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ARTICLE

Optimization of a gene electrotransfer procedure for efficient intradermal immunization with an *hTERT*-based DNA vaccine in mice

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DNA vaccination consists in administering an antigen-encoding plasmid in order to trigger a specific immune response. This specific vaccine strategy is of particular interest to fight against various infectious diseases and cancer. Gene electrotransfer is the most efficient and safest non-viral gene transfer procedure and specific electrical parameters have been developed for several target tissues. Here, a gene electrotransfer protocol into the skin has been optimized in mice for efficient intradermal immunization against the well-known telomerase tumor antigen. First, the *luciferase* reporter gene was used to evaluate gene electrotransfer efficiency into the skin as a function of the electrical parameters and electrodes, either non-invasive or invasive. In a second time, these parameters were tested for their potency to generate specific cellular CD8 immune responses against telomerase epitopes. These CD8 T-cells were fully functional as they secreted IFNγ and were endowed with specific cytotoxic activity towards target cells. This simple and optimized procedure for efficient gene electrotransfer into the skin using the telomerase antigen is to be used in cancer patients for the phase 1 clinical evaluation of a therapeutic cancer DNA vaccine called INVAC-1.

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INTRODUCTION

In vivo gene electrotransfer (EGT)[1,2] possesses promising applications in the field of DNA vaccination as a cost-saving and practical alternative to peptide-based and viral vector-based immunization.[3]

In order to induce strong and specific cellular and humoral immune response triggered by DNA vaccination, electric pulses (EP) delivery has to be performed using a tissue-specific procedure that enables the effective EGT. First, DNA has to be brought close to the target cells by injecting it at the expected site of vaccination and second, EP are delivered to transfer the DNA into the cell. Electrotransfer is a multistep process that relies on two different effects of the EP: the cell membrane permeabilization and the electrophoretic transport of the nucleic acids towards and/or across the plasma membrane.[4] Cell membrane permeabilization can be achieved using one or several short (about 100 of microseconds) and intense (about 1,000 volts per centimeter) pulses, called high voltage (HV) pulses, that reversibly permeabilize the cell membrane. A defined lag time later, electrophoretic movement of the DNA is achieved by the application of one or several long (about several hundred of milliseconds) and less intense (about 100 volts per centimeter) pulses, called low voltage (LV) pulses. These pulses are intended to drive the DNA molecules towards the electropermeabilized cells.[5]

Different target tissues are under investigation for efficient vaccination using the EGT strategy. Among them skin tissue and particularly the intradermal (ID) route is regaining interest. Some reasons include the ease of accessibility, the large surface available for the treatment, the ease of histological and clinical evaluation and the presence of numerous antigen-presenting cells (APCs).[2,6] Indeed, skin APCs are capable of efficiently processing the antigens of interest, and then migrate to secondary lymphoid organs to present the antigens onto MHC class I and II molecules in order to prime naive or memory CD8 and CD4 T-cells, respectively. This will generate specific cellular immune responses which are of real interest to fight cancer.[7–9]

Cancer DNA vaccines are intended to break immune tolerance against tumor-specific or tumor-associated antigens.[10] Thus, they aim at generating a pool of antigen-specific effector and memory T-cells and B-cells which will migrate to the tumor site and contribute to tumor destruction *via* several effector mechanisms such as direct cytotoxic activity and cytokines secretion.[11] In the case of cancer vaccines, the stimulation of tumor-specific T-cell response possesses several theoretical advantages. First, T-cells can home into antigen-expressing tumor deposits, no matter where they are located in the body, even in deep tissue beds.[12] Moreover, among immune cells that are relevant in antitumor immune responses, CD8 cytotoxic T-lymphocytes (CTLs) have been identified as the

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most powerful effectors,[9] as are Th1 cytokines such as IFNγ.[11] As a consequence, an efficient cancer vaccine should target class I MHC-restricted peptides derived from tumor antigens, in order to generate antitumor CTLs, which will exhibit cytolytic activity towards tumor cells. Thus, one of the ultimate goals of an efficient cancer DNA vaccine combined with EGT technology must be the generation of a strong cellular CD8 immune response against the tumor antigen encoded by the plasmid of interest.

In muscles, a large number of electrical parameters have been explored, including the combinations of HV and LV pulses which have been finely tuned to obtain safe and efficient EGT.[1,4,13–15] In skin, several kinds of electrodes and EP have been explored[1,2] but the combinations of HV and LV pulses after ID DNA injection have been analyzed only in a few studies.[16–18] To our knowledge, immune responses against an antigen of interest have only been monitored by Roos *et al*.[19] Therefore, in the present study, we have investigated the impact of different EGT parameters after ID administration of a DNA. First, a generic approach was set up using a *luciferase*-encoding plasmid in order to determine the best EGT parameters. Second, these latter were tested in a CD8 response-monitoring DNA vaccination model using the INVAC-1 plasmid that encodes a modified form of the human telomerase reverse transcriptase gene (*hTERT*). Telomerase is a well-known tumor antigen overexpressed in 85% of human cancers and is consequently of great interest for vaccination purposes against cancer.[20,21]

RESULTS

Gene electrotransfer in the dermis achieves high *in vivo* transgene expression and the induction of hTERT-specific CD8 T-cells

C57BL/6J mice were injected intradermally on both flanks with either pCMV-luc (encoding *luciferase*) or INVAC-1 (encoding a modified form of *hTERT*) plasmids, followed or not by the application of EP (1 HV pulse of 1,000 V/cm and of 100 µs followed 1,000 ms later by 1 LV pulse of 140 V/cm and of 400 ms).[1] Erythema was observed neither after mice shaving nor during or after the EGT procedure. Two parameters were measured after EGT according to the plasmid used: the *luciferase* expression 48 hours after EGT of pCMV-luc and the frequency of the IFNγ+ hTERT-specific CD8 T-cells 14 days after EGT of INVAC-1. Both the *luciferase* expression (Figure 1a) and the frequency of IFNγ+ hTERT-specific CD8 T-cells (Figure 1b) were significantly increased when EP were applied directly after ID DNA injection into the dermis ($P < 0.01$ and $P < 0.05$, respectively), in comparison with animals which received ID DNA injection without EP. Thus, electrotransfer is essential to reach significant levels of *luciferase* expression after ID injection of pCMV-luc and to induce significant levels of hTERT-specific CD8 T-cell responses after immunization with an ID injection of INVAC-1.

Determination of the best electrodes for efficient gene electrotransfer in the dermis and generation of intense CD8 immune responses

Different types of electrodes can be used for EGT in the skin.[2] Here, three different electrodes (plate electrodes, finger electrodes and needle electrodes) were tested in order to determine which one was the most suitable for efficient EGT and for the generation of an intense specific cellular immune response in mice. Actually, the EP delivered using invasive needle electrodes or the non-invasive plate electrodes significantly enhanced *luciferase* expression in C57BL/6J mice compared to the animals which received the plasmid without EP ($P < 0.05$ and $P < 0.001$, respectively) (Figure 2a). However, there was a better homogeneity in the response for the group of mice in which the gene electrotransfer was performed with plate electrodes. By contrast, results were not statistically significant when invasive finger electrodes were used.

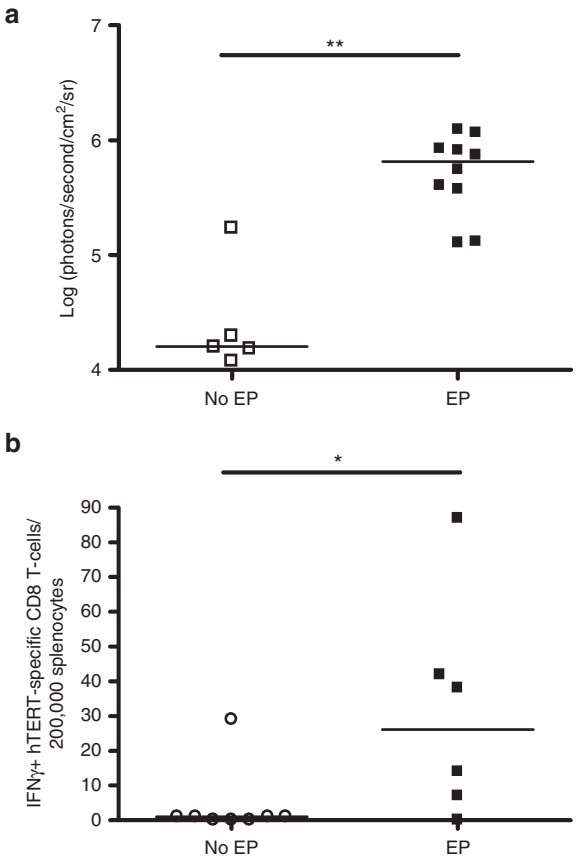

Figure 1 Comparison of pCMV-luc gene transfer into the dermis and INVAC-1-mediated ID vaccination efficiencies with or without EP using plate electrodes. (**a**) Representation of bioluminescence intensities in C57BL/6J mice 2 days after pCMV-luc ID injection followed or not by EP, $n = 5$ mice for pCMV-luc ID injection alone, $n = 10$ (from 5 mice, 2 treatments per mouse) for pCMV-luc ID injection+EP. (**b**) Frequency of hTERT-specific IFNγ+ CD8 T-cells detected in C57BL/6J mice vaccinated 14 days before with 25 µg of INVAC-1 followed or not by EP, $n = 8$ both for INVAC-1 ID injection alone or $n = 6$ for INVAC-1 ID injection+EP. Bars represent median values, $^*P < 0.05$, $^{**}P < 0.01$, Mann–Whitney–Wilcoxon test.

Similar results were obtained in immunogenicity studies in HLA-B7 mice. The highest median frequency of IFNγ+ specific CD8 T-cells was obtained when plate electrodes were used in mice vaccinated using EGT of INVAC-1 injected *via* the ID route. This difference was statistically significant in comparison with control group in which a phosphate-buffered saline (PBS) injection followed by EP application was performed ($P < 0.05$) (Figure 2b).

In summary, plate electrodes displayed the best ability to electrotransfer both pCMV-luc to produce luciferase and INVAC-1 to generate significant levels of hTERT-specific CD8 T-cells.

Localization of luciferase after ID injection followed by gene electrotransfer

EGT is known to be very efficient in muscles.[1] In order to make sure that *luciferase* was only electrotransferred into the skin after an ID injection of pCMV-luc, a skin flap was opened in the flank of C57BL/6J mice at the site of the treatment. The bioluminescence for both the skin flap and for the underlying muscles was

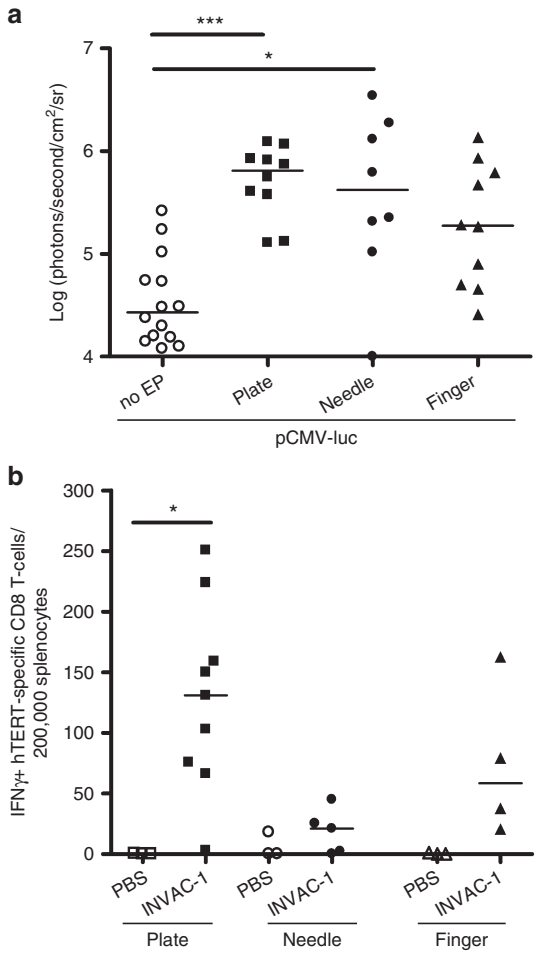

Figure 2 Choice of the best electrodes for pCMV-luc electrotransfer into the dermis and INVAC-1–mediated ID vaccination. (**a**) Representation of bioluminescence intensities 2 days after pCMV-luc electrotransfer in C57BL/6J mice using the three types of electrodes, $n = 14$ mice for pCMV-luc ID injection alone, $n = 8–10$ (from four to five mice, two treatments per mouse) for pCMV-luc ID injection+EP. (**b**) Frequency of hTERT-specific INFγ^+ CD8 T-cells detected in HLA-B7 mice vaccinated intradermally 14 days before with INVAC-1 using the three types of electrodes, $n = 3$ mice for PBS immunization control and $n = 4–9$ mice for INVAC-1–mediated immunization. Bars represent median values, $^*P < 0.05$, $^{***}P < 0.001$, Kruskal–Wallis test with Dunn's multiple comparison test.

measured 4 days after EGT performed with the plate electrodes. We confirmed that the transgene expression occurred only in the skin and that no expression was detected in the underlying muscles (Figure 3).

Determination of the best HV pulse for efficient gene electrotransfer in the dermis

The first optimization of the EGT electrical parameters consisted in determining the most efficient amplitude of the HV pulse (100 µs duration) among the following field amplitudes: 600 V/cm; 800 V/cm; 1,000 V/cm; 1,200 V/cm; 1,400 V/cm; and 1,600 V/cm. The intensity of the LV pulse (400 ms duration) was kept constant at 140 V/cm and the lag between HV and LV pulses was set at 1,000 ms. This evaluation was performed in C57BL/6J mice using the *luciferase* reporter gene.

Among the tested amplitudes, C57BL/6J mice electrotransferred with field amplitudes of 1,200 V/cm; 1,400 V/cm; or 1,600 V/cm presented the most significant enhancements of *luciferase* expression as compared to control mice ($P < 0.001$) (Figure 4). In particular, the highest median bioluminescence was obtained in the group treated with a field amplitude of 1,400 V/cm. There was also a better homogeneity in the results for this group as compared to other groups. However, there was no statistical difference between *luciferase* expressions obtained in the 1,200; 1,400; and 1,600 V/cm groups.

Determination of the best HV-LV pulses combination for efficient gene electrotransfer in the dermis and generation of intense CD8 immune responses

The influence of different HV-LV combinations was evaluated in C57BL/6J mice by monitoring the *luciferase* expression and the cellular CD8 immune response after electrotransfer of intradermally injected pCMV-luc and INVAC-1 plasmid, respectively. Regarding the HV pulse, 1,000 V/cm or 1,400 V/cm were chosen to be combined with various LV pulses. Pulses of 1,000 V/cm are the reference for EGT following subcutaneous injection of plasmids[1] whereas 1,400 V/cm was optimal for EGT following ID administration of the plasmid, as determined previously. Hence, directly after ID injection of pCMV-luc or INVAC-1, one HV pulse (100 µs duration) of 1,000 V/cm or 1,400 V/cm was applied followed by one LV pulse (400 ms duration) of either 60 V/cm, 100 V/cm, 140 V/cm, 180 V/cm, or 220 V/cm. The ten HV-LV pulses combinations were referred to as "P1" to "P10" (Table 1).

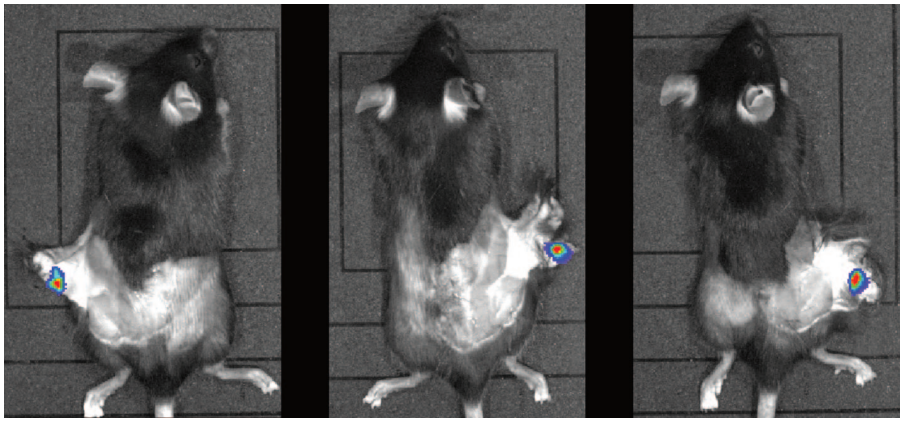

Figure 3 Localization of *luciferase* expression after ID injection and electrotransfer of pCMV-luc into the dermal layer of C57BL/6J mice using plate electrodes. Bioluminescence intensities were evaluated in the skin flap and in the underlying muscles 4 days after ID injection of pCMV-luc and EGT, $n = 3$ mice.

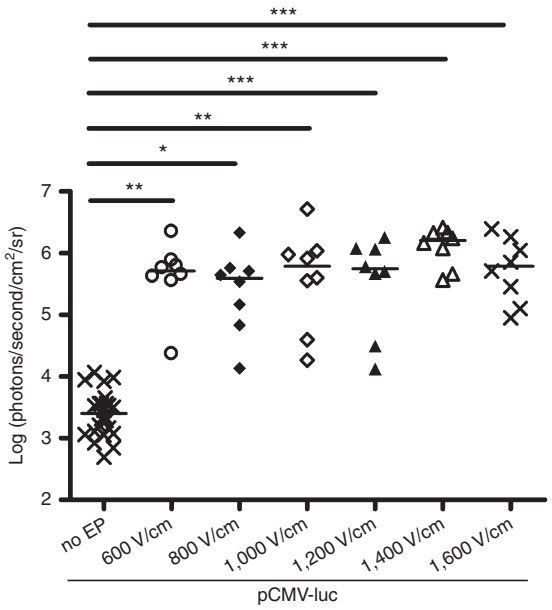

Figure 4 Determination of the optimal HV pulse intensity for intradermally injected pCMV-luc electrotransfer in C57BL/6J mice using plate electrodes. Bioluminescence intensities were evaluated 2 days after pCMV-luc electrotransfer performed in C57BL/6J mice. One single HV pulse of different intensities in combination with one LV pulse of 140 V/cm were compared, $n = 24$ mice for pCMV-luc ID injection alone, $n = 8$ (from 4 mice, 2 treatments per mouse) for pCMV-luc ID injection+EP. Bars represent median values, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Kruskal–Wallis test with Dunn's multiple comparison test.

Due to technical limitations, the Cliniporator was not able to deliver constantly 220 V/cm during 400 ms. Thus, the results obtained when P5 and P10 conditions were used did not generate reliable data and were excluded for data analysis.

The P4, P8, and P9 combinations of HV-LV pulses generated the highest median bioluminescence intensities (Figure 5a). All of these three combinations displayed very high statistical differences when compared to pCMV-luc ID injection without EP ($P < 0.001$). In particular, P9 showed the best median bioluminescence intensity, the highest value for the minimum bioluminescence intensity and the lowest point dispersion.

P4, P8, and P9 HV-LV pulses combinations were then tested for ID vaccination with INVAC-1. The hTERT-specific CD8 T-cell responses of these groups were compared to the P3 combination which was previously published for EGT following subcutaneous administration of the DNA.[1] When analyzing the data from the immunogenicity assay, P8 and P9 combinations appeared to be the best ones, since they enabled the generation of significant frequencies of IFNγ$^+$ specific CD8 T-cells in comparison with mice that received PBS followed by EP application ($P < 0.01$ and $P < 0.001$, respectively) (Figure 5b). Even though the difference between P8 and P9 groups was not statistically significant, P9 displayed a higher median frequency of hTERT-specific CD8 T-cells.

Given bioluminescence and immunogenicity data analyses, the best HV-LV pulses combination appeared to be P9, *i.e.*, one HV pulse (100 µs duration) of 1,400 V/cm followed by one LV pulse (400 ms duration) of 180 V/cm, when administering the DNA intradermally and delivering the EP to the skin with plate electrodes.

The Cliniporator being CE marked for the delivery of a maximum of 1,000 V and because the plate electrodes chosen for the clinical evaluation of INVAC-1 DNA vaccine are separated by a 8-mm gap, the field amplitude that can be used in clinical trials must not exceed 1,250 V/cm. As a consequence, we compared both the *luciferase* expression and the frequency of hTERT-specific CD8 T-cells in mice in which EGT was performed with P9 or with one HV pulse (100 µs duration) of 1,250 V/cm followed by one LV pulse (400 ms duration) of 180 V/cm. This last combination will be further referred as Pd. No statistically significant difference was observed between the two groups, in both bioluminescence (Figure 5c) and immune-monitoring assays (Figure 5d).

Table 1 Combinations of HV-LV pulses evaluated in bioluminescence and in ELISpot assays

Electric fields (V/cm)	LV = 60	LV = 100	LV = 140	LV = 180	LV = 220
HV = 1,000	P1	P2	P3	P4	P5
HV = 1,400	P6	P7	P8	P9	P10

Induction of hTERT-specific CD8 T-cell–mediated killing after INVAC-1-based ID vaccination

We performed an evaluation of the cytolytic strength of the hTERT-specific CD8 T-cells using carboxyfluorescein succinimidyl ester (CFSE)-labeled and peptide-pulsed splenocytes as target cells, as mentioned in the Materials and Methods section. Indeed, the cytotoxic activity of CTL is mandatory to kill tumor cells. In order to finally validate the EGT Pd parameters that could be used in clinical trials, we compared the cytotoxic activity of hTERT-specific CD8 T-cells in C57BL/6J mice immunized through the ID route using INVAC-1 combined with two distinct EGT parameters: one HV pulse of 700 V/cm and of 100 µs followed 1,000 ms later by one LV pulse of 100 V/cm and of 400 ms (optimal parameters for EGT in muscle,[1] further referred as Pm) or Pd parameters. In the context of a single immunization with INVAC-1 plasmid, we showed that EGT performed with Pd was associated with a twofold increase in the lysis of target cells bearing class I MHC-restricted telomerase peptides (50% of specific lysis), as compared to EGT performed with Pm (25% of specific lysis) (Figure 6), even though this difference was not statistically significant due to high standard deviations. Thus, INVAC-1–mediated ID vaccination using efficient EGT parameters can generate hTERT-specific CTL.

DISCUSSION

DNA vaccination is an effective approach to generate efficient immune responses against a defined antigen by transfecting the gene encoding it directly into target cells.[22] Moreover, DNA vaccines are promising tools for immunotherapy strategies, particularly in the field of cancer management.[7,23] EGT using DNA molecules remains the most efficient and the safest way to deliver a transgene into tissues.[24] Thus, it is considered as an interesting alternative to viral vectors for clinical applications of DNA vaccines.[3,22,25]

In our study, we sought to optimize an *in vivo* EGT protocol for efficient telomerase-based DNA immunization into the dermis. In a first step, we optimized the pCMV-GFP electrotransfer in order to obtain intense *luciferase* expression and, in a second time, our aim was to obtain a high frequency of hTERT-specific CD8 T-cells following EP-mediated DNA vaccination with INVAC-1.

Our results support previous ones demonstrating that EGT into cutaneous tissues increased up to 100-fold the transgene expression in comparison with DNA injection alone and led to an intense antigen-specific immune response.[18,26] Our electrotransfer protocol

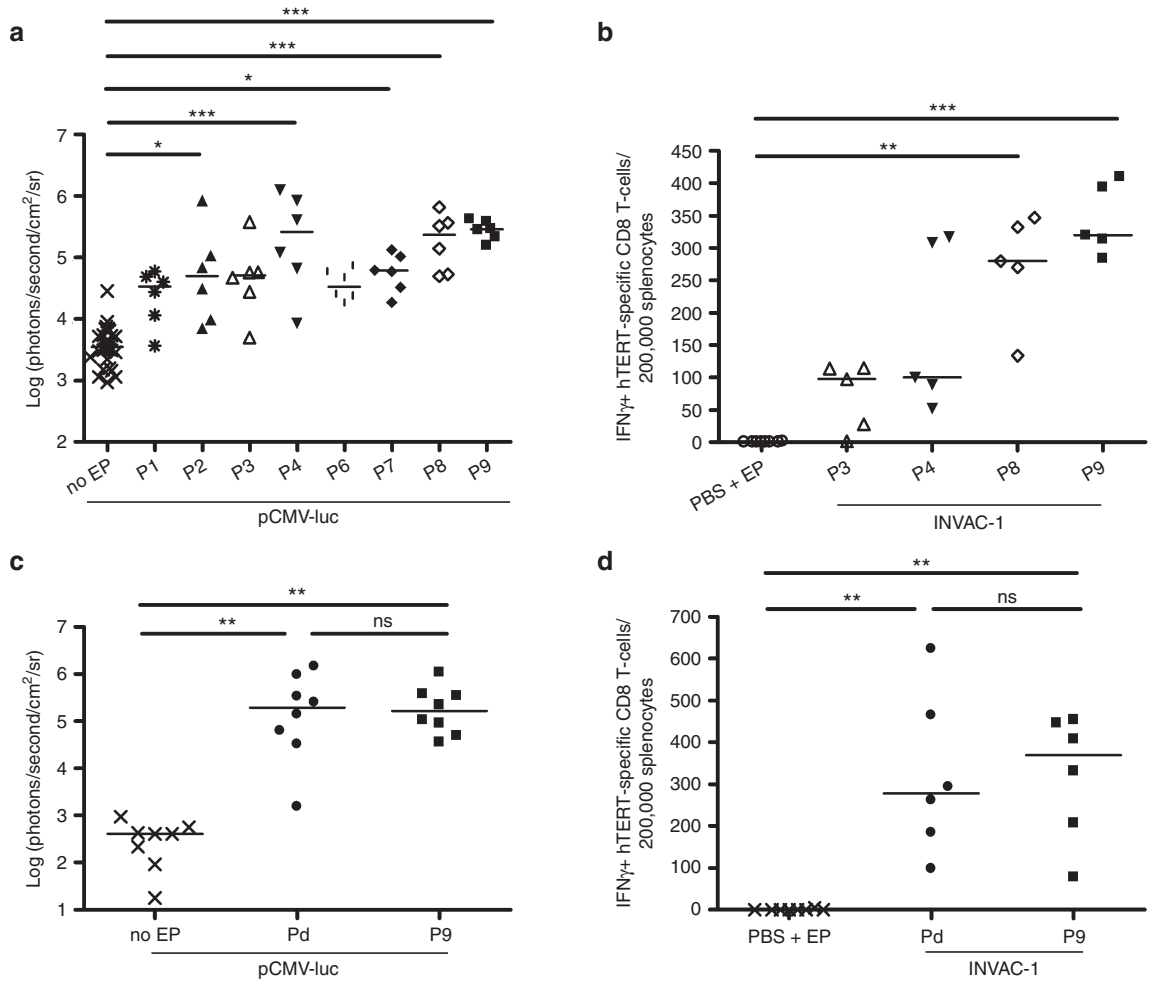

Figure 5 Choice of the best HV-LV pulses combination for pCMV-luc dermal electrotransfer and INVAC-1–mediated ID vaccination. (**a**) Bioluminescence obtained in C57BL/6J mice 2 days after pCMV-luc ID injection upon various HV-LV pulses combinations, $n = 30$ mice for pCMV-luc ID injection alone and $n = 6$ (from 3 mice, 2 treatments per mouse) for pCMV-luc ID injection+EP delivered onto the skin. (**b**) Frequency of hTERT-specific IFNγ$^+$ CD8 T-cells detected in C57BL/6J mice vaccinated 14 days before with INVAC-1 according to various combinations of HV-LV pulses, $n = 8$ mice for PBS immunization control and $n = 5$ mice for INVAC-1–mediated immunization. (**c**) Comparison of the bioluminescence intensities in C57BL/6J mice in which EGT was performed 2 days before with P9 or Pd, $n = 4$ mice for pCMV-luc ID injection alone, $n = 8$ (from four mice, two treatments per mouse) for pCMV-luc ID injection+EP. (**d**) Comparison of the frequency of hTERT-specific IFNγ$^+$ CD8 T-cells in C57BL/6J mice immunized intradermally 14 days before by using INVAC-1 combined with P9 or Pd, $n = 4$ mice for PBS immunization control and $n = 6$ mice for INVAC-1–mediated immunization. Bars represent median values. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Kruskal–Wallis test with Dunn's multiple comparison test.

was optimized by testing several electrotransfer parameters including different types of electrodes as well as various combinations of HV and LV pulses intensities.

We determined that non-invasive plate electrodes generated both an intense expression of the *luciferase* gene and a specific cellular CD8 immune response against telomerase epitopes. This finding was robust since a similar observation was done in dogs immunized using a canine *TERT*-based DNA vaccine (data not shown). Many preclinical studies used plate electrodes on small rodents for ethical and practical reasons and very few compared non-invasive and invasive electrodes for EGT efficiency.[2,16] Indeed, invasive electrodes designed for human tissues may not be appropriate for small laboratory animals. In order to ensure that all needles penetrate the skin, the experimenter must put enough pressure when digging them into the tissues, which can eventually harm dramatically the animals. Moreover, the use of invasive electrodes may mechanically damage the target tissue and very intense electric fields may occur during electric pulse application at the level of the needles,[27] potentially leading to the death of cells in contact with or very close to the electrodes. Overall, this may explain the low efficiency of invasive electrodes in our study. Moreover, plate electrodes generate more homogeneous electric field distribution than invasive electrodes[28] and may achieve a more robust electropermeabilization of target cells. For these reasons, most ongoing clinical trials using electroporation deal with non-invasive electrodes in order to minimize patients' discomfort and safety concerns. Several optimization procedures have been made in order to improve electroporation using non-invasive plate electrodes. Some of them include the use of conductive gel to improve the contact with the skin[29] or a particular handling in order for the rows of the plate electrodes to be largely in contact with the target tissue.[30] However, it should be noted that the use of noninvasive electrodes involves to overcome the insulating properties of the stratum corneum. In that configuration, a higher electric field should be used with non-invasive electrodes than with invasive electrodes in order to ensure the permeabilization of the target cells located underneath the stratum corneum. Aside from practical and safety issues in favor of the use of non-invasive plate electrodes, we demonstrated here that these were more efficient for EGT of *luciferase* and immunization with INVAC-1 than the two

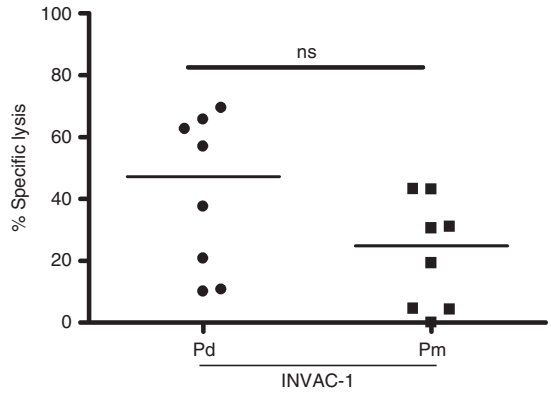

Figure 6 Evaluation of the hTERT-specific cytotoxicity of CD8 T-cells in immunized mice as a function of the dermal electrotransfer parameters used. C57BL/6J mice were intradermally immunized with INVAC-1 using Pd or Pm parameters. 14 days later, splenocytes from naïve mice were recovered, stained with CFSE, pulsed with p660 peptides and eventually injected i.v. in immunized mice. 15 hours later, spleens from immunized mice were recovered and CFSE-labeled splenocytes were quantified by flow cytometry, $n = 8$ mice. Bars represent median values, ns for not statistically significant, Mann–Whitney–Wilcoxon test.

other invasive electrodes, both procedures being performed after ID injection of the DNA. This finding is supported by *in silico* electric field modelization showing that non-invasive electrodes permeabilize very efficiently the surface of tissues (in our case, the skin tissue) while invasive electrodes are intended to be used for electroporation of deeper tissues.[31] Conversely, one study reported that invasive electrodes were more potent than non-invasive ones to induce gene expression following EGT in porcine skin.[16] It should be noted that this comparison was made using the same HV pulse intensity (*i.e.*, 1,000 V/cm), which may not have been high enough to overcome the insulating properties of the stratum corneum in the case of the use of non-invasive electrodes. Moreover, although high transgene expression is an important factor for mounting intense immune responses, it is not the only factor. As a demonstration, there was no obvious difference in our study between plate and needle electrodes in terms of *luciferase* expression whereas the contrast was marked in terms of specific cellular immune response intensity.

Regarding EGT electrical parameters, we tested various HV-LV combinations. Indeed, although different pulse parameters are used amongst the different groups working in this field, it is accepted that the combination of one HV and one LV leads to an intense transgene expression.[4,14,15] We determined that an injection of *luciferase* gene into the dermis followed by one HV of 1,400 V/cm and of 100 µs followed by one LV pulse of 180 V/cm and of 400 ms resulted in a higher luciferase production than the other combinations. This result was supported by the ELISpot assay since this combination led to the highest frequencies of hTERT-specific CD8 T-cells secreting IFNγ. Our group previously showed that optimal electrotransfer of *luciferase* into subcutaneous tissues required one HV of 1,000 V/cm and of 100 µs followed by one LV pulse of 140 V/cm and of 400 ms.[1] However, one should take in account that this latter work was performed with a subcutaneous injection of DNA (targeting the hypodermis, a fat layer located beneath the skin tissues) whereas the present work was performed with injections into the dermis. Given that dermis-resident fibroblasts, a population mostly represented in this layer of the skin, present smaller radius than adipocytes located in the subcutaneous tissues,[6] it could be hypothesized that adipocytes are more easily electropermeabilized than fibroblasts.[13] Consequently, it is not surprising that, after

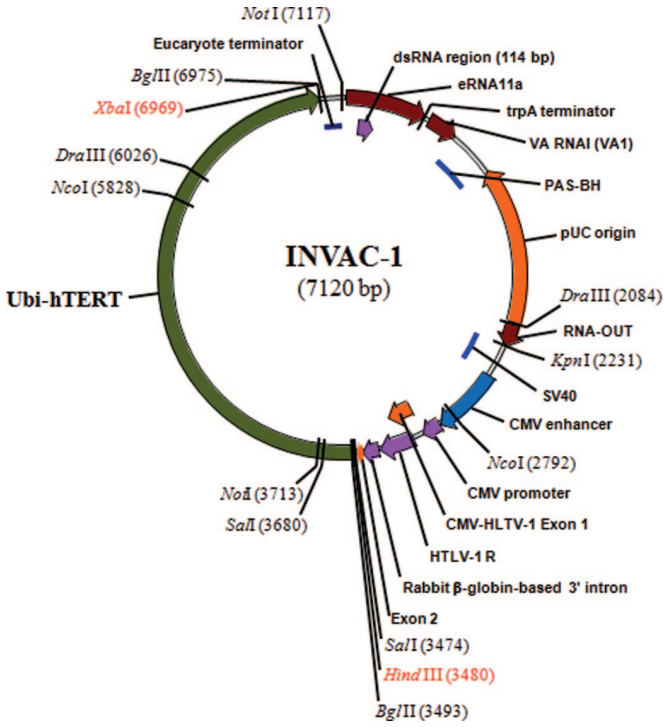

Figure 7 INVAC-1 plasmid map. Bases 1–3478: NTC8685-eRNA41H-HindIII-XbaI vector (NTC); Bases 3479–3484: HindIII cloning site (NTC/Invectys); Bases 3485–6967: Ubi-Telomerase transgene (Invectys); Bases 6968–6973: XbaI cloning site (Invectys/NTC); Bases 6974–7120: NTC8685-eRNA41H-HindIII-XbaI vector (NTC).

an ID injection, a higher electric field amplitude has to be used to transfer DNA into the dermis resident cells. Besides, we demonstrated in this study that plate electrodes were more suitable than invasive ones when using nonoptimized electrical parameters. The major concern when using plate electrodes consists in applying an electric field intense enough to overcome the insulating barrier represented by the stratum corneum. Here, the optimum electrical parameters for EGT following ID DNA injection (one HV pulse of 1,250 V/cm followed by one LV pulse of 180 V/cm) were more intense than those used for the determination of the best electrodes (one HV pulse of 1,000 V/cm followed by one LV pulse of 140 V/cm). It is thus expected that these optimum electrical parameters would further favor the choice of plate electrodes over invasive ones since the stratum corneum insulating property would be overcome more efficiently.

These optimal parameters were obtained in mice using non-invasive plate electrodes spaced 5 mm apart to fit with an ID injection of a 50 µl volume in mice. For an injection of 100 µl of a plasmid solution, as intended to be used in humans, the 8-mm-spaced-plate electrodes must be used. The voltage must be set according to the distance between the two rows of the electrodes. In that case, the HV is limited to 1,250 V/cm (instead of 1,400 V/cm) because Cliniporator settings for human use are limited to 1,000 V for safety reasons (CE mark). The CE mark also covers the 8-mm-spaced-plate electrodes. Consequently, we compared the optimal parameters that were discussed previously with the ones of maximum intensity that can be used while keeping the Cliniporator CE mark (Pd parameters). As a matter of fact, both parameters were equally efficient in inducing an intense *luciferase* expression and the difference in terms of hTERT-specific CD8 T-cell response was not statistically different between the two groups.

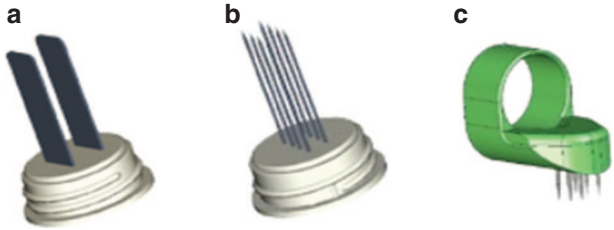

Figure 8 Electrodes used in this study. (**a**) Non-invasive plate electrodes. (**b**) Invasive needle electrodes. (**c**) Invasive finger electrodes.

Finally, we showed that immunized mice generated hTERT-specific CTL endowed with killing capacity towards target cells bearing class I MHC-restricted hTERT peptides. Moreover, this cytotoxic activity was strongly dependent on the electric parameters used for EGT.

In summary, we demonstrated that our parameters for EGT into the dermis greatly enhanced the *luciferase* expression *in vivo* and allowed the induction of intense specific CD8 T-cell responses when using an ID injection of DNA vaccine encoding *hTERT* as a tumor antigen. The present study highlights the need to define optimal EGT parameters to allow a successful immunization when using DNA vaccination protocols in a defined target tissue. The determined EGT parameters are being used in regulatory toxicology and safety studies of INVAC-1-based DNA vaccination and are intended to be used for its clinical evaluation. A prime/boost strategy will be investigated in order to mount robust, intense and cytotoxic hTERT-specific cellular immune responses in cancer patients. Moreover, it is expected that our EGT parameters could be used for efficient DNA vaccination using other types of antigens, should they be tumor or pathogen antigens.

MATERIALS AND METHODS

Mice

HLA-B7 mice are transgenic mice expressing the HLA-B*0702 class I molecule. They are knock-out for mouse class I $H2D^b$ and $H2K^b$ molecules. They were described by Rohrlich *et al.*[32] and were obtained from the Pasteur Institute internal breeding. Female C57BL/6J mice (6–8 weeks old) were purchased from Janvier (Saint-Berthevin, France) or Harlan (Gannat, France) laboratories.

Animals were housed at specific pathogen-free animal facilities of the Pasteur Institute or of Gustave Roussy. All animal experiments were performed in strict compliance with the ethical guidelines issued by the European Committee (Directive 2010/63/EU) and animals were handled in strict accordance with good animal practice. The experimental protocols (#2013_0026 and #2012_007) were respectively approved by the registered committees on ethics in animal experimentation, CETEA–Institut Pasteur and CEEA #26.

Plasmids

pCMV-luc (Plasmid Factory, Bielefeld, Germany) is a double stranded plasmid DNA of 6,233 bp encoding the firefly *luciferase* reporter gene under control of the cytomegalovirus promoter (pCMV).

INVAC-1 is a double stranded plasmid DNA of 7,120 bp encoding a modified sequence of the telomerase protein fused to the Ubiquitin protein sequence. The encoded telomerase protein is enzymatically inactive, due to the deletion of three amino acids inside the catalytic site of hTERT (delta VDD), but can still induce immune responses against telomerase epitopes *in vivo*. The ubiquitin-telomerase insert is cloned into the NTC8685-ERNA41H-HindIII-XbaI expression vector designed by Nature Technology Corporation (Lincoln, Nebraska). The presence of the ubiquitin increases the addressing of the TERT protein to the proteasome and increases the MHC class I presentation pathway of TERT-derived peptides.[33,34] The DNA sequence coding for the TERT protein was deleted of 47 amino acids in the N-terminal region, which encodes the nucleolar localization signal. Figure 7 represents INVAC-1 plasmid map.

EP generator and electrodes

EGT was performed using the Cliniporator (IGEA, Carpi, Italy) delivering HV pulses and LV pulses. Voltages were set up according to the distance between the two rows of the electrodes. Different types of electrodes were used: (i) non-invasive plate electrodes (P30-8B, IGEA) consisting in two metallic plates, 1 mm thick and 5 mm apart (Figure 8a), (ii) invasive needle electrodes (N-30-4B, IGEA) consisting in two rows of four long needles, 4 mm apart (Figure 8b), and (iii) invasive finger electrodes (F-05-OR, IGEA) consisting in two rows of three short needles, 4 mm apart (Figure 8c).

In vivo gene electrotransfer

Prior to the ID injections, mice were anesthetized either with 2% isoflurane/oxygen mixture gas anesthesia (Abbot, Suresnes, France) or with a mix solution (intraperitoneal route) of 2% xylazine (Bayer Santé, Loos, France) and 8% ketamine (Merial, Lyon, France) in PBS according to individual animal weight. After shaving, ID injection was performed on the lower part of the flank (bilateral injections) with 29 G insulin specific needles. Each animal, either HLA-B7 or C57BL/6J mouse, was vaccinated with a single dose of DNA, corresponding to 100 µg (unless otherwise specified) of INVAC-1 plasmid (50 µg in 25 µl PBS per flank). Regarding *luciferase* electrotransfer, 5 µg in 25 µl PBS per site were injected (three sites per mouse: two electroporated and one control non-electroporated).

Immediately after the ID injection, EGT was performed using one HV pulse (100 µs duration) followed 1,000 ms later by one LV pulse (400 ms duration). Electrodes were placed in such a way they surrounded the bleb formed by the plasmid injection and were in contact with it. Both finger or needle electrodes were pressed for about 5 mm into the skin. Large amount of conductive gel (NM Médical, Asnières-sur-Seine, France) was used for the plate electrodes in order to improve the contact between the metallic plates and the skin. Negative controls of vaccination included the application of EP in mice injected with PBS only and negative controls of gene transfer consisted in the absence of EP application on mice injected with pCMV-luc.

In vivo bioluminescence imaging and electrotransfer localization

Two days after pCMV-luc electrotransfer, mice were injected intraperitoneally with 0.15 mg beetle luciferin (Promega, Lyon, France) per gram of body mass. Twenty minutes after the injection, animals were anesthetized using a 2% isoflurane/oxygen mixture gas anesthesia and the luciferase-driven biochemoluminescent reaction was detected using the *In Vivo* Imaging System IVIS 50 (Xenogen, Waltham, MA). In order to validate the EGT in the skin, three mice were killed by cervical dislocation 4 days after EGT and 20 minutes after luciferin injection. Then, the electropermeabilized skin area was removed from the animals. Bioluminescence intensities were assessed in the skin flap and in the underlying muscles.

HLA-B7 and H2 restricted peptides

hTERT peptides restricted to HLA-B*0702 class I molecules have been previously described.[35,36] Other peptides were predicted by *in-silico* epitope prediction in order to bind mouse MHC class I, $H2K^b$, $H2D^b$ using four algorithms available online: Syfpeithi (http://www.syfpeithi.de/), Bimas (http://www-bimas.cit.nih.gov), NetMHCpan and SMM (http://tools.immuneepitope.org/main/). All synthetic peptides were purchased lyophilized (>90% purity) from Proimmune (Oxford, UK). Details of peptides sequence according to B7 or H2 restriction are shown below:

HLA-B7-restricted hTERT peptides:

- HLA-B7: RPSLTGARRL (p351)
- HLA-B7: RPAEEATSL (p277)
- HLA-B7: LPSDFKTIL (p1123)

H2-restricted hTERT peptides:

- $H2D^b$: RPIVNMDYV (p660).
- $H2K^b$: HAQCPYGVL (p429)

For availability reasons, HLA-B7-restricted hTERT peptides were used in a first assessment in ELISpot assays with transgenic HLA-B7 mice to compare EGT efficiency into the dermis using different types of electrodes. Then, H2-restricted hTERT peptides were used in ELISpot assays with C57BL/6J mice to determine the best electrical parameters for EGT into the dermal layer. The p660 peptide was also used in the *in vivo* CTL killing assay performed in C57BL/6J mice.

IFNγ ELISpot assay

IFNγ cytokine was chosen since it is strongly produced by cytotoxic CD8 T-cells, one of the most efficient antitumor effector cells.[9] An IFNγ ELISpot kit (Diaclone, Eurobio, Courtaboeuf, France) was used according to the manufacturer's instructions with some adjustments. Briefly, 14 days after ID injection of INVAC-1 and EGT, spleens were recovered and a ficoll-purified lymphocytes solution was prepared at 2×10^6 cells/ml in complete RPMI culture medium (RPMI medium supplemented with 10% heat-inactivated Fetal Calf Serum, 1% sodium-pyruvate, 1% penicillin-streptomycin and 0,1% β-mercaptoethanol). All the components mentioned above were purchased from Life technologies (Saint-Aubin, France). Lymphocytes were incubated in triplicates at 2×10^5 cells/well in the presence of the relevant H2 peptides (p660 and p429) or B7 peptides (p351, p277 and p1123) at 5 µg/ml. Positive controls with 10 µg/ml phorbol 12-myristate 13-acetate (PMA)-ionomycine and negative controls with serum-free culture medium were performed. Plates were incubated for 19 hours at 37 °C. Spots were revealed following the manufacturer's instructions and were counted using the Immunospot ELISpot counter and software (Cellular Technology, Bonn, Germany).

In vivo CTL killing assay

The *in vivo* CTL killing assay was performed using labeled and peptide-pulsed syngenic splenocytes as targets.[37] Briefly, labeling was performed using CFSE (Life Technologies) for 15 minutes at room temperature. $CFSE^{high}$ splenocytes (labeled with 5 µmol/l CFSE) from naïve C57BL/6J mice were pulsed with class I MHC-restricted p660 peptides at 5 µg/ml during 90 minutes and non-pulsed $CFSE^{low}$ splenocytes (labeled with 0.2 µmol/l CFSE) served as control. A mix containing an equal number of both fractions (high and low), that represented 6×10^6 total cells, was injected through the retro-orbital vein into immunized or control mice 14 days following, respectively, INVAC-1 or PBS injection and EP application. After 15 hours, single-cell suspensions from spleens were analyzed by flow cytometry (Myltenyii, Bergisch Gladbach, Germany). The disappearance of peptide-pulsed cells was determined by comparing the ratio of pulsed (high CFSE fluorescence intensity) to non-pulsed (low CFSE fluorescence intensity) populations in INVAC-1-immunized mice versus control non-immunized mice. The percentage of specific killing per test animal was calculated according to the following calculation:

$$[1 - [\text{mean } (CFSE^{low}PBS/CFSE^{high}PBS)/(CFSE^{low}INVAC\text{-}1/CFSE^{high}INVAC\text{-}1)]] \times 100$$

"Mean ($CFSE^{low}$PBS/$CFSE^{high}$PBS)" represents the mean of the ratios of the percentages of unpulsed cells stained with 0.2 µmol/l CFSE to the percentages of peptide-pulsed cells stained with 5 µmol/l CFSE that were detected in spleens from control non-immunized mice.

"$CFSE^{low}$INVAC-1/$CFSE^{high}$INVAC-1" represents the ratio of the percentage of unpulsed cells stained with 0.2 µmol/l CFSE to the percentage of peptide-pulsed cells stained with 5 µmol/l CFSE that were detected in spleens from the considered INVAC-1-immunized mouse.

Statistical analysis and data handling

GraphPad Prism software was used for data handling, analysis and graphic representations. A Mann–Whitney–Wilcoxon test or a Kruskal–Wallis test with Dunn's multiple comparison test was used, depending on the experiment. Significance was set at $P < 0.05$.

CONFLICT OF INTEREST

INVAC-1 plasmid is developed by the company INVECTYS. L.M.M. has a consulting activity for IGEA and holds several patents on the electroporation technologies.

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