HHS Public Access

Author manuscript

Cancer Gene Ther. Author manuscript; available in PMC 2011 June 01.

Published in final edited form as:

Cancer Gene Ther. 2010 December ; 17(12): 864–871. doi:10.1038/cgt.2010.43.

Plasmid injection and application of electric pulses alter endogenous mRNA and protein expression in B16.F10 mouse melanomas

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Abstract

The application of electric pulses to tissues causes cell membrane destabilization, allowing exogenous molecules to enter the cells. This delivery technique can be used for plasmid gene therapy. Reporter gene expression after plasmid delivery with eight representative published protocols was compared in B16.F10 mouse melanoma tumors. This expression varied significantly based on the pulse parameters utilized for delivery. To observe the possible influence of plasmid injection and/or pulse application on endogenous gene expression, levels of stress related mRNAs four and 24 hours after delivery were determined by PCR array. Increases in mRNA levels for several inflammatory chemokines and cytokines were observed in response to plasmid injection, electric pulses alone, or the combination. This upregulation was confirmed by individual real-time reverse transcription TaqMan PCR assays. Proteins were extracted at the same time points from identically treated tumors and inflammatory protein levels were assayed by ELISA and by a custom multiplex bead array. Increases in inflammatory protein levels generally paralleled mRNA levels. Some differences were observed, which may have been due to differing expression kinetics. The observed upregulated expression of these cytokines and chemokines may aid or inhibit the therapeutic effectiveness of immune-based cancer gene therapies.

Keywords

Electroporation; gene therapy; inflammation; melanoma

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Conflict of Interest Statement

RH has ownership interest in RMR Technologies and owns stock and stock options of Inovio Biomedical Corporation.

Introduction

The use of *in vivo* electroporation (EP) as a therapeutic gene delivery approach has been successfully employed in a variety of applications including cancer therapy and the regulation of protein levels to enhance or reduce protein function. For therapeutic cancer applications, plasmids evaluated for gene therapy encode the same types of genes or cDNAs tested using viral delivery methods, including immune modulators, cell cycle regulators, suicide genes, anti-angiogenic genes and genes encoding toxins or tumor antigens. Diverse delivery protocols varying in pulse parameters and in electrode configurations have been described (1).

Many of the therapeutic studies of *in vivo* intratumor electroporation in experimental cancer models test the delivery of plasmids encoding immune modulators. These studies may demonstrate significant tumor regression, indicating that the delivered genes or cDNAs are potentially effective as antitumor agents. A limited number of these studies have demonstrated long-term, complete tumor regression, including studies delivering plasmids encoding interleukin (IL)-12 (2–6), interferon (IFN) α (7;8), IL-15 (9), and IL-21 (10) as single agents. Complete tumor regression was observed after delivery of combinations of plasmids encoding IL-6 and IL-15 (11), GM-CSF and B7.1 (12), or IL-12 and B7.1 (13). Intratumor electroporation of a plasmid encoding the human IL-12 cDNAs for melanomas has been successful therapeutically in a Phase I clinical trial (14). Intratumor delivery of a plasmid encoding the human IL-2 cDNA to melanomas has also reached clinical trials, although efficacy has not yet been reported (15). These studies support the idea that immune modulators may be efficacious as cancer therapies.

Inflammation induced by the combination of plasmid delivery and electric pulses has been described in several tissues, most commonly muscle. Local inflammatory responses have been observed between 24 hours and seven days after plasmid injection (16) or delivery of pulses alone in rat (17), mouse (18–22), and pig (23) muscle. The combination of vector plasmid and pulses may induce higher levels of inflammation than plasmid alone (20;24;25). In skin, no significant histological changes were observed up to 5–7 days after delivery of pulses alone (26). In another study, minimal to mild inflammation was observed (19). Tumors have also been studied. In B16.F10 mouse melanomas, a strong infiltration of polymorphonuclear cells, monocytes, and some lymphocytes was observed 24 hours after vector plasmid delivery (27). In the RM4 rat bladder cancer model, macrophages were observed in the tumor periphery three days after electrically mediated plasmid delivery (28).

When plasmid DNA is present, the observed inflammation may be due in part to the induction of an inflammatory response to CpG motif DNA. The mammalian TLR9 receptor recognizes double stranded DNA that is not CpG methylated as a danger signal (29). Since plasmid DNA is produced bacterially and is not CpG methylated, an inflammatory immune response may be produced in response to its introduction, particularly to B and plasmacytoid pre-dendritic cells. Secreted immune modulators may include IFNγ, IL-1β, IL-6, IL-8, IL-10, IL-12, IL-18, tumor necrosis factor (TNF) α, interferon-gamma-inducible protein 10 (IP-10), macrophage inflammatory protein (MIP)-1β and granulocyte monocyte colony

stimulating factor (GM-CSF). This inflammatory response can be reduced or eliminated by deleting CpG motifs from the plasmid (30).

Approximately 50 different electroporation protocols for *in vivo* plasmid delivery to tumors have been described. These studies have differed in the electrode for delivery, tumor type, animal model, reporter protein, and the time point chosen for assessment. No direct comparison of reporter gene expression after plasmid delivery using these protocols has been reported. One purpose of this study is to clarify the relationship between electroporation parameters and transgene expression by using a single commercially prepared reporter plasmid in a single tumor model at a single time point. In addition, mRNA and protein levels of endogenous genes regulated by intratumor plasmid vector DNA injection, by the delivery of two different types of electric pulses to the tumor, and to the combination will be quantified. Endogenous genes regulated by delivery may be important considerations when designing therapeutic antitumor protocols.

Materials and Methods

Tumor and animal model

All procedures were approved by the Animal Use and Care Committee of the University of South Florida College of Medicine. 10^6 B16.F10 (ATCC CR6475) mouse melanoma cells in 50 μl PBS were injected subcutaneously in the left flank of female 7–8 week old C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME). Tumors were allowed to grow eight days to a diameter of approximately four mm before plasmid delivery.

Plasmid DNA preparation

gWizLuc, which encodes the luciferase gene driven by the CMV promoter, and gWizBlank, a plasmid vector that does not encode a transgene, were commercially prepared (Aldevron, Fargo, ND) and suspended to a concentration of 1 μg/μl in sterile injectable saline. Endotoxin levels were <0.1 EU/μg plasmid.

EP delivery

Mice were anesthetized using a mixture of 2.5% isoflurane and 97.5% O_2. Tumors were injected with 50 μl plasmid DNA and pulsed immediately as described in each figure at a frequency of 1 Hz using a T820 Electrosquare porator. Delivery was performed with caliper electrodes moistened with electrode paste or with a 6-needle array per each published protocol. An autoswitcher (BTX, San Diego, CA) was used for delivery with the 6-needle array. At the time points indicated after delivery, mice were humanely sacrificed, tumors removed and snap frozen on dry ice.

Reverse transcription PCR assays

For PCR assays, RNA was extracted from tumors using Trizol (Invitrogen, Carlsbad, CA), purified using RNeasy columns (Qiagen, Valencia, CA), and quantified RNA from four tumors per group was pooled. mRNA levels were determined using Mouse Stress and Toxicity SYBR PCR arrays (SABiosciences, Frederick, MD, USA) after reverse transcription per manufacturer's instructions. Relative quantification was performed by

comparison to the housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $\Delta\Delta C_t$ method. TaqMan one step RT-PCR was performed for GM-CSF, Cxcl10, IL-1α, IL-1β, IL-6, β-actin and GAPDH to confirm changes in mRNA levels (TaqMan Gene Expression Assays, Applied Biosystems, Foster City, CA). Since untreated tumors did not express GM-CSF endogenously, total lung RNA was used as a positive control.

Protein Assays

For luciferase quantification, the tumors were weighed, homogenized and enzyme activity in extracts was normalized to the tumor weight (31). For enzyme-linked immunosorbent assays (ELISAs) and multiplex bead arrays, tumors were homogenized in PBS containing protease inhibitors. Homogenates were centrifuged to remove cell debris. BCA assays (Pierce Biotechnology, Rockford, IL) were performed and samples normalized to 1 mg/ml total protein. ELISAs were performed per manufacturer's instructions (R&D Systems, Minneapolis, MN). A custom multiplex bead array including mouse IL-1α, IL-1β, IL-4, IL-6, IL-10, MIP-1α, MIP-1β, TNFα, and GM-CSF was constructed and samples assayed using a Bio-Plex System (Bio-Rad, Hercules, CA) per manufacturer's instructions.

Statistical analysis

Analysis of reporter gene expression after electrically mediated plasmid delivery to B16.F10 mouse melanomas was completed using analysis of variance (ANOVA). A post-hoc Dunnett's test was used to adjust for multiple comparisons to one control group (injection of gWizLuc without electroporation). Analysis of cytokine protein expression was completed using ANOVA. A post-hoc Fisher's Least Significant Difference test was used to adjust for multiple comparisons.

Results

Reporter gene expression

Eight published electroporation protocols for intratumor delivery were compared in the subcutaneous B16.F10 mouse melanoma model using a plasmid encoding luciferase as a reporter (gWizLuc, Aldevron, Fargo, ND). These protocols varied with respect to electrode, and pulse amplitude, length and number. At 24 hours, luciferase expression was determined (Figure 1). Using this specific reporter and time point, four delivery protocols produced expression that was significantly higher than expression from plasmid injection. Two protocols suggested increased expression, while two protocols exhibited no substantial expression above plasmid injection alone.

Endogenous mRNA levels

To determine if *in vivo* electroporation can effect endogenous gene expression, two very different pulse protocols from Table 1 were compared. One protocol included six relatively short (100 μs) pulses at a field strength of 1300 V/cm using a six-needle array electrode (EP5)(2). Delivery with this protocol produced low-level and reproducible reporter gene expression. A second protocol varied markedly in that a single relatively long (100 ms)

pulse at 667 V/cm was delivered with a caliper electrode (EP6) (32). Delivery with this protocol produced high levels of reporter gene expression.

Gene expression was observed after delivery of saline or plasmid vector DNA with each pulse protocol and compared to untreated tumors and tumors injected with plasmid DNA without electroporation. Four and 24 hours after delivery, mice were sacrificed and tumors excised. Pooled RNA from tumors in each experimental group (n=4) was subjected to two step real-time SYBR RT-PCR performed using Mouse Stress and Toxicity SYBR PCR arrays (SABiosciences, Frederick, MD, USA). The gene groups contained in the array included oxidative or metabolic stress, heat shock, proliferation and carcinogenesis, growth arrest and senescence, necrosis or apoptosis, and inflammation. Each array was then repeated with a second group of four tumors. The fold changes in expression were determined by comparison to the housekeeping genes β-actin and GAPDH.

No downregulated mRNA levels were observed. The inflammation group contained the highest number of upregulated mRNA levels across the delivery groups. Within this group, mRNAs for Ccl21b, Ccl3/MIP-1α, Ccl4/MIP-1β, Cxcl10/IP-10, IL-1 α, IL-1 β, IL-6, IL-18, iNos, and Serpine1/PAI-1 were upregulated in response to at least one delivery condition, while mRNA levels for Csf2/GM-CSF, Lta/TNFβ, MIF, and NFκB1were unchanged.

In response to plasmid DNA injection alone, the array results indicated that most of the mRNA levels were upregulated by four hours after delivery. This is likely a response to CpG motif DNA. By 24 hours, expression was considerably reduced. Saline injection followed by electroporation protocol EP5 alone appeared to induce upregulation for Ccl21b and Serpine1/PAI-1, while saline injection with protocol EP6 appeared to upregulate Ccl21b, Cxcl10/IP-10, IL-1 α, IL-1 β, IL-6, iNos, and Serpine1/PAI-1 to higher levels. The detected increases in relative levels of mRNAs were tested by a one step TaqMan RT-PCR for a subset of targets, including IP-10, IL-1α, IL-1 β, IL-6, and Serpine1 (Table 2). The specific PCR quantifications for IP-10, IL-1α, IL-1β, and IL-6 in general tended to support the increases in mRNA levels detected by the PCR array. However, increased levels of serpine1 observed with the PCR array were the not confirmed with the individual PCR.

Endogenous protein levels

To determine if the observed changes in mRNA levels resulted in concomitant changes in protein levels, a subset of proteins was assayed using ELISAs and multiplex bead arrays. For these experiments, deliveries were performed and samples taken as described for the RNA experiments. For each delivery group, four tumors were assayed individually.

Using ELISAs for mouse IL-1β and IL-6, low endogenous levels of each cytokine were present in untreated tumors (Table 3). Four hours after delivery, IL-1β protein levels in tumors from the group receiving plasmid injection tended to be higher than the levels in untreated tumors. Protein levels in tumors receiving pulse protocol EP5 with saline also increased, but this increase appeared to be greater after delivery of saline with pulse protocol EP6. In groups receiving the combination of plasmid DNA and either electroporation protocol, levels were significantly higher than in untreated tumors. At four hours, IL-6 protein levels also increased after plasmid injection, but this increase was not statistically

greater than untreated tumors. Similar to IL-1β, the increase in IL-6 levels tended to be higher after saline delivery with protocol EP6 than with EP5. With the combination of plasmid and electroporation, these levels were statistically discernable with either electroporation protocol. For both cytokines, increased levels were reduced by 24 hours.

Multiplex bead arrays were performed for mouse MIP-1α, MIP-1β, IL-1α, IL-1β, and IL-6 per manufacturer's instructions (Table 4). Three cytokines that may be upregulated in response to CpG motif DNA (IL-4, IL-10, TNFα) were also tested. At the tested time points, IL-4 and TNFα were not detected in untreated tumors and were undetected or detected at insignificant levels in the delivery groups (data not shown). Detectable endogenous levels of the remaining protein targets, particularly IL-1α and MIP-1α, were present in untreated tumors. After plasmid injection, levels of MIP-1α, IL-1α and IL-10 reached statistically significance. When saline was delivery with EP5 pulses, only IL-1α levels increase significantly at four hours. No other statistically significant increases in protein levels to pulses alone were observed. However, with the combination of EP5 and plasmid DNA, significant increases in IL-1β, IL-6, MIP-1α, and MIP-1β proteins were observed at four hours. When plasmid DNA was combined with EP6, MIP-1α, IL-6, and IL-10 were significantly increased at four hours.

Discussion

Using reporter gene expression 24 hours after electrically mediated delivery, the level of transgene expression varied up to 92 fold over plasmid injection alone after delivery with different electroporation protocols. For some groups, no statistical increase in reporter gene expression was observed after delivery. For these protocols, reporter gene expression may not be detectable at this time point. Alternatively, the reduction in transmembrane potential necessary for membrane permeability may not have been reached. These results clearly indicate that the level of therapeutic transgene expression can be controlled by the electroporation protocol with which the plasmid is delivered. Although the expression levels varied significantly, it is difficult to make any generalizations concerning the relative usefulness of each pulse type. The therapeutic gene delivered and its desired level of expression should influence the pulsing protocol used for delivery, along with other methods used in the control of transgene expression, such as the use of alternative plasmid promoters.

Increases in levels of several inflammatory mRNAs were observed particularly after plasmid injection, application of pulse protocol EP6, or the combination of plasmid DNA with either pulse protocol. While changes in mRNA levels were confirmed using individual PCRs, particularly at greater than 10-fold increase on the array, the precise fold change in expression was not necessarily confirmed. Lower levels of upregulation were not necessarily reproducible, particularly at the 24 hour time point. This may be due to the differences in specific sequence targets and detection chemistries between the two PCR detection methods.

While proteins tended to be upregulated when the mRNA levels were upregulated, the specific levels did not necessarily correlate. It appeared that any manipulation of the tumor induced short-term IL-1α expression. For IL-1β, the array very closely reproduced the results of the ELISA assay. Interestingly, while the pattern of expression was similar for

IL-6, the multiplex quantified levels were clearly lower than the ELISA quantified levels. After the array was performed, the ELISA was repeated on the same samples and the ELISA values were confirmed. This discrepancy between the two protein assays may be due to differing target affinities of the antibodies used in the respective assays. However, each assay indicated that IL-6 in particular was upregulated synergistically in response to combination delivery.

IL-10 levels were significantly increased at four hours after any delivery containing plasmid DNA, indicating that a CpG motif DNA effect was responsible. The expression of the chemokines MIP-1α and MIP-1β tended to be longer than cytokines expression, continuing through four and 24 hours. While MIP-1α, MIP-1β, IL-1β, IL-6, and IL-10 protein levels were increased after delivery by both electroporation protocols, expression tended to be higher after plasmid delivery with EP5 than with EP6. This conflicts with the observed mRNA levels, where detected mRNA was much higher after combination delivery with EP6 than with EP5. These discrepancies may be due to the particular time points chosen for the assays; changes in protein levels may occur at different time points. Also, protein levels may not necessarily directly depend on mRNA levels. For example, both mRNA and protein stability may affect protein levels (33) and protein synthesis may be regulated (34).

In a study of pulse delivery alone in mouse muscle, increased levels of several mRNAs encoding proinflammatory modulators, including MIP-1α, MIP-1β, IP-10, IL-6, iNos and GM-CSF were observed by microarray two and four hours after delivery (21). With the exception of GM-CSF, the data presented here confirm this upregulation in a tumor model at four hours. As in tumors, the cytokine mRNA levels decreased to background by 24 hours. However, a difference in chemokine mRNA levels was observed. In muscle, these levels decreased to background more quickly than in tumor tissue. In a study of electroporation delivery of a low concentration of plasmid DNA to rodent muscle, no changes in expression of mRNAs involved in an inflammatory response were observed by microarray analysis at time points of four hours, 48 hours, and 3 weeks. However, an infiltration of mononuclear cells was observed histologically at 48 hours (35), suggesting the presence of an inflammatory response. Cytokine protein expression after electroporation of plasmid to mouse lung in bronchial alveolar lavage fluid was tested 24 hours after delivery. No upregulation of IL-6, TNFα, IFNγ, or IL-12 was observed (36). The IL-6 results presented here confirm this observation, since by 24 hours all observed increases in mRNA and protein levels had decreased to insignificance. In an array analysis of electroporation pulses to a human melanoma cell line, no significant increase in inflammatory gene expression was observed (37). This is not unexpected due to the absence of immune cells in this *in vitro* study.

The CMV promoter is commonly used for gene therapy because it induces high levels of transcription in many tissues. *In vitro*, transcription from this promoter is cytokine regulated, particularly by IFNγ and TNFα (38–43), although other cytokine regulators have been described. In mice, regulation of CMV promoter expression has been correlated to serum TNFα (44) and IL-12 levels (45). Lung expression has been correlated to TNFα, IL-12, and IFNγ expression in bronchoalveolar lavage fluid (46). The effect on expression may be dependent on the tissue type (45). In this study, low but consistent levels of TNFα were

observed in all tumor groups at the tested time points, limiting any conclusions concerning differential regulation in this model. IL-1β upregulates CMV-driven transcription (42;43) in some cell types. Although the combination of plasmid with EP5 induced higher levels of IL-1β than EP6, reporter gene expression driven by the CMV promoter was considerably lower. This does not support IL-1β upregulation of the promoter, however there are many additional variables that would affect transcription. IL-10 has been observed to downregulate CMV-regulated transcription (43). In the case of IL-10, EP5 induces higher levels of IL-10 than EP6, and this downregulation could account for the reduced luciferase activity. Once again, there are many additional variables to be considered.

Inflammation induced by electroporation acts as a vaccine adjuvant after muscle delivery of antigen encoding plasmids (21;23;47–50). Changes in CD3+ peripheral blood lymphocyte levels have also been observed in macaques after plasmid DNA immunization in combination with electroporation (51). It is possible that the induction of these immune modulators can also increase the effectiveness of cancer immunotherapies by the induction of a specific antitumor immune response (52). This endogenous cytokine induction may enhance NK cell activation, producing a direct anti-tumor effect. Antigen presenting cells could potentially be stimulated, which would aid in the induction of an immune response by the protein expressed from the delivered therapeutic gene. The electric pulses may induce tumor cell damage that may make tumor antigens available to the immune response. Interestingly, an observable (8;9;53–57) or statistically significant (27;58) effect on tumor growth has been observed after electroporation delivery of non-coding control plasmids when compared to untreated tumors. This effect may be due to the induction of gene expression, including the immune modulators described here. It is also possible that some induced cytokines may work in opposition to the induction of a specific immune response, for example the suppressive cytokine IL-10. The production of appreciable levels of these immune modulators should be considered when designing any electroporation based antitumor therapy.

Acknowledgments

The work in this study was supported by NIH 1 R21 CA106860 (LH).

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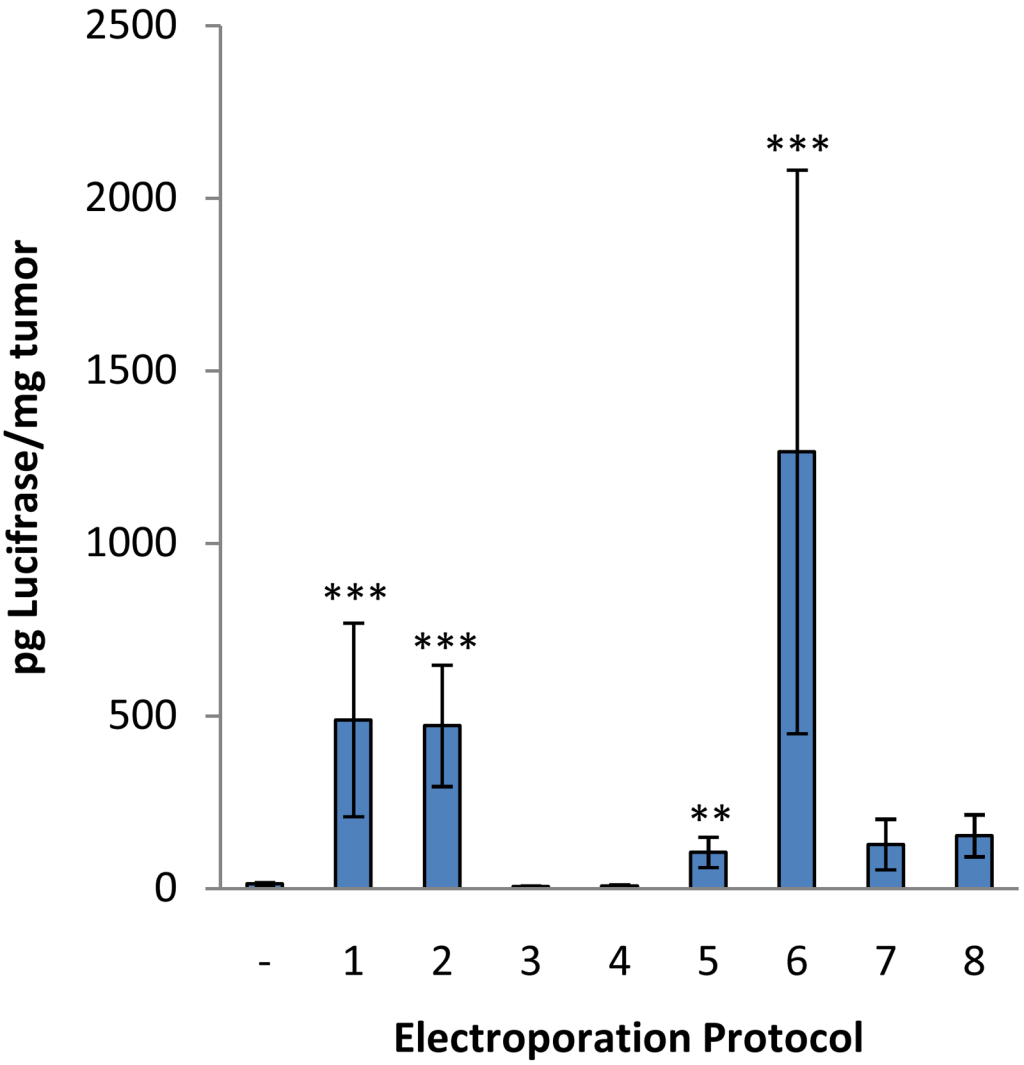

Figure 1. Reporter gene expression 24 hours after electrically mediated plasmid delivery to B16.F10 mouse melanomas

50 μg gWizLuc (Aldevron, Fargo, ND) was delivered to melanomas using various electroporation protocols: 1, ten 50 ms 800 V/cm pulses with a caliper electrode (59); 2, eight 20 ms 500 V/cm pulses with a caliper electrode (54); 3, eight 50 ms 66 V/cm pulses with a 6-needle array (5); 4, six 50 ms 167 V/cm pulses with a caliper electrode (60); 5, six 0.1 ms 1300 V/cm pulses with a 6-needle array (2); 6, one 100 ms 667 V/cm pulse with a caliper electrode (32); 7, two 20 ms 450 V/cm pulses with a caliper electrode (3); 8, five 10 ms 400 V/cm pulses with a caliper electrode (55). Activity was normalized to tumor weight. **p<0.01, ***p<0.001 with respect to injection alone, n=12–18 per group. Values represent mean ± standard error.

Cancer Gene Ther. Author manuscript; available in PMC 2011 June 01.

Table 1

Mean fold changes in mRNA expression by reverse transcription and PCR array with respect to untreated control tumors after plasmid delivery to B16.F10 mouse melanomas

	Time Point (h)	Ccl21b	Ccl3/MIP-1α	Ccl4/MIP-1β	Cxcl10/IP-10	IL-1α	IL-1β	IL-6	IL-18	iNos	Serpine1/PAI-1
gWizBlank only	4	2.2	111.4	512.0	19.0	26.0	76.1	8.9	35.5	48.5	7.7
	24	15.5	14.4	46.9	6.5	4.8	7.5	1.3	73.5	12.0	11.7
Saline + EP5	4	11.7	2.4	2.5	2.8	6.1	6.7	3.4	1.5	3.1	18.4
	24	4.4	3.7	2.5	2.8	3.4	3.3	1.6	6.6	3.1	15.7
Saline + EP6	4	11.5	7.1	31.5	25.6	23.8	62.9	36.1	6.8	15.2	102.2
	24	3.7	2.7	2.7	2.1	3.4	7.5	1.7	5.9	1.5	22.6
gWizBlank+ EP5	4	1.7	4.3	6.3	46.8	10.2	16.6	32.0	2.8	17.8	1.9
	24	13.7	10.4	29.3	11.1	3.5	7.3	1.7	26.5	18.7	40.1
gWizBlank+ EP6	4	−2.0	6.1	81.6	28.8	11.3	71.0	28.8	10.2	9.8	132.5
	24	1.1	3.2	28.8	3.4	1.5	7.7	−2.1	6.3	6.5	16.6

EP5, six 100 μs 1300 V/cm pulses delivered with a 6-needle array electrode; EP6, one 100 ms 667 V/cm pulse delivered with caliper electrodes

Table 2

Fold changes in mRNA expression by reverse transcription and TaqMan PCR with respect to untreated control tumors after plasmid delivery to B16.F10 mouse melanomas

	Time Point (h)	Cxcl10/IP-10	IL-1α	IL-1β	IL-6	Serpine1/PAI-1
gWizBlank only	4	24.8	7.7	7.6	39.0	0.7
	24	3.2	1.0	1.1	1.2	0.3
Saline + EP5	4	1.4	1.5	2.0	3.6	1.2
	24	2.0	0.2	1.7	2.4	0.9
Saline + EP6	4	13.7	14.8	68.6	128.0	2.5
	24	0.3	2.0	6.5	2.3	0.8
gWizBlank + EP5	4	35.8	4.5	7.9	26.0	1.1
	24	2.1	2.7	4.2	3.9	1.1
gWizBlank + EP6	4	21.4	14.4	40.0	128.0	1.7
	24	2.6	3.2	9.1	5.6	1.0

Table 3

Cytokine expression measured by ELISA after plasmid delivery to B16.F10 mouse melanomas (mean±SD)

	Time Point (h)	IL-1β (pg/mg total protein)	IL-6 (pg/mg total protein)
Untreated		4.5±5.9	21.2±9.8
gWizBlank only	4	112.5±50.0	245.9±126.8
	24	1.9±2.2	32.1±14.5
Saline + EP5	4	12.0±5.8	32.6±9.9
	24	3.0±3.7	10.1±8.7
Saline + EP6	4	57.6±24.5	141.2±106.5
	24	7.8±4.7	10.1±8.8
gWizBlank + EP5	4	219.0±126.4***	1644.7±868.7***
	24	8.6±5.9	45.0±3.5
gWizBlank + EP6	4	156.3±111.7*	996.8±865.0**
	24	10.0±7.0	49.2±20.8

Table 4

Cytokine expression measured by multiplex bead array after plasmid delivery to B16.F10 mouse melanomas (mean±SD)

	Time Point (h)	Ccl3/MIP-1α (pg/mg total protein)	Ccl4/MIP-1β (pg/mg total protein)	IL-1α (pg/mg total protein)	IL-1β (pg/mg total protein)	IL-6 (pg/mg total protein)	IL-10 (pg/mg total protein)
Untreated		67.9±27.6	10.9±6.6	44.2±25.6	11.1±10.0	2.5±2.5	2.4±1.2
gWizBlank only	4	453.4±38.1**	130.7±159.5	214.0±115.3***	104.3±31.8	47.3±18.3	27.8±16.0**
	24	422.5±136.5*	159.8±120.1	32.4±8.7	14.2±4.9	4.6±2.5	13.5±3.1
Saline + EP5	4	30.0±26.0	4.3±3.8	151.8±44.4*	13.9±7.4	6.3±2.1	2.2±2.0
	24	157.7±121.2	18.2±14.9	8.3±1.7	3.1±3.6	0.7±0.9	4.3±1.7
Saline + EP6	4	43.4±18.8	3.0±5.2	73.1±36.0	34.9±12.4	61.1±65.1	1.5±0.5
	24	28.5±28.0	0.7±1.2	8.4±4.0	7.6±10.0	1.4±1.5	0.4±0.7
gWizBlank + EP5	4	874.8±339.3***	369.8±221.1***	114.3±41.2	221.6±130.2***	544.1±202.3***	47.7±19.1***
	24	430.4±139.4**	119.7±56.5	10.0±6.2	8.0±7.3	6.4±2.2	8.0±4.1
gWizBlank + EP6	4	398.2±111.0*	206.4±110.8	100.5±73.8	110.0±79.1	299.0±312.4*	24.1±3.4**
	24	226.2±61.4	75.6±44.9	13.3±10.2	6.1±7.2	7.1±3.4	9.7±2.8