Ear Pinna: A Privileged DNA Electroporation Site for Inducing Strong Th1 Immune Responses

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Abstract: DNA vaccination appears a very attractive approach for inducing immune responses towards the encoded antigen, but studies in large animals and in humans revealed weaknesses of such responses. In this study, we evaluated a new approach based on a new device combining DNA vaccination with electroporation (EP) at the ear pinna site. Under optimal EP conditions, the expression of the DNA encoded antigen and the induced immune responses were considerably increased. Very interestingly, DNA vaccination using EP at the ear pinna induced much stronger cellular immune responses than at the flank skin although antigen expression was similar at both sites. As compared to vaccination at the ear pinna without EP, IFN-γ but not IL-4 production by splenocytes from immunized mice was significantly enhanced. In contrast, IL-4 but not IFN-γ production was increased by EP at the flank skin. The vaccination site of the ear pinna combined with EP route even provided therapeutic effects in a mouse tumor model.

In conclusion, this study highlights the ear pinna as a privileged site for the induction of strong Th1 polarized cellular immunity against a defined antigen when combining DNA vaccination with EP.

Keywords: Antigen expression, immunization, electroporation, vaccination, tumor therapy.

INTRODUCTION

Genetic immunization using naked DNA [1, 2] offers considerable advantages over conventional vaccines:

(i) high stability of plasmid DNA and relative temperature insensitivity making them highly suitable for mass production and easy transportation in both industrialized and developing countries,

(ii) low manufacturing costs,

(iii) lack of infection risks that are associated with attenuated viral vaccines,

(iv) capacity to target multiple antigens (Ags) on one plasmid by inserting several open reading frames from one or more genes and

(v) absence of intrinsic immunogenicity allowing successful boost after DNA vaccination without producing a heterologous immune response.

It has been shown to be effective and safe for inducing protective immunity in preclinical models of infectious diseases [3-6], cancer [7-10] and autoimmune diseases [11-13].

After over a decade of active research, DNA vaccines are now reaching the commercial market with recent approvals of West Nil virus in horses [14], infectious hematopoetic necrosis virus in salmon [15] and melanoma in dogs [16-18]. However, despite more than 200 clinical trials to date [19], no plasmid DNA products have received approval by FDA for use in humans. There have been, however, numerous preclinical and clinical studies for most types of cancer [20].

One important problem for all DNA vaccines relates to the translation of therapy data from small animals to large animals [21] and to humans [22-26]. The efficacy in humans has been disappointing [24, 26, 27], partly due to the difficulties in scaling up DNA vaccine dose and injection volume for human application [28]. Low vaccine dose results in poor Ag expression and reduced immunogenicity [29].

In contrast to conventional vaccines, DNA vaccine must be delivered intracellularly in order to elicit production of the antigen with minimal toxic effects. Cellular uptake of DNA appears then to be a significant limiting factor for transfection efficiency *in vivo*. To overcome this problem, different delivery approaches have been used to enhance the level of transfection and consequently of antigen expression from plasmids [30]. Instead of injection of naked DNA plasmids [31], they can be combined with liposomes [30] or applied by gene guns [32], by microseeding or puncture [33], by micromechanical disruption methods [34, 35]. Viral vector systems can also be used [36, 37]. All these methods were capable to improve the induction of Ag specific immunity [38-42].

In this study, we employed electroporation (EP) because during the past few years great potential of this new technol-

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ogy was demonstrated [43, 44]. It has been evaluated in studies involving delivery of plasmid DNA *in vivo* to different types of tissues. The transfection efficiency was 10-1000 folds greater than that of naked DNA injection with remarkable reduction of an inter-animal variability [45]. EP is based on the creation of pores in cell membranes through the application of electrical pulses for allowing the cellular entry of macromolecules such as DNA [46]. The exact mechanism remains elusive. Both active [47-49] and passive mechanism involving simple diffusion of DNA through the membrane [50] have been proposed. The vaccination efficiency of EP is also due to an induced inflammation process which leads to the recruitment of DCs, macrophages and lymphocytes to the injection site [51, 52]. Recent technological developments in the fields of EP involve devices that are capable of delivering series of pulses controlling the pulse length, the electric field strength and various other parameters.

Intramuscular (i.m.) [53] and intranasal injections are commonly used routes of administration for DNA vaccines [54-57]. But DNA immunization has also been effective in eliciting an immune response [1, 2] with various other administration routes such as intraperitoneal, intravenous, oral, ocular, and transdermal/topical administration [58].

Methods based on electric pulse for increasing DNA delivery into cells have mostly been applied to skin. This is an attractive site for DNA vaccination since this is the most accessible somatic tissue for gene transfer and it can be monitored conveniently. More importantly, for vaccination purposes, it is an active immune surveillance tissue which is especially rich in specialized cells enhancing immune responses [59] such as dendritic cells (DCs): epidermal Langerhan´s cells (LCs) and dermal DCs [60]. By targeting the skin, DNA immunization attempts to produce an immunologically efficacious response [1, 61]. Upon skin application, DNA vaccines allow for protein expression in a variety of cells, including keratinocytes, LCs, and dermal DCs, which are located in the two main areas of the skin, the epidermis and the dermis [61]. After maturation, DCs can migrate to local lymph nodes where presentation of antigens to T cells can occur and initiate a variety of immunological responses [62, 63]. In mouse models, intradermal (i.d.) injection is usually applied for vaccination purposes to abdominal or flank skin. Interestingly, in our previous studies with the highly metastatic lymphoma ESb tumor, it was shown that the ear pinna is a privileged site (compared to subcutaneous (s.c.) tumor inoculation) for the induction of antitumor immunity, preventing the outgrowth of an otherwise lethal dose of tumor cells [64]. Further studies corroborated the superiority of the ear pinna in comparison to i.m. and i.d. immunization sites also for DNA and RNA based vaccines [65, 66].

In this study, we compared the efficiency of EP combined with DNA vaccination at different immunization sites in term of antigen expression and of immune responses against the plasmid antigen. After having optimized the conditions of DNA EP, we demonstrate that intradermal DNA injection in combination with EP at the ear pinna but not at the flank skin of mice induces Th1 polarized immune responses which exert strong anti-tumor effects.

MATERIALS AND METHODS

Cells. All cell lines were obtained from the tumor cell bank of the German Cancer Research Center (DKFZ, Heidelberg, Germany). Cell culture media were supplemented with 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin (all purchased from Gibco Invitrogen, Karlsruhe, Germany). P815, a mastocytoma, and its lacZ-transfected variant P13.1 were cultivated in RPMI-1640 medium with supplements (as indicated above) as well as β-mercaptoethanol at a final concentration of 50 μM. The medium for lacZ transfected cells was supplemented with 200 μg/mL G418 to maintain stable lacZ gene expression. DA3, a mammary carcinoma cell line, and DA3-EpCAM (DA3 transfected with human EpCAM gene) were cultivated in RPMI-1640 medium complemented with supplements (as indicated above) and with β-mecarptoethanol (50 μM).

Mouse tumor models. Female DBA/2 mice were purchased from Charles River WIGA (Sulzfeld, Germany) and used at 6-8 weeks of age. These mice were used for the optimization of the voltage conditions, the analysis of antigen expression and the *ex vivo* analysis of the cytotoxic response against the target tumor cells.

Therapy experiments were performed with female Balb/c mice which were also obtained from Charles River WIGA. During tumor challenge, mice were injected s.c. with 1×10^7 DA3-EpCAM or DA3 tumor cells into the flank. For therapeutic immunization DNA plasmids were injected 1, 2, 3 and 4 weeks after tumor inoculation. All mice were monitored twice a week for tumor development and were killed when the mean tumor diameter reached 20 mm, in accordance with the guidelines of the animal house facility of our institute.

DNA plasmids. The plasmid coding for the luciferase under the control of a CMV promoter (CMV-luciferase) was kindly provided by Dr. Daniel Scherman (INSERM, Institut National de la Santé et de la Recherche Médicale, Paris, France). The plasmid pCMV SPORT-βgal was purchased from Invitrogen (Karlsruhe, Germany). A plasmid encoding 1.5-kb of the human EpCAM gene (extracellular and transmembrane domain) was kindly provided by Dr. Frank Momburg (DKFZ, German Cancer Research Center, Heidelberg, Germany). To prepare the plasmid (leading to EpCAM expression under control of a CMV promoter), the EpCAM DNA fragment was cloned into pTandem-1 (Novagen, Darmstadt, Germany) to prepare CMV-EpCAM. All plasmids were grown in Escherichia coli (Top 10) and purified using the Qiagen Endo-free Gega Prep Kit (Qiagen, Hilden, Germany). The DNA stocks having a 260:280 ratio from 1.8 to 2.0, were prepared in Endo-free water (B. Braun Melsungen AG, Melsungen, Germany) and stored at -20°C. For *in vivo* injections, the DNA was diluted and adjusted to 0.5 mg/mL or 1 mg/mL in Endo-free PBS (PromoCell, Heidelberg, Germany) just before use.

DNA immunizations. Mice were anesthetized by intraperitoneal (i.p.) injection of Rompun (4.5mg/kg BW) (Bayer, Leverkusen, Germany) and Ketanest (45 mg/kg BW) (Bayer, Leverkusen, Germany). DNA with the amount of 25 μg/50 μL (for gene expression and immune responses) or 50 μg/50 μL (for the therapeutic tumor model), dissolved in PBS, was injected into ear pinna (i.e.) or shaved flank skin by using a

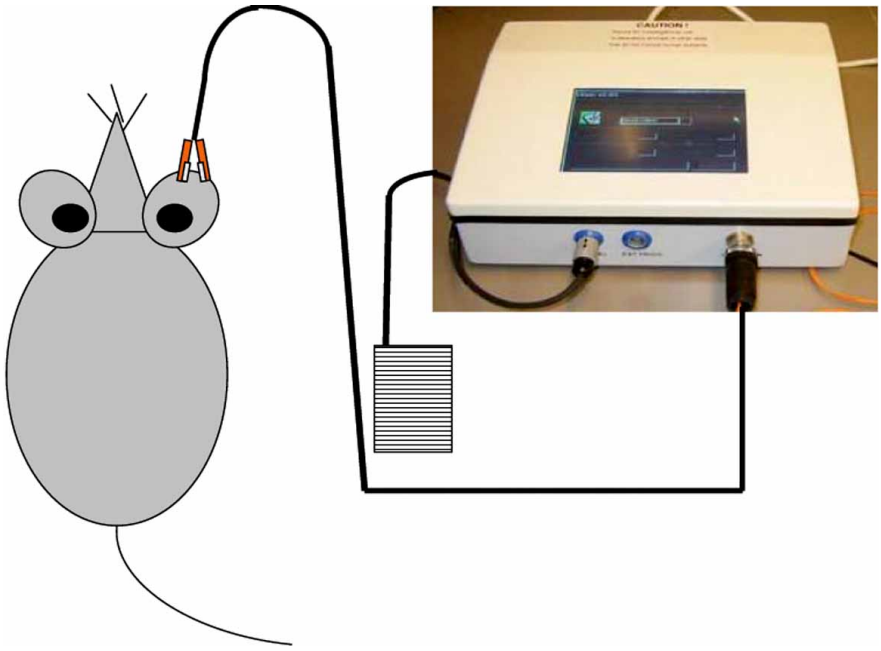

Fig. (1). EP device.
The device ELGEN1000 EP-based DNA delivery system (Inovio) was used through all this study. It is composed of two parts: the pulses generator and the caliper electrodes. A pedal allows an easy control of the pulse generator by a foot. After DNA injection with 50 μL volume, Ultrasound gel was applied to the local injection site. Caliper electrode was placed at the injection site, with 1 mm distance between the two electrodes. EP was performed by pressing the pedal followed by a triple beeps which indicated successful EP. Electrodes are connected to a pulse stimulator which generates the electric signals necessary to enhance the intradermal delivery of the vaccine using the optimal parameters suggested by the Inovio company and indicated in the Table **1**.

BD Insulin syringe (29-gauge, BD Biosciences, Heidelberg, Germany).

Immediately after DNA application, EP was performed using the ELGEN1000 DNA delivery system (Inovio, San Diego, USA) connected to caliper electrodes (as shown in Fig. **1**). Various voltages (from 40 to 120 V/cm) and other optimal parameters as timing and sequence of pulses (see Table **1**) which are important for effective DNA delivery were applied as suggested by the provider of the device.

Table 1. Electroporation Parameters

Time :	20 ms
Volt :	optimized in this study
Number of sequences :	5
Number of trains :	1
Pulse Delay :	100 ms
Train Delay :	100 ms
Current Limit :	1000 mA

In vivo imaging of mice. The IVIS100 imaging system (Xenogen, Alameda, USA) was used for imaging mice. D-luciferin potassium salt (SYNCHEM, Huddersfield, UK), the firefly luciferase substrate, was diluted to a final concentration of 30 mg/mL in PBS. Imaging of mice was made 5 min after the i.p. injection of 100 μL D-luciferin solution. Bioluminescent color images were acquired by Living Image 2.50 software overlay (Xenogen, Alameda, USA) and analyzed by Igor Pro 4.09A software. Bioluminescence signals are expressed in units of photons per second per cubic centimeter per steradian (p/sec/cm^2/sr).

ELISA. Blood samples were collected from the retro-orbital plexus of mice 2 weeks after DNA immunization. Plasma were prepared and stored at -20°C. Titers of anti-β-gal antibodies were assessed by ELISA. Briefly, 96-well plates were coated overnight with purified β-gal protein (Sigma, Taufkirchen, Germany) with PBS, the plates were then blocked with 2% milk in PBS for 30 min at 37°C. Plasma samples were serially diluted in this buffer and applied to the plates for 2 h at room temperature. After further washing steps with 0.05% Tween-20/PBS, bound antibodies were detected with a peroxidase-conjugated goat anti-mouse IgG+M immunoglobulin (1:5000, Dianova, Hamburg, Germany). The plates were then washed and finally developed with TMB substrate (KPL, Gaithersburg, USA) and optical density was then read at 450 nm. A monoclonal mouse anti-β-gal antibody (Ab) (Sigma, Munich, Germany) was used as a positive control. Titers were calculated by the formula from the Ab curves when the OD at 450 nm was equal to 0.5 OD unit. Levels of IFN-γ and IL-4 were also measured by ELISA using the High Sensitivity ELISA Ready-SET-Go Kits (eBioscience, San Diego, Germany) according to manufacturer´s instructions. TGF-β was also quantified using the Duo Set ELISA Development kit mouse plasma TGF-β1 (R&D Systems, Wiesbaden-Nordenstadt, Germany).

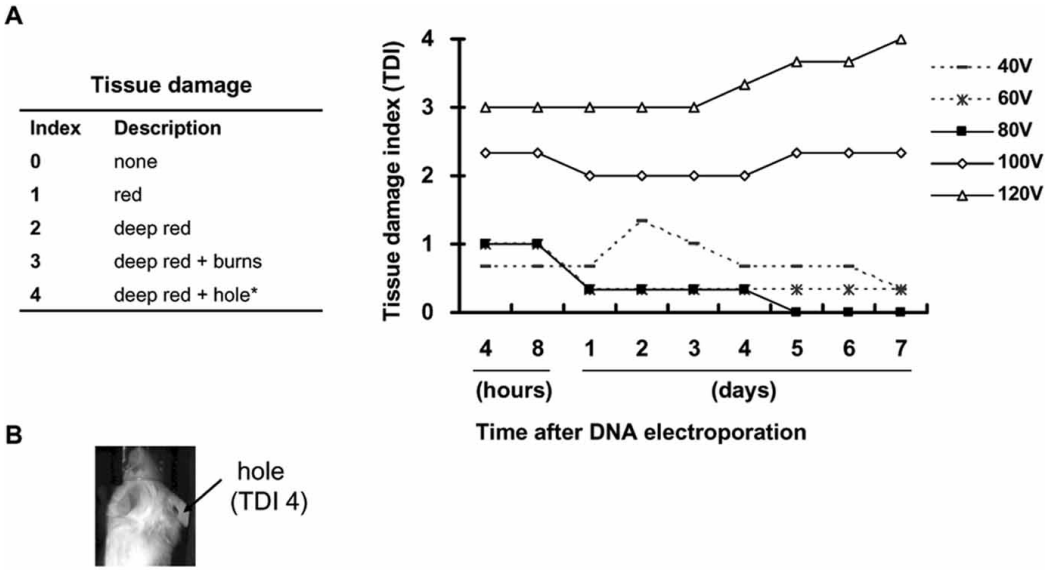

Fig. (2). Analysis of the local tissue damage induced by EP using different voltages.
A. Tissue damage in mice after EP. DNA was applied into the ear pinna of DBA/2 mice and EPs were performed using the EP device which is described in Fig. (**1**). Different voltages varying from 40 to 120V were used. The damage level was quantified using indexes evaluating local tissue damages at the ear pinna site as defined in the left table. It was monitored during 7 days following EP. n=3 in each group.
B. A representative mouse with a burned hole in the ear after EP by 120 V. EP in the ear using a voltage of 120 V induced high tissue damage. This can be observed by the apparition at day 7 of a burned hole in the ear pinna of the mouse as shown on the photography.

In vitro re-stimulation and cytotoxicity assays. Two weeks after lacZ DNA immunization, mice were killed. Splenocytes were prepared and then re-stimulated *in vitro* for 5 days in RPMI medium containing 10% FCS and 0.5 μg/ml of the synthetic nonamer TPHPARIGL in 5×10^6/mL. This peptide represents the naturally processed H-2Ld-restricted CD8 T cell epitope of β-gal spanning amino acids 876–884. This peptide was synthesized by R Pipkorn (DKFZ, Heidelberg, Germany). Supernatants of these cultures were collected on day 2 or on day 5 for the determination respectively of IFN-γ and IL-4 by ELISA. Re-stimulated spleen cells were used as the effector cells to test their cytotoxic activity in a standard 4 h ^{51}Cr release assay at different effector : target ratios against 5×10^3 ^{51}Cr-labeled P13.1 ($lacZ^+$) and P815 ($lacZ^-$) cells, respectively as described [67, 68]. The amount of ^{51}Cr released was measured in a gamma counter and the percentage of lysis was calculated from the formula:

((experimental cpm - spontaneous cpm)/(maximum cpm - spontaneous cpm))×100. Spontaneous release was always below 30%.

STATISTICS

The statistical significance of results from experimental groups in comparison to control groups was determined by the Student's t test unless otherwise specified. All tests were two-tailed and $p<0.05$ was considered to be statistically significant.

RESULTS

Optimization of DNA EP to Skin

All the EPs were performed using the ELGEN1000 DNA delivery system which is represented in Fig. (**1**) and using the parameters summarized in Table **1**. We performed a panel of experiments by testing different voltages (40, 60, 80, 100, 120 V/cm) for DNA EP at the site of the ear pinna. First, we monitored the tissue damage at the site which has been electroporated. In order to evaluate such effects in a quantitative manner, we defined the tissue damage index (TDI) as indicated in Fig. (**2A**). EP of DNA using a low voltage (80 V or lower) induced a transient and mild tissue inflammation corresponding to redness in ears which vanished within 1 week. The use of higher voltages (as 100 V and 120 V) induced more severe irreversible tissue damages such as deep red spots and burns at the site of EP persisting during 3 days after EP. Thirty percent or 100% of the mice showed a punctured skin (Fig. **2B**) at the site of EP when using 100 V or 120 V respectively (data not shown). Serious tissue damage was also seen when EP was performed at the flank skin when using 100 V and 120 V (data not shown). Tissue damage was still visible 1 year after EP (data not shown). We conclude that EP should be performed using a voltage lower than 100 V.

We next analyzed the effect of EP on Ag expression following DNA plasmid injection. Mice were injected with a plasmid CMV-luciferase coding for the firefly luciferase at the ear pinna (i.e.) or at the flank skin (i.d.) in combination with EP (right) or not (left) as described in the schema of the Fig. (**3A**). The expression of the firefly luciferase as indication of the antigen expression was monitored during 1 week by *in vivo* imaging (Fig. **3B**). Quantitative analysis of the measured signal showed that Ag expression improved by a factor of 10 in the ear pinna and by a factor of 100 in the flank skin after DNA application with EP when the three voltages of 80, 100 and 120 V were used. In contrast, the use of 40 and 60 V during EP led to a lower improvement of antigen expression at the flank skin and no improvement at

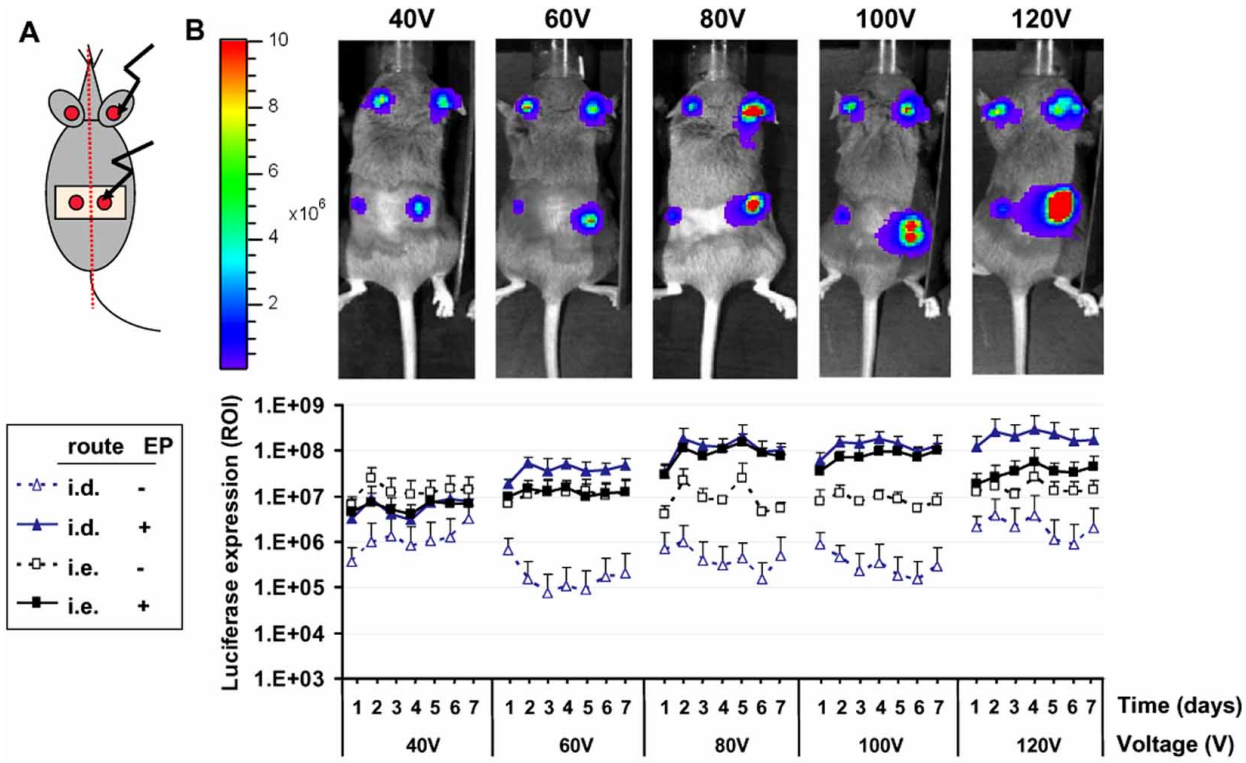

Fig. (3). Ag expression after DNA EP using different voltages.

A. DNA transfer into ear and flank skin. The plasmid CMV-luciferase encoding the firefly luciferase gene under the control of the CMV promoter was injected (25 μg/50 μL) intradermally to the ear pinna and flank skin. And EP was performed (right side of the mouse) or not (left side).

B. *In vivo* luciferase expression. Using the experimental design indicated in A, DNA was applied to DBA/2 mice and electroporated using different voltages comprised between 40 V and 120 V. Bioluminescent signal was monitored over 7 days with the IVIS100 imaging system using an exposure time of 10 s. One representative mouse of each group (n=3 for each) is shown. Luciferase signal was quantified in pseudo-photon unit (p/sec/cm^2/sr) as described in material and methods. One representative experiment out of three is shown.

the ear pinna. These results showed that DNA EP using a voltage of 80, 100 or 120 V efficiently improved Ag expression in the skin after DNA injection. We decided to use 80 V for all the further EPs.

Long-term Ag Expression after DNA Injection and EP

To analyze long-term effects on Ag expression after DNA injection combined with EP, mice were immunized with the plasmid CMV-luciferase. Left ear and flank skin were not electroporated whereas the right ones were (as indicated in Fig. **3A**). Luciferase activity was monitored over 350 days after EP. We observed that EP improved the Ag expression at both application sites. An increase of antigen expression corresponding to factors between 50 and 500 was observed when vaccination was performed at the flank site. In contrast, the antigen expression was only increased by factors between 5 and 20 when the ear pinna was used as vaccination site (Fig. **4**). Although we observed that the signals were 5 to 50 times higher at the site of the ear pinna as compared to the flank skin without EP, similar levels of Ag expression were obtained for both routes after DNA EP (Fig. **4**).

High levels of Ag expression were seen for 2 weeks after DNA EP at both sites. After 4 weeks, Ag expression decreased by a factor of 10 to 100, but maintained at a low but stable level during 350 days (Fig. **4**). These results reveal that EP can be used to increase Ag expression for long terms.

Humoral and Cellular Immune Responses after EP

We then analyzed if EP can improve immune responses against the target Ag encoded by the plasmid *in vivo*. For these investigations, we used a DNA plasmid with the LacZ gene coding for bacterial beta-galactosidase (β-gal). We applied this plasmid either to the ear pinna (i.e.) or to the flank skin (i.d.). Humoral responses were analyzed via ELISA, testing the levels of antibodies specific for the β-gal protein in the plasma of mice. EP induced an increase of anti-β-gal antibody levels by a factor 46 when immunization was performed at the flank skin. In contrast, the increase of the antibody titer towards β-gal was only increased by a factor of 5 when the ear pinna was used (Fig. **5A**). However, a higher Ab titer was induced by DNA immunization at the ear pinna in comparison to the flank skin, no matter if EP was performed or not.

Although enhancement of immune responses by EP is probably also due to an increase of antigen expression by other cells such as keratinocytes, we believe that DCs play a major role [94] in the induction of a strong immune response at the ear pinna site either by cross-priming or by direct priming. DCs very probably migrate to the draining lymph nodes and trigger the immune response. The role of DCs has been demonstrated during EP-based vaccination. DCs from draining lymph nodes were shown to contain DNA originating from the injection site [103].

Our data show also that the ear pinna route skews the immune response towards Th1 whereas the application of DNA to the skin from the flank skews the immune response towards Th2. Gene gun immunization has been shown to skew immune responses towards Th2. In contrast, EP is more efficient for induction of Th1 immune responses [104-106]. Consequently, the use of EP as a method of antigen delivery and ear pinna as the site of DNA application might explain the Th1 polarization of the immune response. Further studies need to be performed in order to identify the cells that are involved and understand why the use of this route leads to the generation of such a strong Th1 type cellular immune response.

Mechanisms of tolerance and immune escape are drawbacks to cancer vaccination. TGF-β, one of the immunosuppressive cytokine which is often secreted in large amounts by malignant cells such as the DA3 tumor cell line used in this study. This molecule acts on nontransformed cells present in the tumor mass as well as on distal cells in the host to suppress antitumor immune responses creating an environment of immune tolerance [107]. A strong immune reactivity is then required to reverse the immunosuppressive effects induced by this cytokine. We observed in this study that EP after DNA injection also down-regulated peripheral TGF-β levels in tumor-bearing mice. It is not clear how the down-regulation was achieved. It could be the outcome from the inhibition of tumor growth by DNA EP, because less tumor derived suppressive factors might be secreted by smaller tumors.

The goal of active therapeutic vaccination against cancer is to induce a strong effective immunity against tumor cells and to establish immunological memory that is able to maintain continuous surveillance against emergent cancer cells. EP was found to improve the kinetics of immune responses by requiring less time than conventional injection to reach a maximal immune response [108, 109] and favoring the induction of long-term memory [110] but also the consistency of the immune responses induced when compared with injection without EP. Because these initial results seem promising, vaccination trials based on ear pinna immunization with autologous irradiated tumor cells electroporated with DNA should be considered for the future. Efficacy and tolerability of EP was demonstrated in phase I clinical trials performed in collaboration with some companies [111] where in some studies the EP technology was used for intratumoral delivery of plasmid DNA [112]. The increase of knowledge about immune responses generated from the site of the ear pinna and the potential of DNA EP delivery for generating strong cellular immunity give hope to the field of DNA-based cancer therapy.

In conclusion, the results presented here show that the ear pinna is a privileged site for DNA EP. Compared to traditional DNA application to the flank skin in mice, the strategy of DNA EP at the ear pinna induced similar Ag expression but led to the induction of very strong cellular immunity toward the Ag encoded by the plasmid with a strong Th1 polarization. By this method, therapeutic anti-tumor effects were also achieved in a mouse tumor model. Such increases in DNA vaccine potency provide encouragement that such an immunization could be tested in large animals.

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