massachusetts, Worcester, MA), we generated a subclone (DC-1) that can be efficiently transfected by using Lipofectamine 2000 (Invitrogen). The TC-1 tumor cell line was produced by retroviral transduction of primary human lign epithelial cells with E6, E7, and activated human c-Ha-ras gene (16). DC-1 and TC-1 cells were grown in RPMI 1640 (Sigma-Aldrich, St. Louis, MO). All cells were cultured at 37°C in a humidified incubator with 5% CO₂.

**Characterization of apoptosis and necrosis.** 293 cells were transfected by using Lipofectamine 2000 with 2 μg of either pCMV or VSV-G plasmid. Every 24 h after transfection for a period of 5 days, fusion was monitored by phase-contrast microscopy, and the extent of cell death was quantified by staining with annexin V conjugated to phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) (eBioscience, San Diego, CA), followed by analysis using a Becton Dickinson FACSCAN flow cytometer (BD Biosciences, San Jose, CA) with FlowJo software (Tree Star, Ashland, OR).

**Preparation and culture of bone marrow-derived DCs (BMDCs).** Bone marrow cells were flushed from tibiae and femurs of C57BL/6 mice. After lysis of red blood cells, the cells were harvested and stained with 7-AAD (eBioscience, San Diego, CA), followed by analysis using a Becton Dickinson FACSCanto flow cytometer (BD Biosciences, San Jose, CA) at the site of inoculation by four electrodes 2 mm in length surrounding the central needle.

**Histological analysis of muscle tissue.** Mice received intramuscular electroporation with 2 μg of either empty pCMV vector or VSV-G plasmid. At days 1, 4, and 7 after immunization, the animals were sacrificed, and muscle was excised from the hind leg. As a control, electroporation with blue dye was performed to visualize diffusion of the injected chemicals and to confirm that tissues were properly resected around the inoculation site. Tissues were then sectioned, embedded in paraffin, and stained with hematoxylin and eosin (H&E; AML Laboratories, Rosedale, MD). Samples were analyzed for areas of necrosis and inflammation by a pathologist at the Johns Hopkins Hospital, Baltimore, MD.

**Characterization of antigen-specific T-cell-mediated immune responses.** Mice (three per group) were vaccinated by intramuscular electroporation with 2 μg of either pCMV or VSV-G plasmid in a 1:1 mass ratio. Electroporations with 1 μg of VSV-G (mixed with 1 μg of pCMV) or 2 μg of pCMV alone were performed as controls. Animals were boosted at the same dose and regimen 7 days later. At 14 days after the initial vaccination, the mice were sacrificed, the splenocytes were harvested, and 10⁵ cells per group were seeded in 24-well plates. Splenocytes were mixed with 1 μg of GolgiPlat (BD Biosciences, Franklin Lakes, NJ)/ml to arrest the secretory pathway and incubated with either E7 peptide (amino acids 49 to 57) or medium alone. After 15 h, splenocytes were stained with anti-mouse CD8-PE MAb (eBioscience), Cells were then permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained with anti-mouse IFN-γ-FITC MAb (eBioscience). Flow cytometry was performed to quantify the number of activated E7-specific CD8⁺ T-cell precursors. Analysis was restricted to the lymphocyte population as gated by forward- and side-scatter properties.

**Tumor treatment experiments.** A total of 5 × 10⁵ TC-1 tumor cells were injected subcutaneously into the flanks of mice (five animals per group). After 7 days, when the tumor reached 3 mm in diameter, the mice were vaccinated by intramuscular electroporation with 2 μg of total CRT/E7 and either empty pCMV vector or VSV-G plasmid at a 1:1 mass ratio. Electroporations with 1 μg of VSV-G (mixed with 1 μg of pCMV) or 2 μg of pCMV alone were performed as controls. Animals were boosted on day 14 after TC-1 challenge at the same dose and regimen, and tumor sizes were measured every 5 days. The percent survival of the mice at each time point was also monitored.

**Statistical analysis.** Data expressed as means ± the standard deviations are representative of at least two independent experiments. Comparisons between individual data points were performed by using a Student t test, and all P values of <0.05 were considered significant. Differences in survival between groups in the tumor treatment experiments were assessed by the Kaplan-Meier method and the log-rank test. Statistical analysis was conducted by using SigmaPlot (Systat Software, San Jose, CA).

**RESULTS**

VSV-G plasmid successfully controls cancer progression and prolongs survival in the context of antigen-specific DNA vaccination. Based on our hypothesis that VSV-G would amplify the CD8⁺ T-cell responses produced by DNA vaccination, we reasoned that this strategy could be of value as a treatment for cancer or viral infection. One week after TC-1 tumor challenge, the animals were vaccinated intramuscularly at defined intervals by electroporation technology with CRT/E7 in conjunction with either VSV-G plasmid or empty vector. Studies in our laboratory have established that the use of electroporation relative to injection substantially improves...
the transfection of plasmid DNA into myocytes (data not shown). The tumor size was measured every 5 days. As shown in Fig. 1A, vaccination with empty vector, VSV-G, or CRT/E7 alone was unable to control cancer progression and, after 3 weeks, the tumors were all larger than 100 mm², with minimal deviations observed between groups. In contrast, vaccination with CRT/E7 and VSV-G together generated a strong and persistent therapeutic effect, and 25 days after challenge, tumor size remained at \( \approx 30 \) mm². These prominent differences in tumor size corresponded to length of survival. As depicted in Fig. 1B, mice administered with empty vector or VSV-G alone all died \( \approx 40 \) days after tumor cell injection. Immunization with CRT/E7 had a mild protective effect, since 40% of the animals were still alive at day 40, although none survived to day 50. However, 100% of mice vaccinated with CRT/E7 and VSV-G together remained alive at 60 days. Importantly, there were no overt side effects of the combined treatment in any animals.

Thus, introduction of VSV-G in the context of antigen-specific DNA vaccination is able to successfully control the growth of otherwise refractory tumors and prolong the survival of diseased hosts.

Coadministration with VSV-G plasmid amplifies antigen-specific CD8\(^+\) T-cell responses generated by DNA vaccination. Because VSV-G could restore the therapeutic efficacy of CRT/E7 against advanced cancer, we were prompted to determine whether the magnitude of the CD8\(^+\) T-cell responses was strengthened by this combinatorial regimen. CD8\(^+\) T cells constitute a critical arm of immunity against cancer in our TC-1 tumor model. In this context, mice were vaccinated by intramuscular electroporation with CRT/E7 in combination with either VSV-G plasmid or empty vector, and the levels of E7-specific CD8\(^+\) T cells elicited were probed in the spleen. Administrations of VSV-G or empty vector alone were performed as negative controls. As shown in Fig. 2, splenocytes...
harvested from mice vaccinated with CRT/E7 and VSV-G, compared to all other groups, contained higher numbers of E7-specific, gamma interferon (IFN-γ)-secreting, CD8+ T-cell precursors. Specifically, we observed a 7-fold increase in T-cell responses elicited when VSV-G was introduced. As expected, vaccination with VSV-G or empty vector alone failed to produce E7-specific immunity. Our data therefore indicate that VSV-G amplifies antigen-specific CD8+ T-cell responses raised by DNA vaccination, a phenomenon that underlies the strong therapeutic potency of this FMG. We did not observe the production of CD4+ (i.e., T_{H1}, T_{H2}, or T_{H17}) responses against E7. Furthermore, we did not detect CD8+ or CD4+ T-cell-mediated immunity against VSV-G in our experiments (data not shown).

**VSV-G rapidly induces extensive cell fusion and death.** We next sought to elucidate the mechanisms responsible for the immunostimulatory and therapeutic function of VSV-G we observed against E7. It has been consistently shown in a variety of models that VSV-G induces syncytium formation and eventual death in cells that express it (1, 2). We therefore expected...
to be able to see this phenomenon in a standard cell line using our VSV-G DNA construct. To confirm and further explore the fusogenic effects of VSV-G, we characterized the morphology and kinetics of cell death induced by this protein. 293 cells were transfected with VSV-G and then analyzed by phase-contrast microscopy. As evident from Fig. 3A, at 24 h these cells exhibited conglomerated structures and fused into large multinucleated syncytia. At 72 h, nearly all of the cells fragmented into small membranous particles and detached from the culture plate (data not shown). In comparison, control-transfected cells appeared healthy and did not aggregate throughout (Fig. 3A). The type and kinetics of cell death induced by VSV-G were then assessed by staining with the phosphatidylserine-binding protein annexin V and the DNA-intercalating dye 7-AAD. After 24 h, a large fraction of VSV-G-transfected cells (~30%) had already died (7-AAD−), and most of those (~95%) had done so through nonapoptotic mechanisms (annexin V−7-AAD−) (Fig. 3B and C). In contrast, more than 90% of the cells remained viable in the control-transfected group (7-AAD−). Similar results were obtained by transfection of a variety of other models—derived from both mouse and human sources—with VSV-G, indicating that its function is likely preserved across different species and cell types, which is consistent with previous reports of the wide tropism of this protein (17). These data demonstrate that VSV-G incites rapid, extensive fusion and cell death through a nonapoptotic pathway.

Intramuscular electroporation with VSV-G plasmid incites a strong inflammatory response. Because VSV-G induces cell death, we hypothesized that this viral protein might induce acute inflammation when administered in vivo. In principle, this would lead to the accumulation of DCs, macrophages, and lymphocytes at the inoculation site and could facilitate antigen cross-presentation in a highly immunogenic context. As shown in Fig. 3D, muscle derived from mice that received electroporation with VSV-G plasmid exhibited leukocyte infiltration within 4 days, as detected by H&E stain. By 7 days, the tissue had healed and the inflammation had fully resolved, as the histology appeared normal. In contrast, in mice administered with control plasmid, only mild muscle damage, attributable to the electroporation, was observed after 1 day, and by day 4 the tissue was completely normal. The presence of a small number of leukocytes could only be detected at day 1. Altogether, these results demonstrate that VSV-G provokes extensive tissue disruption in vivo, which in effect instigates an acute and transient inflammatory response.

VSV-G mediates concentrated antigen transfer to DCs. Because VSV-G induces cell death with release of membranous particles, we reasoned that small molecules such as antigen could be cross-presented to DCs in a concentrated manner through this pathway. One route by which antigen can be concentrated is in membrane vesicles originally termed syncytiosomes, as has been suggested by previous studies (2).
Syncytiosomes are 60 to 90 nm in diameter, with shapes and sizes that resemble exosomes. Therefore, we compared the levels of exosomes produced by either VSV-G- or control-transfected 293 cells. It was found that VSV-G-transfected cells secreted a large amount of exosomes (\(0.37 \mu g/\mu L\)) after 72 h (Fig. 4A). In contrast, exosomes were virtually undetectable in medium from control-transfected cells. We reasoned that exosomes derived from VSV-G-expressing cells could package antigen and shuttle it to DCs. To test this, 293 cells were cotransfected with the model antigen GFP and either a control or VSV-G plasmid and then incubated with DCs. After 15 h, cells were collected, stained for CD11c, a DC marker, and analyzed by flow cytometry. As demonstrated in Fig. 4B and C, 293 cells transfected with GFP alone could not pass this antigen to DCs, since the frequency of CD11c⁺ GFP⁺ cells in this group was negligible (\(<1\%)\). However, there was a marked proportion of GFP⁺ cells (\(13\%)\) among DCs incubated with GFP- and VSV-G-expressing 293 cells. Therefore, VSV-G mediates concentrated antigen transfer to DCs, a property that is critical to the effectiveness of DNA vaccines.

BMDCs are activated by incubation with VSV-G-transfected cells. In addition to promoting antigen release to DCs, successful DNA vaccines must also efficiently activate these cells in a concerted manner, a process accompanied by upregulation of essential costimulatory molecules and migration to secondary lymphoid organs. We predicted that, because it causes nonapoptotic death, VSV-G may spur the production of inflammatory factors by cells which express it. These factors could stimulate the maturation of DCs that have recently absorbed antigen, effectively conferring upon them the ability to prime cognate naive CD8⁺ T cells. To test this possibility, 293 cells were transfected with either VSV-G or control vector and incubated with BMDCs. Cells were subsequently isolated and costained for CD11c and the DC activation markers MHC class II, CD80, CD86, or CD40. Notably, BMDCs incubated with 293 cells transfected with VSV-G, relative to the control

**FIG. 4.** Flow cytometry analysis to demonstrate antigen transfer to DCs mediated by VSV-G. (A) Protein concentration assays showing induction of exosome release by VSV-G. 293 cells were grown to confluence in 175-cm² flasks and transfected with 30 µg of either pCMV or VSV-G plasmid. After 72 h, supernatant was collected, and exosomes were purified by sequential spinning in 4°C at 300 × g for 10 min, 800 × g twice for 15 min, and 10,000 × g for 30 min. Exosomes were then pelleted at 100,000 × g for 1 h by using an Optima L-80 XP ultracentrifuge (Beckman-Coulter, Fullerton, CA), washed once, and resuspended in 1× PBS. Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA) (*, \(P < 0.007\)). B, 293 cells were transfected with GFP and either VSV-G or pCMV. At 24 h later, DC-1 cells were added to each well. After 15 h, cells were stained for the DC surface marker CD11c, and flow cytometry was performed. Representative density plots are shown. (C) Bar graph depiction of the number of CD11c⁺ GFP⁺ cells (*, \(P < 10^{-5}\)).
VSV-G, amplified the immune response raised by an already powerful DNA vaccine, CRT/E7, against the E7 oncoprotein of HPV type-16 and successfully controlled the growth of aggressive cancers. Our data suggest that the mechanism for the immunological and therapeutic effects of VSV-G may be mediated through its capacity to enhance antigen uptake by DCs and successively stimulate their complete maturation. In addition, histological examination revealed that administration of VSV-G provokes rapid and massive but transient leukocyte infiltration into the site of inoculation. Collectively, our results demonstrate the feasibility, safety, and promise of incorporating FMGs into the design of a novel class of DNA vaccines for the treatment of viral infection or cancer.

The CRT/E7 vaccine has previously been characterized by Cheng et al. and was shown to consistently induce robust E7-specific CD8+ T-cell-mediated immunity (10). Furthermore, it was demonstrated in the same paper that CRT/E7 is able to eradicate small tumors and prevent metastasis. In the present study, we used a more aggressive challenge model than that used by Cheng et al., delaying treatment until tumor burden was large in order to more closely simulate a clinical situation; this is the likely reason for the limited therapeutic benefit of CRT/E7 observed. In this context, it is notable that VSV-G could restore the efficacy of CRT/E7.

Previous reports have established that VSV vectors encoding specified antigens (including E7) are immunogenic and can be effective in the prevention and treatment of papillomavirus infection (5–7, 19, 20). Our data indicate that the glycoprotein from VSV is at least partially responsible for this effect and can be used to improve immune responses in the context of DNA vaccination. The immunostimulatory function of VSV-G could be due to the inflammatory properties of this protein. We confirmed prior reports that VSV-G mediates rapid cell fusion and nonapoptotic death, with the concomitant release of antigen to DCs. The manner of cell death dictates the extent to which DCs are able to absorb antigens. Notably, we observed that DCs could uptake antigen much more proficiently when mixed with VSV-G-transfected compared to control-transfected, antigen-loaded cells. In addition, a previous study showed that VSV-G enhances antigen cross-presentation to DCs by tumor cells (2). These findings are consistent with a model in which the multinucleated bodies formed by VSV-G-expressing cells abundantly shed membrane vesicles reminiscent of exosomes (2). These vesicles may encapsulate antigen and are in principle studded with VSV-G molecules which can readily mediate fusion with the DC surface. In this process, the antigen may be transported into DCs in a concentrated fashion with an efficiency that surpasses the conventional route of phagocytosis. Notably, we found that transfection with VSV-G strongly induced exosome release (see Fig. 4A).

The delivery of antigens to DCs in exosomes also has important implications for the manner by which these molecules are subsequently processed and the type of immunity that is ultimately generated. Components of dead cells are normally phagocytosed by DCs into endosomes and lysosomes, which targets the antigen to the MHC class II pathway. This consequently biases the T-cell response toward the helper CD4 phenotype. In contrast, because antigen-loaded exosomes derived from VSV-G+ cells may fuse with the DC plasma membrane, the antigen could potentially enter the cytoplasm and be

**FIG. 5.** Flow cytometry analysis to demonstrate BMDC activation by VSV-G-expressing cells. (A) BMDCs were mixed with 293 cells transfected with either VSV-G (blue) or pCMV (red). CD11c+ cells were gated and analyzed for expression of CD80, CD86, CD40, and MHC class II by flow cytometry. Representative histograms are shown. (B) Bar graph depiction of the frequency of BMDCs with high expression levels of the indicated activation markers (*, \( P < 0.04 \); **, \( P < 0.004 \)).

*DISCUSSION*

In this study, we have proven the concept that viral FMGs can be used to couple concentrated antigen transfer to DCs with induction of the acute inflammatory response for the development of DNA vaccines with significantly increased effectiveness. Our strategy generated robust CD8+ T-cell responses capable of treating tumors in a preclinical model. Specifically, combined administration with a model FMG, cells, displayed much higher amounts of CD80, CD86, and CD40, while marginal increases in MHC class II levels were noted (Fig. 5A). Approximately 80% of total BMDCs were fully activated by VSV-G+ cells (Fig. 5B). Collectively, these data demonstrate unequivocally that VSV-G-expressing cells have the capacity to stimulate DCs.
processed and presented through the MHC class I pathway for the stimulation of CD8+ T-cell-mediated immunity. We discovered that, consistent with this paradigm, coadministration with VSV-G could magnify frequency of CD8+ T cells generated by CRT/E7. Nevertheless, we cannot exclude the possibility that the increased antigen transferred to DCs may also be in a free form not contained within exosomes and that it could be taken up into phagosomes or endosomes for presentation on MHC class II molecules. It would be of scientific and clinical interest to further test whether VSV-G has a similar positive effect on a vaccine designed to produce T_{H}1 or T_{H}17 responses. Both of these types of responses have been documented to have important roles in immunity against viruses, bacteria, and cancerous cells (3, 8).

Antigen uptake and presentation by DCs is necessary for the development of CD8+ T-cell-mediated immunity, but it is not sufficient. The proper stimulatory cues (e.g., cytokines or TLR agonists) are also required for the maturation of DCs to a degree that enables them to faithfully prime cognate naive T cells. In the vast majority of cases, such cues are not present, and antigen-loaded DCs instead become paralyzed or tolerogenic (18) states believed to be important in the prevention of autoimmunity but counterproductive from a therapeutic point of view. In this context, the distinct advantage of the approach we have reported here is that it links antigen transfer to DCs with their maturation in full. To our knowledge, the present study shows for the first time that the presence of VSV-G-expressing cells stimulates BMDCs with high efficiency. In the future, it would be interesting to see whether VSV-G is also able to stimulate other subsets of DCs.

The maturation of DCs could be mediated through the secretion of inflammatory factors such as Hsp70 and HMGB1 by VSV-G-transfected cells. These factors are among the most powerful known activators of DCs and are frequently used for the purpose of vaccination (9, 13). In addition, Sancho et al. have recently identified a C-type lectinlike molecule, CLEC9A (also known as DNGR-1), as an abundantly expressed receptor for necrotic cells on CD8+ conventional and plasmacytoid DCs required for cross-presentation of antigen (24). Signaling through CLEC9A leads to activation of the tyrosine kinase Syk, which can mediate downstream maturation of DCs (24). In the future, it will be intriguing to assess the potential role of CLEC9A, as well as other similar molecules, in the enhancement of the adaptive immunity by viral FMGs.

Besides BMDC activation, we have also shown by histology that the immunostimulatory factors induced by VSV-G provoke intense local inflammation. Although intramuscular electroporation itself is known to cause mild inflammation by disruption of the plasma membrane, this phenomenon was strongly accentuated by the introduction of VSV-G. This represents another attractive facet of our proposed strategy since it serves to draw DCs to the location where antigen is most abundant. Indeed, a dense leukocyte infiltrate was observed within 4 days at the site of inoculation. We have recently performed immunohistochemistry to characterize the leukocyte population recruited to regions of muscle inflammation and found it to consist of substantial quantities of DCs, lymphocytes, macrophages, and NK cells (data not shown). Therefore, the delivery of VSV-G together with antigen into tissue concentrates key subsets of cells in the vaccination area. These cells create and coordinate a cytokine milieu conducive to the activation of T cells, wherein a powerful immune response may be launched.

A concern associated with the electroporation of VSV-G into muscle lies in the potential for excessive, intolerable tissue damage or the onset of autoimmune. Because our approach relies on the delivery of VSV-G plasmid DNA, however, we expect expression of the encoded fusogenic protein to be relatively short-lived and its effects to be temporary. Indeed, although extensive damage was observed shortly after electroporation, the tissue regenerated completely after 1 week. In addition, at this time point, inflammation seemed to be entirely resolved, since the vast majority of leukocytes dissipated and became undetectable. Moreover, long-term observation of VSV-G-treated mice showed no overt symptoms of autoimmunity or disability. Thus, it appears that the method we describe induces a transient and naturally reversible, albeit strong, immunological stimulus and thus should represent a safe vaccination strategy.

Most importantly, this proof-of-principle study provides impetus for the integration of viral FMGs into DNA vaccines and, more generally, for the exploration of strategies which couple antigen transfer to DCs with induction of the acute inflammatory response. Although this method was evaluated here in the context of HPV type 16-associated cervical cancer, the approach we describe is in theory useful as a treatment for any viral infection or cancer with defined antigens. In the near future, it will be essential to apply this technique to a broad spectrum of disease models in order to build a framework for translation into the clinical arena.

ACKNOWLEDGMENTS

We are grateful to Shiwen Peng and Shaw-Wei Tseng for helpful discussions and critical reviews of the manuscript. We also thank Archana Monie for assistance with the preparation of the manuscript and Lucy Wangaruro for excellent secretarial support.

This study was funded by a Johns Hopkins University Provost’s Undergraduate Research Award (in 2009), NCI SPORE in Cervical Cancer grant P50 CA098252, and NIH grant 1 RO1 CA115245-01.

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