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Skin-specific promoters for genetic immunisation by DNA electroporation

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ABSTRACT

This study aimed at restricting expression of DNA vaccine to specific cell types by using skin-specific promoters (i) to contribute to the understanding of the mechanism of intradermal DNA vaccines and (ii) to address safety concerns associated with DNA vaccines. The expression and immune response after delivery of plasmids encoding luciferase by intradermal DNA electroporation were assessed. Two ubiquitous promoters, CMV and CAG, and three tissue-specific promoters were studied. Keratin 14 promoter restricts gene expression to keratinocytes of the epidermal basal layer, CD11c promoter to dendritic cells and fascin promoter only to mature dendritic cells. The use of plasmids with tissue-specific promoter resulted in significant, but very low protein expression, as compared to that obtained with ubiquitous promoter plasmids. Immunisation with both ubiquitous promoter plasmids elicited humoral and cellular anti-luciferase immune response. No immune response was observed after delivery of CD11c plasmid while fascin and keratin 14 plasmids induced IFN-gamma response suggesting that the targeting of skin-specific cells could be a suitable approach but only for the treatment of pathologies or pathogens requiring mainly cellular and not humoral immune response.

1. Introduction

Intradermal DNA electroporation is one of the most efficient non-viral methods for the delivery of gene into the skin. Previous studies have demonstrated that a combination of a short high-voltage pulse (HV) and a long duration low-voltage pulse (LV) was efficient for DNA electroporation in the skin [1,2] and that intradermal electroporation was suitable to deliver DNA vaccine when a Th1-oriented response is desired [3].

Various cell types of the skin are involved in the development of immune response. Langerhans cells (LC) are located in the suprabasal portion of the epidermis where they are considered immature. Although they represent less than 5% of the total cells in the epidermis, they cover almost 25% of the skin area. Because of their long dendrites and their horizontal orientation, LC create an almost continuous network that enables them to capture most antigens that enter through the skin. Dermal antigen-presenting cells (APC) that are present in higher number than LC are less characterized [4,5]. LC and dermal APC differ in their anatomic location, antigen recognition, processing machinery and migratory capacity. The relative contribution of the different cutaneous dendritic cell subtypes to particular immune responses is not yet fully understood [6]. In addition to their role in the maintenance of the keratin barrier, keratinocytes are biochemically active [7]. They produce a wide range of cytokines, chemokines and anti-microbial peptides upon activation by various stimuli through pattern recognition receptors, such as the toll-like receptors (TLR). These cytokines shape the local microenvironment to help maintain the appropriate balance of skin immune responses and stimulate the maturation and migration of LC [8].

Delivery of DNA into the skin could induce direct-presentation of the encoded antigen by APC or cross-presentation after uptake by keratinocytes [9]. However, the involvement of each mechanism in the development of the response is still controversial.

The use of tissue-specific promoters is an attractive approach in delivering genes to targeted cell types [10]. The optimisation of targeted expression systems is an interesting strategy for gene therapy and vaccine due to safety consideration. Indeed the use of CMV expression signals on vaccine plasmid may induce recombination events and form new chimera of CMV which infects a large part of the population [11]. Moreover specific-promoter plasmids are advantageous tools to study the role of the different cell types of the skin.

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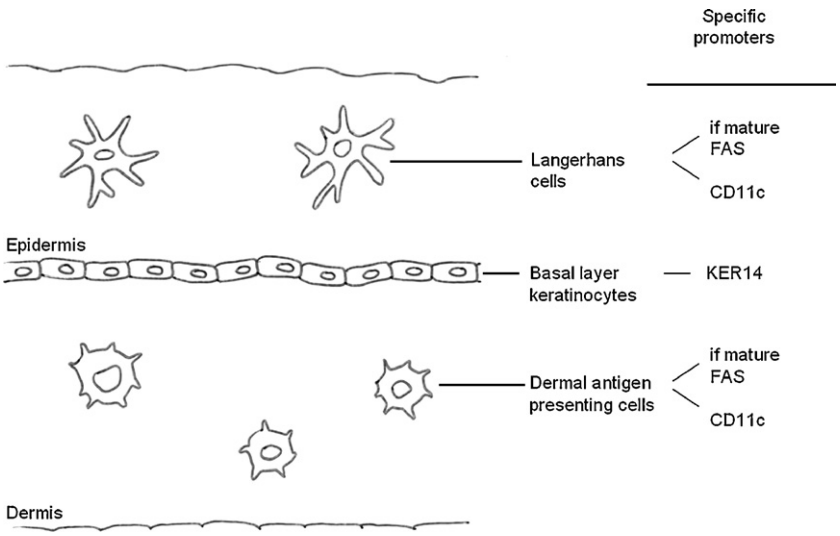

Fig. 1. Specificity of expression of promoter specific plasmids in skin cells.

This study aimed at restricting expression of DNA vaccine to specific cell types by using skin-specific promoters (i) to contribute to the understanding of the mechanism of intradermal DNA vaccines and (ii) to address safety concerns associated with DNA vaccines [11].

We specifically targeted keratinocytes of the epidermal basal layer or dendritic cells. First, we determined the expression of the plasmids with ubiquitous or tissue-specific promoters after electroporation into the skin, the ear pinna, which contains more dendritic cells than the skin [12], and the muscle. Then, we immunised mice with these plasmids and we assessed both humoral and cell-mediated immune response.

Two ubiquitous promoters and three tissue-specific promoters were assessed (Fig. 1), both encoding the luciferase, a weakly immunogenic protein inducing cellular and humoral immune response after electroporation [3]:

(i) The cytomegalovirus (CMV) promoter, which is commonly used to drive expression of the transgene because of its strong, constitutive activity in a wide range of cell types.
(ii) The CMV–actin–globin (CAG) promoter, which is a composite promoter that combines the human cytomegalovirus immediate-early enhancer and a modified chicken beta-actin promoter and first intron. The CAG promoter is a very strong and ubiquitous promoter.
(iii) The keratin 14 promoter (KER14), which restricts gene expression to the keratinocytes of the epidermis basal layer [10,13].
(iv) The fascin promoter (FAS), which restricts gene expression to the mature dendritic cells. The fascin protein is expressed by dendritic cells and represents an important structural component, required for the formation of dendrites. Fascin is not produced by immature dendritic cells but is strongly upregulated during maturation [14,15].
(v) Finally, the CD11c promoter, which dictates the tissue-specific expression of the encoded gene to dendritic cells.

2. Material and methods

2.1. Plasmid DNA

Plasmids were constructed from the pGL3 Luciferase Reporter Vector (Promega Benelux, Leiden, Netherlands). pGL3CAGLUC and pGL3CMVLUC plasmids were generously provided by E. Zeira, Jerusalem and pGL3CD11cLUC plasmid was kindly provided by P. Fournier, Heidelberg. For the construction of pGL3FASLUC, fascin promoter PCR fragment from B16 was cloned into pCR2.1 vector by TA cloning (Invitrogen, France). T7 promoter and M13 reverse primers were used to amplify the sequence which was then digested with SpeI/XhoI and cloned into a NheI/XhoI opened pGL3LUC vector. The same method led up to pGL3KER14LUC from genomic DNA of A375M cells [13]. Inserts were sequenced to verify proper orientation and fidelity of PCR. In pGL3SAFLUC plasmid, the fascin promoter was inverted. This non-functional plasmid has been used as a control. The sequence of each promoter is available as supplementary data.

Plasmids were prepared using Endo-Free Qiagen Gigaprep kit, according to the manufacturer's protocol. The quality of resulting plasmids was assessed by the ratio of light absorption (260 nm/280 nm) and by 1% agarose gel electrophoresis. Light absorption at 260 nm was used to determine DNA concentration. All plasmid dilutions were done in phosphate buffer saline (PBS). Plasmids were stored at −20 °C before use.

2.2. Animals

For the expression studies, we used female NMRI mice, 6 weeks old (Université catholique de Louvain, Brussels, Belgium). Shaved mice were anesthetized with 40 μl of a mixture of ketamine 50 mg/ml (Ketalar, Pfizer, Brussels, Belgium) and xylazine 5.6 mg/ml (Sigma, Bornem, Belgium). For the vaccination studies, female BALB/c mice, 6 weeks old at the beginning of the experiment were used (Janvier, Le Genest St Isle, France). They were anesthetized with 20 μl of the ketamine/xylazine mixture. The skin of the abdomen was depilated 1 day prior to the experiments with a depilatory cream (Veet for sensitive skin, Belgium), in order to thoroughly remove all the hair.

All experimental protocols in mice were approved by the Ethical Committee for Animal Care and Use of the faculty of Medicine of the Université catholique de Louvain.

2.3. Plasmid injection and electroporation

For electroporation into the ear, we injected two volumes of 15 μl into the external side of the ear pinna using a Hamilton syringe with a 30-gauge needle. The ear was placed between 2 mm spaced electrodes. Then, a short HV pulse (700 V/cm 100 μs), immediately followed by a LV pulse (200 V/cm 400 ms) was applied approximately 1 min after plasmid injection [3]. For expression

and immunisation studies, the doses injected were 12 and 50 μg, respectively.

For intradermal electroporation, the plasmids were injected into the dermis using a Hamilton syringe with a 30-gauge needle. We injected 15 μl intradermally in two different sites, with a distance of about 5 mm, a cutaneous fold was performed and the sites of injection were placed between plate electrodes, 2 mm spaced [3]. Then, we applied HV–LV pulses (700 V/cm 100 μs, 200 V/cm 400 ms) [1]. The dose injected into the skin was 12 μg.

For electroporation into the muscle, we injected 1 μg in a volume of 30 μl into the tibial cranial muscle, and we placed the leg between 4 mm spaced electrodes. We delivered 8 square-wave electric pulses (200 V/cm 20 ms 2 Hz) [16].

For all experiments, conductive gel was used to ensure electrical contact with the skin (EKO-GEL, ultrasound transmission gel, Egna, Italy). The pulses were delivered by a Cliniporator system (Cliniporator, IGEA, Carpi, Italy) using 2 mm or 4 mm spaced plate electrodes (IGEA, Carpi, Italy).

2.4. Luciferase assay

Two days after the electroporation, the mice were sacrificed and the electroporated areas of the skin, the ear pinna or the tibial cranial muscles were removed. The samples were cut into pieces and homogenized in 1 ml cell culture lysis reagent solution (CCLR, Promega Benelux) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) using a Duall® tissue grinder (Cofraz, Essene, Belgium). After centrifugation at 12,000 × *g* for 10 min at 4 °C, we assessed the luciferase activity of 10 μl of the supernatant (diluted in CCLR if needed) after the addition of 50 μl of Luciferase Assay Substrate (Promega), using a TD-20/20 luminometer (Promega). The results were expressed in relative light units (RLU). The limit of detection, defined as the mean background + 3* background standard deviation, was 0.08 RLU. Dilutions of purified firefly luciferase protein (Sigma L4899) were used as standard. Based on standard curve performed for each experiment, 10000 RLU represent the luciferase activity of approximately 330 pg of luciferase protein [3].

2.5. Immunisation studies

BALB/c mice were injected with 0.5, 5 or 50 μg of plasmid encoding luciferase into the ear pinna and electric pulses were applied as described. Two boosts were similarly applied 2 and 4 weeks after the priming. Two weeks after the last boost, blood samples were collected by retro-orbital puncture and sera were separated by centrifugation at 700 × *g* for 20 min at 4 °C. Anti-luciferase antibodies were measured by ELISA [3,16]. Titres were defined as the highest dilution to give an optical density of 0.2 at 492 nm [3].

For cytokine assays, mice were sacrificed, and their spleens were removed aseptically. Splenocytes were adjusted to a concentration of 5×10^6 cells/ml and cultured 500 μl per well in 48-well tissue culture plates (Becton Dickinson, Belgium) in RPMI 1640 medium supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% sodium pyruvate, 5×10^{-5} M 2-mercapto-ethanol and 10% MEM (Gibco, Merelbeke, Belgium). Cells were stimulated by the addition of 10 μg of luciferase recombinant protein (Promega) per well. Unstimulated cells were used as control. Cells were incubated at 37 °C in a humidified 5% CO_2 incubator and supernatants were collected either after 48 h for interferon-gamma (IFN-γ) assay or after 72 h for interleukin-4 (IL-4) assay. We measured cytokine concentrations in the supernatants using mouse IFN-γ and IL-4 DuoSet ELISA development kits (R&D Systems Europe Ltd., Abingdon, UK) according to the manufacturer's protocols [3].

2.6. Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using the software GraphPad Prism 5 for Windows.

3. Results

3.1. Luciferase expression after electroporation of ubiquitous and tissue-specific plasmids into the ear pinna, the skin and the muscle

We measured luciferase expression after DNA electroporation of each plasmid. Electroporation into the ear pinna, the skin and the muscle did not result in the same expression [3]. Luciferase expression values after electroporation of the CMV promoter plasmid into the ear pinna, the skin and the tibial cranial muscle were 373, 1171 and 112,110 relative light units, respectively. Consequently, in order to easily compare expression after electroporation into these tissues, we considered that the ubiquitous CMV promoter plasmid gave an expression of 100%. Electroporation of a plasmid under the control of the CAG promoter gave the same expression as a CMV promoter plasmid in all the studied tissues (Fig. 2). The use of plasmids with tissue-specific promoter resulted in significant, but very low expression, as compared to that obtained with ubiquitous promoter

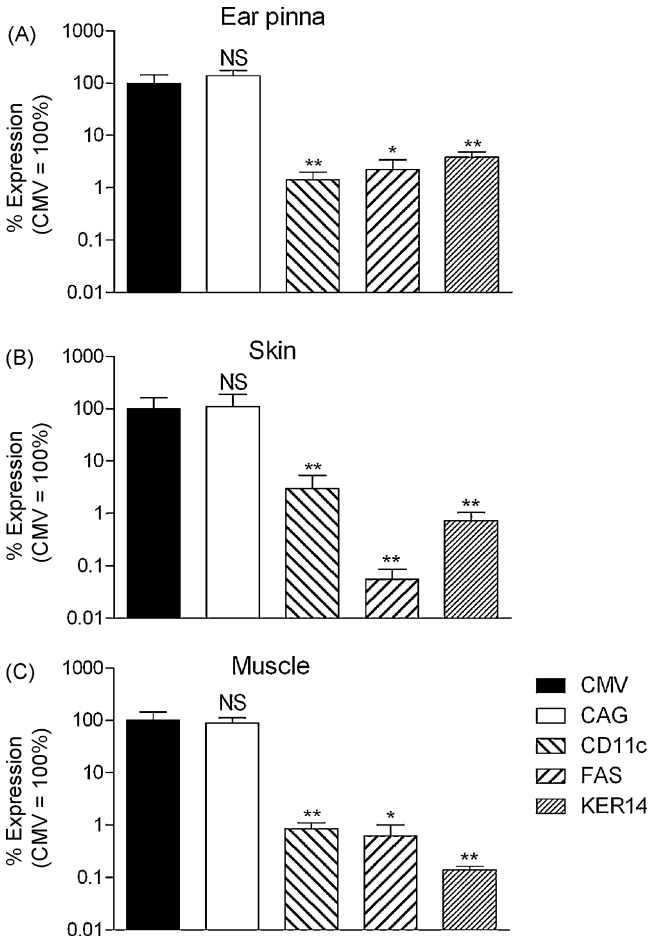

Fig. 2. Luciferase expression after electroporation of plasmid with ubiquitous and tissue-specific promoters into the ear pinna (Panel A), the skin (Panel B) and the muscle (Panel C). The plasmid doses were 1 μg for the muscle and 12 μg for the skin and the ear pinna. We considered that electroporation of pGL3CMVLUC plasmid resulted in 100% luciferase expression. Bars represent the mean values (±SEM) of luciferase activity determined biochemically from tissue sample, $n = 5$. Statistical analysis: one-way ANOVA and Tukey's post test. $^*P < 0.05$ and $^{**}P < 0.01$ versus CMV.

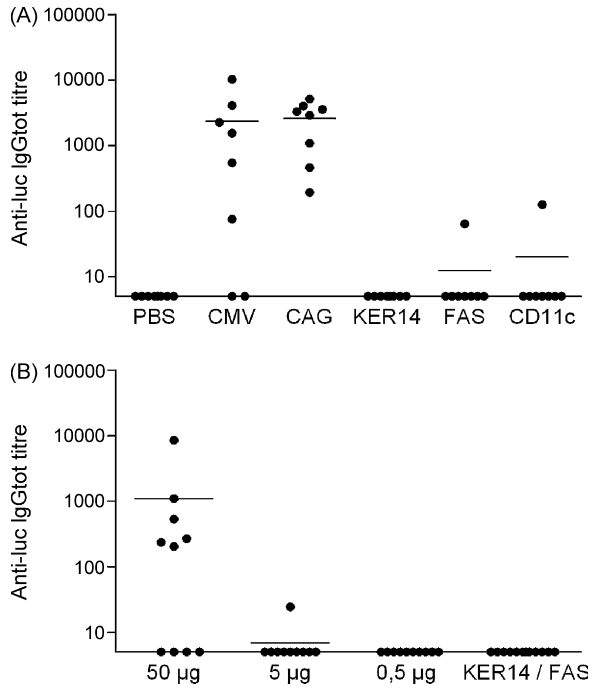

Fig. 3. Total anti-luciferase IgG titres after immunisation by intra-pinna electroporation of 50 μg plasmid encoding luciferase under the control of CMV, CAG, KER14, FAS or CD11c promoters, $n = 8$ (Panel A). Total anti-luciferase IgG titres after immunisation by intra-pinna electroporation of 50 μg, 5 μg or 0.5 μg pGL3CAGLUC plasmid or of 50 μg KER14 promoter plasmid co-injected with 50 μg FAS-promoter plasmids, $n = 10$ (Panel B). Circles represent individual titres 6 weeks after the first immunisation and lines represent the mean values.

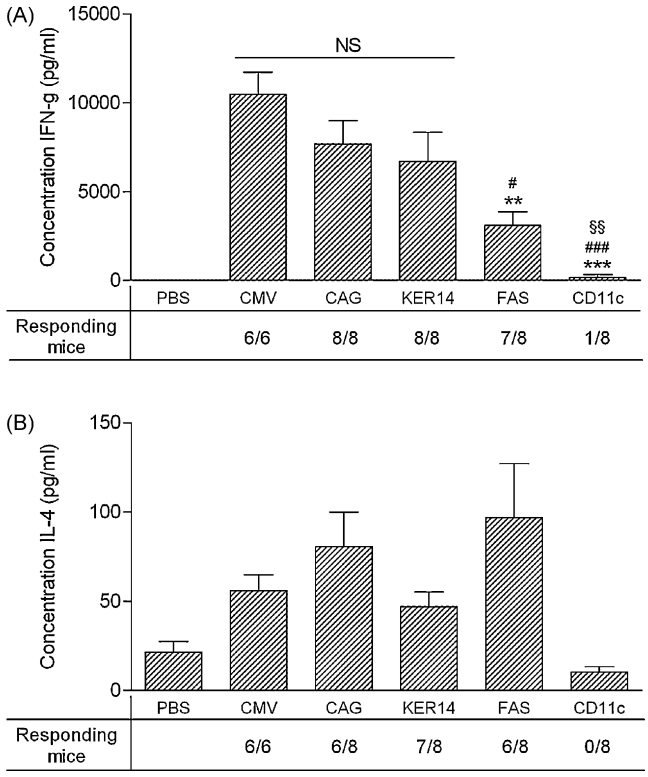

Fig. 4. Concentrations of IFN-γ (Panel A) and IL-4 (Panel B) after immunisation by intra-pinna electroporation of 50 μg plasmid, determined after mice sacrifice and luciferase-stimulated splenocytes culture. Bars represent the mean values (±SEM), $n = 8$. Statistical analysis: one-way ANOVA and Tukey's post test. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus CMV, $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus CAG, $^{\S\S}P < 0.01$ versus KER14.

plasmids. The reporter gene expression level using CD11c, FAS and KER14 promoters were similar after DNA electroporation into the ear pinna. After delivery into the skin, the luciferase expression of FAS-promoter plasmid was lower. No expression was obtained after delivery of the pGL3SAFLUC control plasmid (data not shown).

3.2. Humoral immune response after electroporation of plasmids into the ear pinna

We immunised mice with plasmids encoding luciferase by intra-pinna DNA electroporation. One priming and two boosts were delivered. Anti-luciferase IgGtot titres were measured 2 weeks after the last boost. As expected [3], immunisation with 50 μg of pGL3CMVLUC and pGL3CAGLUC resulted in high total anti-luciferase IgG titres. Anti-luciferase titres were similar for these two plasmids (*t*-test, *p*-value: 0.87). Immunisation with 50 μg of tissue-specific plasmids failed to induce significant humoral immune response compared to control mice (Fig. 3A). Co-injection of 50 μg pGL3FASLUC and pGL3KER14LUC did not elicit significant humoral immune response (Fig. 3B). To check if the absence of humoral immune response resulted from the low expression of luciferase, we studied the influence of plasmid dose on the immune response. We showed that humoral immune response was strongly influenced by the dose. Immunisation with 50 μg pGL3CAGLUC plasmid resulted in significant IgGtot titres but a dose of 5 μg was not sufficient to elicit humoral response (Fig. 3B).

3.3. Cell-mediated immune response after electroporation of plasmids into the ear pinna

In order to evaluate the cellular immune response, splenocytes were isolated and the concentrations of IFN-γ and IL-4 in the supernatant of luciferase-stimulated cultures were measured. The splenocytes stimulation with the luciferase antigen resulted in cytokine secretion for more than 75% of the mice immunised with CMV, CAG, KER14 and FAS-promoter plasmids. Immunisation with the CD11c plasmid elicited only low secretion of IFN-γ and IL-4 for one out of eight of the treated-mice (Fig. 4). Immunisation with keratinocyte specific plasmid gave the same IFN-γ response than the two ubiquitous plasmids (Fig. 4A). The IFN-γ concentration was lower for the mice immunised with fascin promoter plasmid. However, we observed IFN-γ response for almost all mice. No significant difference was shown between IL-4 concentration after immunisation with CMV and CAG, KER14, FAS or CD11c promoter plasmid.

4. Discussion

The use of specific promoters could be an interesting approach for DNA immunisation to limit gene expression to target cells and address safety concerns associated with DNA vaccines [11]. Beyond their potential clinical application, they offer the possibility to study and to better understand the role of the different cell types on the development of immune response after DNA vaccine delivery. In this work, three tissue-specific promoters were studied: keratin 14 promoter targeting keratinocytes of the epidermal basal layer and fascin and CD11c promoters targeting mature and immature dendritic cells. Plasmids were delivered using DNA electroporation.

First, we studied the luciferase expression after delivery of ubiquitous and tissue-specific promoter plasmid into the ear pinna, the skin and the muscle. We found that the expression was more than 25-fold lower when tissue-specific promoter plasmids were used. Intramuscular delivery of FAS and CD11c promoter driven plasmids resulted in luciferase expression, presumably originating from

Table 1
Effect of skin-specific promoters on intradermal DNA vaccination by electroporation and gene gun.

Specific promoter	Immune response	
	Electroporation	Gene gun
FAS	Cellular	Cellular [14,15]
CD11c	No response	No response [18], humoral [19]
KER14	Cellular	Cellular, humoral [17,18]

transfected muscle dendritic cells. Surprisingly, the expression was very low when FAS-promoter plasmid was delivered into the skin while this plasmid was expressed in the ear pinna. We hypothesise that abdominal skin contains few mature dendritic cells whereas it is known that the number of LC in ear pinna is higher [12]. DNA electroporation into the ear pinna resulted in similar expression with the three specific promoter plasmids. Therefore, we chose to immunise mice by electroporation into the ear pinna to better compare the immune responses with the different plasmids.

Secondly, we studied the immune response obtained after DNA electroporation with these plasmids (Table 1). Two mechanisms are known to be involved in the development of immune response after DNA vaccine delivery: Plasmid delivery could result in direct-presentation after DNA uptake by APC or in cross-presentation after expression by other cells such as keratinocytes or fibroblasts [9]. The previous in vivo studies which targeted specific cells of the skin for immunisation led to interesting but sometimes inconsistent results and quite different conclusions. In all of these studies, plasmids were delivered using gene gun (Table 1). Cho et al. concluded that cross-presentation was the predominant mechanism for the development of $CD8^+$ T cell responses [17]. This outcome was confirmed and completed by another study showing that immunisation with dendritic cell-restricted DNA vaccines failed to generate protective humoral and cellular response, and underlined the role of B lymphocytes in cross-presentation of antigen [18]. Recently, a third study concluded that targeting of dendritic cells was insufficient to mediate optimal induction of T cell immunity [19]. These three studies targeted dendritic cells using CD11b or CD11c promoters. However, other studies were conducted with plasmids under the control of fascin promoter. The studies of Ross et al. and Sudowe et al. clearly demonstrated that Th1 cellular response was induced by gene gun vaccination targeting dendritic cells [14,15].

In the present study using electroporation for DNA delivery, we observed cellular immune response when fascin or keratin 14 promoter plasmid was used but not after immunisation with CD11c promoter plasmids. Consequently, both direct and cross-priming are capable of triggering cell-mediated response. The choice of the promoter appears to be crucial. The functional properties of dendritic cells are strictly dependent on their maturational state [20]. The targeting of mature dendritic cells by fascin promoter appears therefore as a better approach when cellular response is needed.

Anti-luciferase immunoglobulins were measured only after DNA electroporation of ubiquitous promoter plasmids. Two hypotheses could explain that fact: (i) both dendritic cells and keratinocytes must express the antigen for the development of a significant humoral immune response after DNA electroporation or (ii) larger quantities of the immunogenic protein are needed to trigger this type of response. Our results suggested this latest hypothesis to be the most likely as the humoral immune response was strongly influenced by the plasmid dose suggesting that promoter-specific plasmids failed to elicit humoral response due to the low expression of the encoded protein.

Humoral responses were observed after delivery of specific promoter plasmids by gene gun [17,19]. This difference could be explained by the fact that gene gun immunisation is known to promote the development of Th2 responses [21,22] while DNA electroporation elicits Th1 responses [3,23,24].

To conclude, we showed that dendritic and keratinocyte-specific promoter plasmids resulted in expression of the encoded gene after intra-pinna electroporation. The expression levels were lower than those obtained with the use of ubiquitous promoter plasmids but sufficient to induce anti-luciferase IFN-gamma immune response when fascin or keratin 14 promoters were used. No evidence of humoral immune response was showed after DNA electroporation of skin-specific promoter plasmids. Our results suggested that both direct-presentation and cross-presentation were able to induce cell-mediated immune response. However more extensive immunological studies are needed to support this conclusion. The use of skin-specific promoter plasmids could be an interesting alternative to the use of ubiquitous promoters but only for the treatment of pathologies (e.g. cancer) or pathogens requiring mainly cellular and not humoral immune response.

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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.vaccine.2009.05.022.

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