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Enhancement of Cellular Immune Response to a Prostate Cancer DNA Vaccine by Intradermal Electroporation

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Recently it has become clear that more potent methods for DNA vaccine delivery need to be developed to enhance the efficacy of DNA vaccines. *In vivo* electroporation has emerged as a potent method for DNA vaccine delivery. In a mouse model, we evaluated the CD8$^+$ T lymphocyte response to a prostate cancer DNA vaccine encoding prostate-specific antigen (PSA) after intradermal electroporation. A significantly increased gene expression (100- to 1000-fold) and higher levels of PSA-specific T cells, compared to DNA delivery without electroporation, was demonstrated. Interestingly, investigation of a panel of different electroporation conditions showed that only some conditions that induce high levels of gene expression additionally induced cellular immunity. This suggests that electroporation parameters should be carefully optimized, not only to enhance transfection efficiency, but also to enhance the immune response to the vaccine. This study demonstrates the applicability of intradermal electroporation as a delivery method for genetic cancer vaccines and other DNA vaccines relying on antigen-specific T cell induction.

Key Words: electroporation, prostate-specific antigen, DNA vaccine, CTL, immunotherapy

INTRODUCTION

DNA vaccination was reported to induce humoral and cellular immune responses in murine models [1]. Unfortunately the efficacy of DNA vaccination has not translated as well as desired when evaluated in humans [2]. It is not clear why DNA vaccines are less effective in humans, but one reason could be lower transfection efficacy. We have previously demonstrated that intramuscular vaccination of C57Bl/6 mice with pVax-PSA, a plasmid encoding prostate-specific antigen (PSA), results in potent induction of PSA-specific CD8$^+$ T cells [3,4], which are directed against an immunodominant H-2D^b-restricted epitope, psa65-73 [5]. PSA is a xenogeneic antigen in mice [6] and thus this model does not evaluate the ability to overcome tolerance. However, when pVax-PSA was evaluated in a phase I clinical trial of prostate cancer, the vaccine induced PSA-specific cellular responses at the highest dose level, demonstrating that T cells specific for PSA could be induced in an autologous setting [7]. Moreover, the clinical trial revealed that PSA/DNA vaccination has to be improved to induce stronger immune responses, and one possible strategy is to improve the delivery of the vaccine and subsequent transfection of cells.

To enhance the transfection of DNA vaccines and the subsequent immune response, several nonviral vaccine delivery methods were investigated, including gene gun [8], jet injection [9], poly(lactide–coglycolide) microparticles [10], and *in vivo* electroporation [11–13]. *In vivo* electroporation is considered one of the most efficient nonviral methods of DNA delivery [14] and of special interest are its low cost, safety, and ease of use. Other beneficial qualities are a decreased interindividual variability [12,14] and an increased cellular infiltration at the vaccination site [15,16], which might provide adjuvant function since its presence correlates with better immunity [17]. Furthermore *in vivo* electroporation was shown to induce humoral and cellular immune responses in pigs, goats, cattle [18,19], and nonhuman primates [20], indicating that this DNA delivery method has potential in large animals. Phase I/II studies have also demonstrated the safety and feasibility of this delivery method in the clinic [21,22]; however, it has not yet been tested for the delivery of DNA. When electrical pulses (1300 V/cm, 6 pulses, 99 μs) in combination with chemotherapeutic drugs were delivered to cutaneous and subcutaneous tumors pretreated with a local anesthetic (1% lidocaine), patients experienced muscle contractions and described

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FIG. 1. The needle array electrode used for intradermal electroporation.

the sensation as uncomfortable. However, no residual discomfort was apparent after the pulses had been delivered [21,22].

Muscle is the most commonly targeted tissue for evaluation of electroporation in combination with DNA delivery. Intramuscular electroporation was shown to increase gene expression [11,12,23] and humoral and cellular immunity [13,24–26] as well as enhanced tumor protection [27]. Fewer reports on efficacy of intradermal DNA delivery in combination with electroporation are available. Electroporation of the skin, however, is less invasive compared to muscle and is also more easily accessible to electrodes. The skin additionally harbors epidermal Langerhans cells and other types of dermal dendritic cells, which after DNA/antigen uptake can migrate to lymph nodes where efficient presentation to T cells occurs [28]. The choice of electroporation parameters is very important when performing *in vivo* electroporation. Optimal conditions (electric field strength (amplitude) and number, duration, and interval of pulses) should induce high transfection of DNA, but minimize tissue damage caused by the electric field. However, mild tissue damage can be tolerated and might be beneficial, leading to infiltration of inflammatory cells [17].

In this report we evaluate the induction of PSA-specific $CD8^+$ T cells in mice after intradermal administration of plasmid pVax-PSA in combination with *in vivo* electroporation. Different electroporation conditions were compared based on their ability to enhance reporter gene expression *in vivo* and to induce PSA-specific $CD8^+$ T cell responses. We have found that high gene expression *in vivo* is required but is not sufficient for induction of $CD8^+$ T cell responses after intradermal DNA electroporation. We further demonstrate that DNA vaccination using optimized *in vivo* electroporation conditions significantly increased the levels of PSA-specific T cells, compared to DNA delivery without electroporation.

Results and Discussion

Effects of Electroporation Conditions on Transgene Expression

To determine optimal pulsing conditions for enhancement of gene expression in skin using a needle array

TABLE 1: Different conditions used for *in vivo* electroporation in mouse skin

Electroporation condition	Group 1 Field strength (V/cm)	Group 1 Number of pulses	Group 1 Pulse duration (ms)	Group 2 Field strength (V/cm)	Group 2 Number of pulses	Group 2 Pulse duration (ms)	Schematic of pulsing condition[a]
A	1750	6	0.1	–	–	–	
B	200	6	0.1	–	–	–	
C	1125	2	0.05	–	–	–	
D	275	8	10	–	–	–	× 8
E	1125	2	0.05	275	8	10	× 8 low

[a] The figures are only schematic and not proportional. The pulse interval for electroporation conditions A–B and C–E was 125 and 300 ms, respectively. The pulse interval between group 1 and 2 (condition E) was 500 ms.

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electrode (Fig. 1), we evaluated five different pulsing parameters (Table 1, electroporation conditions A–E). We first tested a previously described parameter for skin electroporation, 1750 V/cm, 6 pulses, 100 μs (condition A) [29], and one parameter of lower field strength, 200 V/cm, 6 pulses, 100 μs (condition B). In line with the previous report [29], condition A significantly increased gene expression (over 200-fold) compared to DNA injection without electroporation (Fig. 2A). The electroporation condition of low electric field strength (200 V/cm), on the other hand, did not enhance gene expression (Fig. 2A). Most probably the combination of low electric field strength and short pulse duration, which causes very limited tissue damage, is not permeabilizing the cells sufficiently for enhanced gene uptake during such short time interval. We further investigated a combination of pulses (condition E) consisting of two groups of pulses, "high amplitude, short duration" and "low amplitude, long duration" pulses. The rationale for this combination of pulses was first described by Andreason and Evans, who suggested that high voltage might be required for initial poration of the cell membrane, while the lower voltage pulses would electrophoretically transfer the DNA into the cell [30]. This theory was later confirmed by others both *in vitro* [31] and *in vivo* [32,33], and transfection efficiency was shown to increase with the duration of the second pulse [31]. In addition to condition E, consisting of a combination of 1125 V/cm, 2 pulses, 50 μs + 275 V/cm, 8 pulses, 10 ms, we evaluated the effect of its two components separately (electroporation conditions C and D). All three conditions (C–E) significantly increased gene expression compared to intradermal injection without electroporation (Fig. 2A). However, the bimodal condition E and the low pulses alone (condition D) were significantly superior to high pulses alone (condition C), inducing gene expression 100- to 1000-fold (Fig. 2A). On the whole, among the five electroporation parameters investigated, conditions A, D, and E induced high and comparable levels of gene expression, while electroporation conditions B and C were inferior (Fig. 2A).

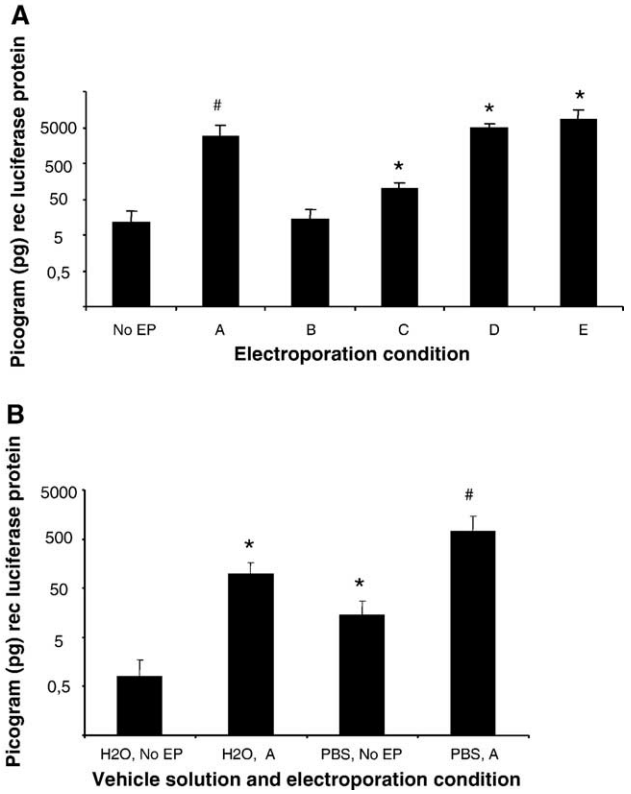

FIG. 2. Comparison of gene expression in mouse skin using different electroporation conditions and different vehicle solutions at time of DNA administration. (A) 10 μg of pVax-luc in 20 μl PBS was injected intradermally alone or in combination with one of electroporation conditions A–E (Table 1). (B) 10 μg of pVax-luc in 20 μl PBS or 20 μl sterile water was injected intradermally and electroporation (electroporation condition A, 1750 V/cm, 6 pulses, 100 μs) was applied. Skin biopsies were removed after 24 h and analyzed for luciferase protein expression. Bars represent the means ± standard deviation ($n = 6$). * and # indicate that the difference between (A) the nonelectroporated group or (B) the H_2O, No EP group and other groups was statistically significant (*$P < 0.01$, #$P < 0.05$).

Effect of the DNA Solvent on Gene Expression Level

When evaluating the effects of *in vivo* electroporation, DNA has been delivered in a variety of vehicle solutions such as sterile water [29,34], saline [24,35], or PBS [15,36–38]. We therefore compared the gene expression level after injection of luciferase DNA in PBS and sterile water. Electroporative delivery of DNA in PBS (electroporation condition A or E (data not shown)) resulted in a higher gene expression level compared to delivery of DNA in sterile water, using the same electroporation conditions (Fig. 2B). When DNA injections were delivered without electroporation the PBS solvent again resulted in an enhanced level of gene expression, compared to injection of DNA in sterile water (Fig. 2B). This difference in gene expression might be due to better stabilization of DNA in PBS, partly because of the potential of calcium ions to facilitate DNA uptake by forming calcium bridges. Furthermore the delivery of DNA in sterile water caused pronounced hemorrhage in the skin, which was evident 24 h to at least 6 days after injection (data not shown). No tissue damage (skin irritation, hemorrhage) was observed after DNA injections in PBS, neither with nor without electroporation (data not shown). Therefore we suggest an isotonic solvent for clinical use considering the higher gene expression and the pain and damaging effects on tissue caused by water.

Enhancement of $CD8^+$ T Cell Responses by *In Vivo* Electroporation

We have further compared the different electroporation conditions based on their ability to enhance PSA-specific $CD8^+$ T cell responses after intradermal administration of

the pVax-PSA plasmid. We first assessed the levels of PSA-specific CD8$^+$ T cells induced by vaccination *ex vivo* in peripheral blood of individual mice after stimulation with the psa65-73 peptide [5] or the irrelevant LCMV-derived peptide GP33 (Fig. 3A). We have previously shown that levels of PSA-specific IFNγ-producing CD8$^+$ T cells correlate with PSA-specific cytolytic reactivity [4,5].

Intradermal immunization without addition of electroporation only occasionally (1/12 mice) induced PSA-specific T cells (Fig. 3B). None of the mice immunized and electroporated under conditions A–C had detectable IFNγ-producing CD8$^+$ T cells after stimulation with the psa65-73 peptide (Fig. 3B). However, mice electroporated under condition D or E mounted a PSA-specific T cell response in peripheral blood (Fig. 3B). The levels of PSA-specific T cells induced in peripheral blood by electroporation conditions D or E were not significantly different. The PSA-specific CD8$^+$ T cell response in peripheral blood to 2 × 10 μg pVax-PSA injected intradermally and electroporated under condition E was superior to 2 × 10 μg pVax-PSA injected intramuscularly and corresponded in its magnitude to 2 × 50 μg pVax-PSA injected intramuscularly [5] (Fig. 3C and data not shown). The kinetics of the PSA-specific CD8$^+$ T cell response in peripheral blood after intradermal injection of pVax-PSA in combination with *in vivo* electroporation (Fig. 3C) correlated with the PSA-specific T cell response kinetics after intramuscular injection of 100 μg pVax-PSA [5] peaking at day 11–15 after immunization.

The PSA-specific CD8$^+$ T cell responses measured in spleen correlated with those found in peripheral blood, showing that among the intradermally immunized mice, only mice electroporated under conditions D and E mounted a strong and consistent PSA-specific CD8$^+$ T cell response, both *ex vivo* (Fig. 4A) and after *in vitro* restimulation (Fig. 4B). In contrast to the results obtained in peripheral blood, the levels of PSA-specific CD8$^+$ T cells in spleen were significantly higher in mice immunized under electroporation condition E than in mice electroporated under condition D (Figs. 4A and 4B). Mice in which DNA was delivered by electroporation condition E had between 1 and 8%

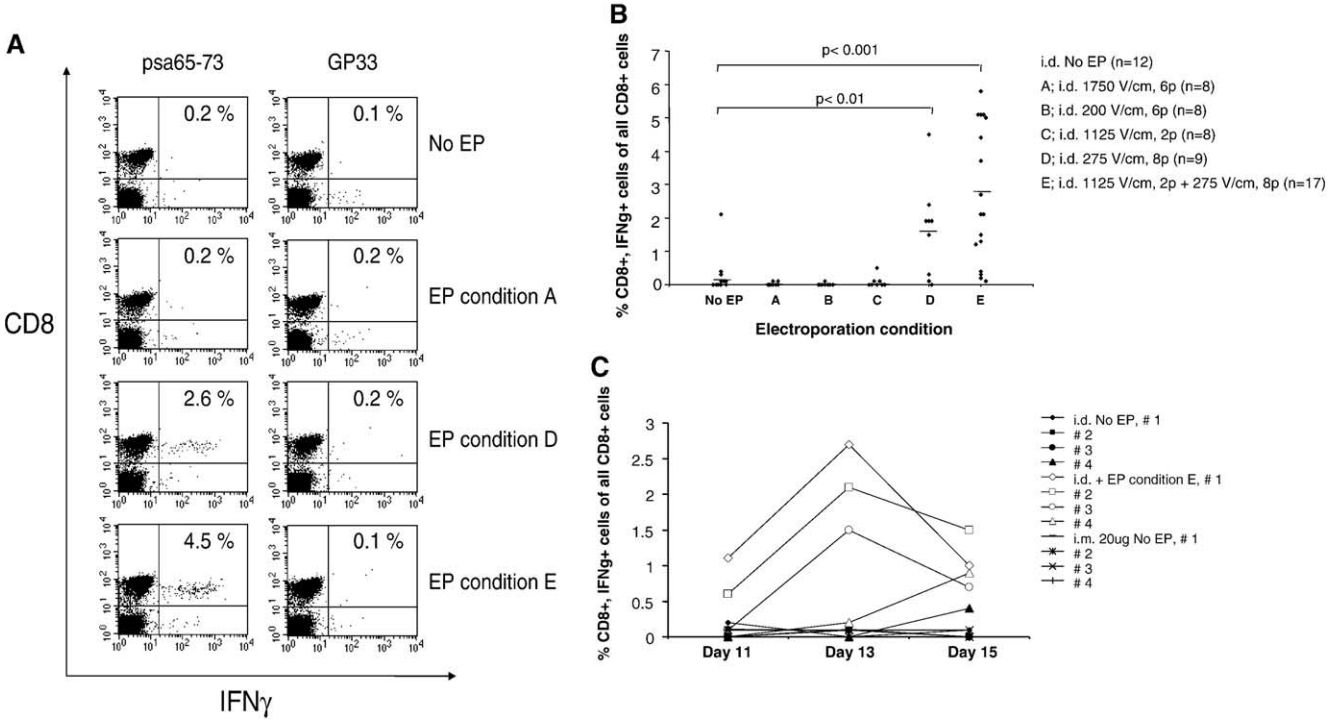

FIG. 3. Monitoring of PSA-specific CD8$^+$ T cells in peripheral blood of mice immunized under different electroporation conditions. C57Bl/6 mice were immunized once with 10 μg pVax-PSA/20 μl PBS intradermally (i.d.) on each flank with or without electroporation (EP) or intramuscularly (i.m.) in each TA muscle. Blood was collected on days 11, 13, and 15 after immunization and the effector cells were stimulated for 4 h with 100 nM PSA-derived peptide psa65-73 or a control peptide GP33. The activated CD8$^+$ T cells were quantified by intracellular cytokine staining for IFNγ and analyzed by flow cytometry. (A) Representative FACS plots showing the frequency of CD8$^+$IFNγ$^+$ T cells at day 13 after i.d. immunization. Percentages CD8$^+$IFNγ$^+$ T cells of all CD8$^+$ T cells are shown in the top right corner of each dot plot. (B) Pooled results from three independent experiments are shown. Background response (0.1–0.3%) to GP33 was subtracted. The *P* value indicates that the difference between groups was statistically significant. (C) Kinetics of PSA-specific CD8$^+$ T cells after DNA delivered i.m. or i.d. ± electroporation.

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PSA-specific CD8$^+$ T cells after 4 h of peptide stimulation (Fig. 4A), and after 5 days of *in vitro* restimulation the frequency increased to 40–90% PSA-specific CD8$^+$ T cells (Fig. 4B). This demonstrates that the PSA-specific CD8$^+$ T cells induced after intradermal electroporation proliferate in response to the immunodominant PSA peptide. Furthermore, all groups with nondetectable IFNγ-producing CD8$^+$ T cells as measured *ex vivo* (Fig. 4A) were still negative for PSA-specific IFNγ-producing CD8$^+$ T cells after *in vitro* restimulation (Fig. 4B). This shows that there were no PSA-specific CD8$^+$ T cells "below the limit of detection" in the *ex vivo* assay.

Both *ex vivo* and after *in vitro* restimulation, the IFNγ-producing CD8$^+$ T cell response in spleen to 2 × 10 μg intradermally injected pVax-PSA and electroporation under condition E was significantly higher than if the same dose was injected intramuscularly (Figs. 4A and 4B) and was equivalent to the CD8$^+$IFNγ$^+$ T cell response to 2 × 50 μg pVax-PSA injected intramuscularly.

High Gene Expression *In Vivo* is Required but Not Sufficient for Induction of CD8$^+$ T Cell Responses

As expected due to the low transfection efficacy, electroporation conditions B and C did not enhance the CD8$^+$ T cell response to the PSA DNA vaccine. Surprisingly, neither did the 1750 V/cm condition A (Figs. 4A and 4B), though it strongly enhanced the gene expression level (Fig. 2A) and previously was shown to enhance humoral immune responses [29]. Thus, although electroporation conditions A, D, and E exhibit no significant differences in induction of gene expression (Fig. 2A), only conditions D and E have the ability to induce PSA-specific CD8$^+$ T cells (Fig. 4B).

The dominant mechanism for priming of CD8$^+$ T cells by antigen-presenting cells (APCs) after DNA vaccination is still a matter of debate [39] and might even vary depending on if DNA is delivered into the muscle or the skin. Two significant studies provide strong evidence that dendritic cells (DCs) in the skin are transfected directly with DNA, when the DNA vaccine is delivered using a gene gun [40,41]. These data, however, are not undisputed [42] and moreover no data are available concerning the involvement of cross- versus direct priming using electroporative DNA delivery into the skin. If cross-presentation, however, were the principal priming mechanism in our model, then the amount of expressed antigen should have significantly affected the number of CD8$^+$ T cells. Hence one would anticipate that the three electroporation conditions A, D, and E, exhibiting similar gene expression, would perform similarly in inducing PSA-specific CD8$^+$ T cells. Since this was not the case and a 100-fold increase in gene expression after electroporation was not sufficient to induce PSA-specific CD8$^+$ T cells using condition A, our results might be more consistent with the notion that direct delivery of DNA into DCs determines the mode of antigen presentation. Nevertheless, electroporation was shown to induce cellular infiltration [17] and the different electroporation conditions might influence the quality and quantity of antigen presentation differently, subsequently affecting cross-presentation and possibly DC function. In that way electroporation may modulate the immune response indirectly in the presence of equal levels of expressed antigen. So the only certain conclusion is that additional experiments will be needed to clarify the contribution of direct versus cross-presentation in our model.

Collectively, our data suggest that efficient gene expression is not the only prerequisite for induction of CD8$^+$ T cells after intradermal vaccination. We propose that to find optimal electroporation conditions for DNA vaccines the focus needs to be on the capacity of electroporation to enhance cellular immunity, especially for cancer vaccines for which IFNγ-producing CD8$^+$ T cells are critical [43,44]. The requirements might be different for the induction of humoral immune responses, for which the induced gene expression level might be of greater importance. This could explain why the electroporation condition A, 1750 V/cm, 6 pulses, 100 μs, was sufficient to induce potent antibody levels [29], but not cellular immunity (Fig. 4B).

Interestingly, we have observed that the bimodal electroporation condition E induced significantly higher levels of PSA-specific CD8$^+$ T cells in the spleen than did the low pulses alone (condition D) (Figs. 4A and 4B). This suggests that even though the low pulses alone are potent enough to permeabilize cell membranes, transfer DNA into the cells, and trigger PSA-specific T cells, the additional high pulses in condition E provide extra adjuvant function. This adjuvant effect might consist of increased activation and migration of APCs, higher transfection of relevant APCs, or increased cellular infiltration, but this has to be evaluated further.

In conclusion, intradermal electroporative delivery of a low-dose PSA/DNA vaccine using a combination of high short and low long pulses (condition E) had the highest potency in our murine model in inducing PSA-specific CD8$^+$ T cells and thus has the potential to enhance the cellular immune response to suboptimal amounts of DNA vaccines, which might be of benefit in larger animals, and for utilization of DNA vaccines in the clinic.

MATERIAL AND METHODS

Animals. Female C57Bl/6 (H-2b) or Balb/c (H-2d) mice (6–8 weeks of age) from Taconic M&B (Bomholt, Denmark) were bred and housed at the animal facility at the Microbiology and Tumor Biology Center at the Karolinska Institute (Stockholm, Sweden). Mice were anesthetized with 4% isoflurane (Baxter Medical AB, Kista, Sweden) and maintained at 2–2.5% isoflurane in a mask during all injections and electroporations. All experiments were approved by the Swedish National Board for Laboratory Animals.

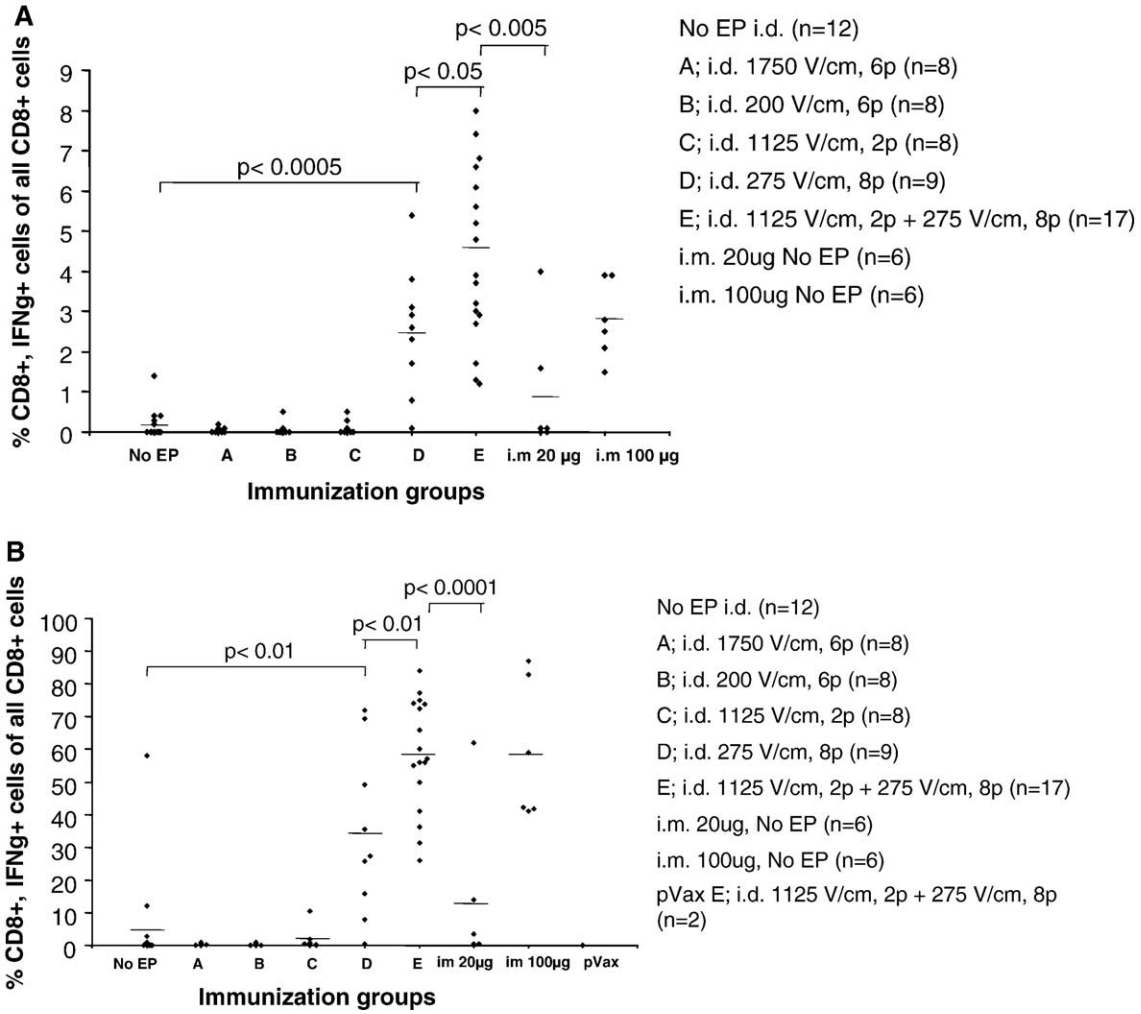

FIG. 4. PSA-specific $CD8^+$ T cells in spleens of mice immunized under different electroporation conditions. C57Bl/6 mice were immunized once with 10 μg pVax-PSA/20 μl PBS intradermally (i.d) on each flank with or without electroporation (EP) or intramuscularly (i.m) with 10 μg pVax-PSA/20 μl PBS or 50 μg pVax-PSA/50 μl PBS in each TA muscle. Sixteen days after immunization the splenocytes were restimulated for 5 days *in vitro* with 1 nM psa65-73 peptide or analyzed *ex vivo* after 4 h with 100 nM PSA-derived peptide psa65-73 or a control peptide GP33. The activated $CD8^+$ T cells were quantified by intracellular cytokine staining for IFNγ and analyzed by flow cytometry. (A) *Ex vivo* detection of PSA-specific $CD8^+$ T cells. (B) Quantification of PSA-specific $CD8^+$ T cells after *in vitro* restimulation. Pooled results from three independent experiments are shown. Background response (0.1–0.3%) to GP33 was removed. The *P* value indicates that the difference between groups was statistically significant.

Plasmids. Plasmid pVax-PSA (3977 bp) was constructed by inserting the gene coding for the full-length human PSA protein (obtained from Dr. Tim Ratliff, Washington University, St. Louis, MO, USA) into vector pVax1 (Invitrogen, Carlsbad, CA, USA). The luciferase-encoding plasmid pVax-luc, 4663 bp, was constructed by inserting the cDNA for firefly luciferase from the pGL2-Basic vector (Promega, Madison, WI, USA) into vector pVax1. Vector pVax1 contains the human cytomegalovirus immediate/early promoter and a polyadenylation signal from the bovine growth hormone gene. Plasmids were amplified in bacteria and purified using the Endotoxin Free Plasmid Purification Kit (Qiagen, Hilden, Germany).

DNA injections and in vivo electroporation. Intramuscular injections were delivered bilaterally into both tibialis anterior muscles with 10 μg DNA/20 μl PBS or 50 μg DNA/50 μl PBS. Intradermal injections with 10 μg DNA/20 μl PBS or sterile H_2O were made on each flank, near the base of the tail, using a 29-gauge insulin-grade syringe (Micro-Fine U-100, BD Consumer Healthcare, Franklin Lakes, NJ, USA). Immediately after intradermal DNA administration, a needle array electrode was placed over the raised skin area of injection and pulses of different voltages were applied or not (nonelectroporated control). The needle array electrode consisted of two parallel rows of four 2-mm pins (1.5 × 4-mm gaps) (Cyto Pulse Sciences, Inc., Glen Burnie, MD, USA). Electroporation was performed using the PA-4000S–Advanced PulseAgile Rectangular Wave Electroporation System and software (Cyto Pulse Sciences, Inc.). Electroporation pulses were monitored and stored using a PCS64i digital oscilloscope (Velleman Components N.V., Belgium).

Luciferase assay. Balb/c mice were euthanized 24 h after DNA administration and skin biopsies removed. Skin biopsies were stored at −80°C until analysis. The skin was homogenized in 500 μl of lysis buffer (BD Biosciences, PharMingen, San Diego, CA, USA), vortexed at room

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temperature for 20 min, and centrifuged. Luciferase activity in cell lysates was measured using the Enhanced Luciferase Assay kit (BD Biosciences, PharMingen) on a Wallac Victor Multilabel Counter (Perkin–Elmer, Life Sciences, Upplands Väsby, Sweden). The bioluminescence of a 50-μl aliquot of each sample was counted for 10 s and recorded as counts per second (cps). Using the Enhanced Luciferase Assay kit, the specific activity of firefly luciferase protein (BD Biosciences, PharMingen) on this luminometer was 170,000 cps/ng luciferase protein. Background luminescence (skin injected intradermally with empty vector, pVax, and electroporated) was subtracted from all samples.

Lymphocyte preparation and in vitro restimulation. C57Bl/6 mice were bled at three different time points between day 11 and day 16 after a single immunization with the pVax-PSA plasmid. One hundred microliters of blood from the tail vein was mixed with 100 μl of CPD-A anticoagulant (Sigma, St. Louis, MO, USA). The erythrocytes were removed using the Ammonium Chloride Lysing Reagent (BD Biosciences, PharMingen) and after washes in handling medium (DMEM supplemented with 10 mM Hepes, 5×10^{-5} M 2-mercaptoethanol, 25 μg/ml gentamicin, and 1% FCS) the cells were resuspended in complete medium (DMEM handling medium + 2 mM L-glutamine, 1% nonessential amino acids, and 5% FCS) and used for *ex vivo* intracellular staining. For analysis of CTL response in the spleen, mice were euthanized and spleens harvested 16–17 days after immunization. Single-cell suspensions were obtained by homogenizing spleens and passing cells through a 70-μm cell strainer. The red blood cells were lysed by Ammonium Chloride Lysing Reagent. The splenocytes were used directly *ex vivo* or set up for 5 days of restimulation with 1 nM synthetic peptide psa65-73 (HCIRNKSVI) (ProImmune, Oxford, UK), in complete medium supplemented with 20 IU/ml human recombinant IL-2 (Proleukin, Chiron Corp., Emeryville, CA, USA) at 2×10^6 splenocytes/ml. The peptide psa65-73 represents an immunodominant H-2D^b-restricted CTL epitope of human PSA [5].

Intracellular cytokine staining for IFNγ. Lymphocytes from blood or spleen were used directly after isolation (*ex vivo*) or after 5 days of restimulation. Briefly, lymphocytes (1×10^6/well) were cultured for 4 h in U-bottom 96-well plates with H-2D^b-restricted synthetic peptides, psa65-73, or the irrelevant LCMV-derived peptide GP33 (KAVYNFATC$_{33–41}$) (ProImmune). After 2 h of incubation at 37°C GolgiPlug Reagent (BD Biosciences, PharMingen) was added to the cells and incubation continued for another 2 h. The lymphocytes were then stained for the surface marker CD8 (rat IgG2a–FITC labeled anti-mouse CD8a; Pharmingen), fixed, and permeabilized with a CytoFix/CytoPerm Plus kit (PharMingen) according to the manufacturer=s instructions and then stained for intracellular IFNγ (rat IgG1–PE labeled anti-mouse IFNγ; PharMingen). Purified rat IgG (Sigma–Aldrich, Stockholm, Sweden) was added to both staining steps to block nonspecific binding. Samples were analyzed using a FACSCalibur flow cytometer (Becton–Dickinson) and CELLQuest software (Becton–Dickinson). Proper compensation during data collection was set using lymphocytes stimulated with PMA and ionomycin (Sigma).

Statistics. Comparison of data from different groups was performed using a two-tailed Student *t* test with a significance level of at least $P \leq 0.05$.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2005.08.005.

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