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## Induction and analysis of antigen-specific T cell responses in melanoma patients and animal model

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## **CHAPTER 3**

# A rapid and potent DNA vaccination strategy defined by *in vivo* monitoring of antigen expression

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### **Abstract**

Induction of immunity after DNA vaccination is generally considered a slow process. Here we show that DNA delivery to the skin results in a highly transient pulse of antigen expression. Based on this information, we developed a new rapid and potent intradermal DNA vaccination method. By short-interval intradermal DNA delivery, robust T-cell responses, of a magnitude sufficient to reject established subcutaneous tumors, are generated within 12 d. Moreover, this vaccination strategy confers protecting humoral immunity against influenza A infection within 2 weeks after the start of vaccination. The strength and speed of this newly developed strategy will be beneficial in situations in which immunity is required in the shortest possible time.

## Introduction

Over the past decade, DNA vaccines have emerged as a promising approach for the induction of immune responses. Generally, current DNA vaccination strategies use a regimen of multiple intramuscular or intradermal administrations, at intervals of 2 weeks or more, and require at least 1 month to achieve immunity<sup>1, 2, 3, 4, 5, 6</sup>. The slow development of T-cell responses after DNA vaccination contrasts sharply with immune responses induced by a physiological antigen encounter, such as viral infection, that build up rapidly and often peak within 10 d<sup>7</sup>. The reason for this slow development of immune responses has remained unclear. It has been postulated that DNA vaccination leads to transfection of few cells and to the expression of relatively small amounts of antigen<sup>8, 9</sup>, therefore requiring a time-consuming prime-boost strategy. But direct evidence for the proposed low antigen expression is scarce and its role in immune induction has not been addressed. To examine whether the slow induction of immune responses could be causally related to the kinetics of antigen expression induced by DNA vaccines, we analyzed *in vivo* levels of antigen expression after intramuscular and intradermal DNA delivery, and correlated these with the induction of T-cell immunity. Based on these data, we developed a short-interval DNA vaccination method that generates functional T- and B-cell responses within a minimal time frame.

## Results

### *Intradermal DNA vaccination by skin tattooing*

To be able to administer DNA to the skin in a controlled manner, over a large surface and only in the upper nonvascularized layers, we made use of a simple tattoo device<sup>10</sup> for intradermal DNA delivery. Histochemical analysis of ‘DNA-tattooed’ skin showed that transfected cells were distributed over the upper layers of the dermis and the epidermis. (Fig. 1a,b). To test the immunogenicity of this method, we vaccinated two groups of mice with a DNA vaccine encoding the influenza A nucleoprotein epitope (amino acids 366–374; NP<sub>366</sub>) fused to the carboxy terminus of a tetanus toxin fragment (d1TTFC-NP)<sup>11</sup>, either by intramuscular injection or by skin tattoo, following the conventional DNA vaccination regimen of three administrations at 2-week intervals. This comparison shows that the intradermal delivery of a DNA vaccine using a tattoo device is an efficient strategy for the induction of T-cell immunity (Fig. 1c).

### *Imaging of in vivo antigen expression*

To monitor antigen expression upon DNA vaccination, we constructed a plasmid encoding the NP<sub>366</sub> epitope fused to the carboxy terminus of firefly luciferase (Luc-NP). The Luc-NP vaccine elicits potent NP<sub>366</sub>-specific T-cell responses (Fig. 1d). After tattoo or intramuscular administration of the Luc-NP vaccine, we used a light-sensitive camera to determine longitudinal *in vivo* antigen expression. Notably, a single intramuscular injection of DNA resulted in high levels of luciferase activity, peaking after 1 week and remaining detectable up to 1 month after injection. The

antigen expression kinetics induced by DNA tattooing were markedly different. First, peak values of antigen expression were at least ten times lower. Second, luciferase activity in the skin peaked after 6 h (data not shown) and disappeared over the next 4 d (Fig. 1e).

We subsequently determined the capacity of both methods to present the vaccine-encoded NP<sub>366</sub> epitope to naive, lymph node-resident T cells. For this purpose, 5 million carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled splenocytes from F5 TCR transgenic mice<sup>12</sup> were injected into mice at different time points after a single intramuscular DNA injection or DNA intradermal tattoo. Luciferase activity was measured on the day of cell transfer, and 3 d later the animals were killed, lymphoid organs excised and the CFSE signal of the F5 cells assessed by flow cytometry. These experiments showed that although antigen production is markedly greater upon intramuscular delivery, presentation of this antigen to naive T cells is markedly more efficient upon intradermal DNA delivery. Specifically, in spite of the high antigen expression in the muscle after intramuscular injection, the fraction of F5 cells in the draining lymph node that had undergone proliferation was marginal in all of the three tested time windows (days 1–4, days 8–11 and days 22–25; Fig. 2a–c). In marked contrast, the priming of F5 T cells after tattooing was very efficient (Fig. 2a). Independent of the number of F5 T cells that were infused (5 million or 1 million; latter not shown), over 80% of the draining lymph node-resident F5 cells had lost CFSE signal on day 4, despite the low antigen level expressed in skin (Fig. 2d). At later time points, NP<sub>366</sub>-specific T-cell activation became substantially less (Fig. 2b,c), consistent with the loss of luciferase signal observed by *in vivo* imaging. Similar results were obtained with spleen-resident F5 cells. No F5 T-cell division could be detected in non-draining lymph nodes (data not shown).

#### *Induction of T-cell immunity by short-interval DNA tattooing*

Based on the transient antigen expression and presentation induced by DNA tattooing as compared to intramuscular injection, we speculated that in the case of dermal DNA vaccination, shortening of the conventional 2-week interval could result in faster T-cell induction. To test this hypothesis, we reduced the interval between consecutive vaccinations to 3 d, resulting in a day 0, 3 and 6 regimen. Notably, this compact vaccination protocol induced profound T-cell responses of 4–8% of total CD8<sup>+</sup> T cells within 12 d after the start of vaccination (Fig. 3a). This regimen of short-interval vaccination does not lead to detectable T-cell responses when the vaccine is administered intramuscularly, consistent with the observation that the duration of antigen expression is not a limiting factor in intramuscular DNA vaccination.

To test the value of this new vaccination regimen for other methods of intradermal DNA delivery, we compared T-cell induction by short-interval gene-gun and short-interval tattoo vaccination, using the previously described Hsp70-HPV-E7 DNA vaccine<sup>13</sup>. Using the short-interval regimen, both methods generated strong T-cell



responses within 12 d against the immunodominant human papillomavirus (HPV) E7<sub>49-57</sub> cytotoxic T lymphocyte (CTL) epitope (E7<sub>49</sub>) (Fig. 3b). Comparison of T-cell responses induced by short-interval DNA tattooing with other previously established vaccination strategies showed that short-interval DNA tattooing yields markedly higher T-cell responses (tenfold or more) than peptide–incomplete Freund adjuvant–CD40-specific monoclonal antibody<sup>14</sup> or peptide-synthetic CpG oligodeoxynucleotide<sup>15</sup> vaccines (Fig. 3c). Furthermore, T-cell induction is efficient for both internal epitopes and those fused at the carboxy terminus (Supplementary Fig. 1 online). The resulting T cells are capable of direct effector function, as indicated by antigen-induced interferon (IFN)- $\gamma$  production (Supplementary Fig. 2 online).

Repetitive application of DNA may conceivably boost T-cell responses by enhancing the absolute amount of antigen expression, or by prolonging the duration of antigen expression. To address this issue, we compared antigen expression levels and T-cell responses induced by three consecutive 4 s tattoos with those induced by one single 16 s tattoo. The cumulative antigen produced by one 16 s application exceeds that of three 4 s applications (Fig. 3d). Whereas three consecutive 4 s applications induce a T-cell response that is readily detectable, a single 16 s application is essentially without effect (Fig. 3e). To further assess whether the observed requirement for repetition reflects a need for continued antigen presence, or is related to the prolongation of nonspecific inflammatory signals, groups of mice were vaccinated at day 0, 3 and 6 with different combinations of a mock vaccine and the Luc-NP vaccine. Replacing either the first or the last Luc-NP vaccine with a mock vaccine lead to a significant drop in the size of the ensuing T-cell response (Student t-test,  $P = 0.002$  and  $P = 0.003$ , respectively; Fig. 3f). Together these results show that for the induction of primary T-cell responses by intradermal DNA vaccination, prolonged antigen expression is crucial

#### *Functional immunity induced by short-interval DNA tattooing*

To assess the ability of short-interval tattoo DNA vaccination to induce therapeutic amounts of tumor-specific T cells, we used the transplantable HPV E6/E7-transformed TC-1 tumor cell model<sup>16</sup>. Three days after subcutaneous injection of  $10^5$  TC-1 cells, we vaccinated B6 mice using the short-interval tattoo method with a plasmid encoding E7<sub>49</sub> attached to the carboxy terminus of green fluorescent protein (GFP-E7; Fig. 4a). Control mice were either given an intramuscular injection in the same regimen with the same plasmid (Fig. 4b), or were tattooed with a control plasmid encoding green fluorescent protein (GFP) only (Fig. 4c). In both control groups, no T-cell responses could be detected after vaccination. In contrast, mice that had been tattooed with GFP-E7 mounted a sizeable T-cell response, up to 15% of the circulating CD8<sup>+</sup> T-cell pool. Notably, the onset of E7<sub>49</sub>-specific T-cell responses coincided with rejection of established subcutaneous tumors, whereas in both control groups tumors grew out rapidly (Fig. 4d). Furthermore, median survival of GFP-E7–tattooed animals was 50 d compared to 17 d in control animals (Fig. 4e).

To assess the value of short-interval DNA vaccination in providing antibody-mediated protection against acute infections, we evaluated its ability to confer protection against influenza A virus infection. Given that protection against reinfection with homotypic influenza A strains is primarily mediated by antibodies<sup>17, 18, 19, 20</sup>, we generated a plasmid that encodes the gene for hemagglutinin of influenza A/HK/2/68, a protein that forms a major target for neutralizing antibodies. Using this DNA vaccine, mice were vaccinated by short-interval DNA tattooing, whereas control groups were either tattooed with a GFP-encoding plasmid, or given intramuscular injection with the hemagglutinin-encoding DNA vaccine. Two weeks after vaccination, we intranasally infected mice with a sublethal dose of influenza A, and determined virus-induced morbidity by measuring body weight loss<sup>1</sup>. In the week after infection, mice that had previously been exposed to influenza A virus showed a minimal weight loss (1% at day 4 after infection). In contrast, mice given tattoo vaccination with the control plasmid or given an intramuscular injection of the hemagglutinin construct showed a sizable (10% and 14%, respectively) drop in body weight. Notably, mice that had received a short-interval hemagglutinin tattoo were largely protected from influenza A-induced morbidity (maximal weight loss of 4%; Student t-test,  $P = 0.039$  versus control vaccine; Fig. 5a). The protection correlated with the induction of neutralizing antibodies (Fig. 5b). Furthermore, intradermal DNA tattooing also conferred long-term (4 months) protection (Supplementary Fig. 3 online). Collectively, these experiments indicate that short-interval intradermal DNA vaccination leads to the rapid and sustained development of both T- and B-cell responses and that such responses can mediate the regression of established tumors and can prevent virus-induced morbidity.

### Discussion

In this study we established a short-interval regimen that induces T- and B-cell immunity within 12 d. Analysis of antigen expression levels showed that after intradermal tattoo, 1/10 to 1/100 of the amount of antigen is produced than after intramuscular injection, and that compared to intramuscular administration, tattoo-induced antigen production occurs over a limited timespan. In spite of this, the presentation of the vaccine-encoded epitope is markedly better upon intradermal tattoo vaccination. The efficiency with which dermally expressed antigens are presented to T cells probably results from the high numbers of antigen-presenting cells (APCs) present in this tissue. Also, the infliction of thousands of perforations could conceivably serve as a potent adjuvant.

Contrary to murine skin dendritic cells (DCs), human skin DCs do not express Toll-like receptor 9 (TLR9), and this may partly explain why DNA vaccines have performed poorly in human trials as compared to mouse model systems<sup>21, 22</sup>. Encouragingly, a comparison of the immunogenicity of tattoo DNA vaccination in wild-type and *Tlr9*<sup>-/-</sup> mice showed that the absence of TLR9 does not measurably affect the immunogenicity of this method (Supplementary Fig. 4 online).

The short-interval repetitive schedule developed here leads to rapid induction of T-cell responses upon either intradermal tattoo or gene-gun vaccination, and may well work for all intradermal vaccination techniques. Because of its simplicity and because the required equipment is orders of magnitude less expensive, DNA tattooing may become the preferred method, in particular in countries with a less developed healthcare system.

In the past years, outbreaks of Ebola virus, severe acute respiratory syndrome and influenza A have been causes for concern. To contain such outbreaks conventional DNA vaccination strategies appear too slow at inducing immunity<sup>23, 24</sup>. The vaccination regimen described here retains the rapid production and the safety of DNA vaccines, but gains the speed of immune induction that is the characteristic of physiological antigen encounters.

### Acknowledgements

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### Methods

#### *Animals.*

We obtained C57BL/6 mice and Balb/c mice from the experimental animal department of The Netherlands Cancer Institute. The Tlr9<sup>-/-</sup> mice were a gift of H. Wagner ((with permission of S. Akira) Institute for Microbiology, Immunology and Hygiene, University of Munich, Germany). We purchased from the US National Cancer Institute the C57BL/6 mice used for the experiments involving a gene gun and kept them in the oncology animal facility of the Johns Hopkins Hospital. We performed all animal procedures according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the Dutch Animal Research Committee.

#### *DNA vaccines.*

We generated DNA vaccines by the introduction of fusion genes in pcDNA3.1. The GFP-E7 fusion gene has been described previously<sup>25</sup>, all other constructs were generated following a similar design. We included the four naturally flanking amino acid residues of NP<sub>366-374</sub> (amino acids GVQI) as a linker at the amino terminus of each epitope in all constructs. We constructed the d1TTFC-NP DNA vaccine with optimized codon usage, in a template-free PCR using overlapping oligonucleotides of 100 bp, spanning the entire first domain of the TTFC gene and the preceding p2 epitope (amino acids 831–1,315<sup>26</sup>). We generated the Luc-NP vaccine, encoding influenza A NP<sub>366-374</sub> epitope fused to the carboxy terminus of firefly luciferase, by genetic linkage of the NP<sub>366-374</sub> fragment to the carboxy terminus of the gene encoding full-length firefly luciferase. The gene encoding influenza A/HK/2/68

hemagglutinin was obtained by RT-PCR of RNA isolated from virus particles, and inserted in pcDNA3.1. to generate the hemagglutinin DNA vaccine. We purchased the construct used for histochemical detection of transfected cells (pVAX:LacZ) from Invitrogen. Replacement of the lacZ gene with chicken ovalbumin generated the construct encoding whole ovalbumin. The generation of the sigE7hsp DNA vaccine was described previously<sup>13</sup>. Sequences were confirmed by sequence analysis. All DNA batches were purified using EndoFree Plasmid kit (Qiagen).

#### *DNA vaccination.*

For intramuscular DNA vaccination, we shaved the hind leg of the mouse and injected it with 100 µg of DNA in 50 µl HBSS (Life Technologies). For intradermal DNA vaccination, we shaved the left hind leg of the mouse, applied a droplet of 20 µg DNA in 10 µl HBSS to the skin and used a sterile disposable 11-needle bar (Radical Clean Magnum11, Eurl Toupera) mounted on a rotary tattoo device (Cold skin, B&A Trading) to apply the vaccine. Needle depth was adjusted to 0.5 mm, and the needle bar oscillated at 100 Hz. DNA vaccines were applied to the skin by a 16 s tattoo. Gene-gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad) according to the manufacturer's protocol. At various time points after immunization, we drew approximately 20 µl of peripheral blood for analysis of T-cell responses.

#### *Detection of NP- and HPV-specific T cells in peripheral blood.*

Peripheral blood lymphocytes were stained with phycoerythrin-conjugated antibody to CD8β (BD Pharmingen) plus allophycocyanin-conjugated H-2D<sup>b</sup>/NP<sub>366-374</sub>-tetramers or allophycocyanin-conjugated H-2D<sup>b</sup>/E7<sub>49-47</sub> tetramers, at 20° C. for 15 min in FACS buffer (PBS, 0.5% BSA and 0.02% sodium azide). We washed cells three times in PBA and analyzed them using flow cytometry. Live cells were selected based on 7-AAD exclusion. We performed IFN-γ assays using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. Peripheral blood lymphocytes were stimulated for 4 h at a 100 nM peptide concentration.

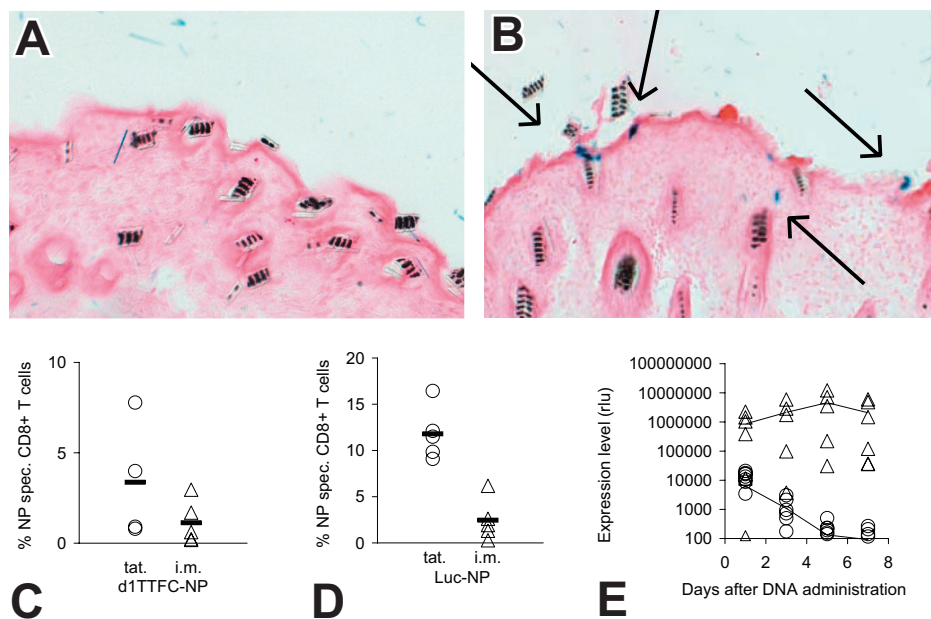
#### *Intravital imaging.*

We anesthetized mice with isoflurane (Abbott Laboratories). We intraperitoneally injected an aqueous solution of the substrate luciferin (150 mg/kg, Xenogen) and 18 min later the luminescence produced by active luciferase was acquired during 30 s in an IVIS system100 CCD camera (Xenogen). Signal intensity was quantified as the sum of all detected light within the region of interest, after subtraction of background luminescence.

#### *Influenza A infection and detection of influenza A-specific antibodies.*

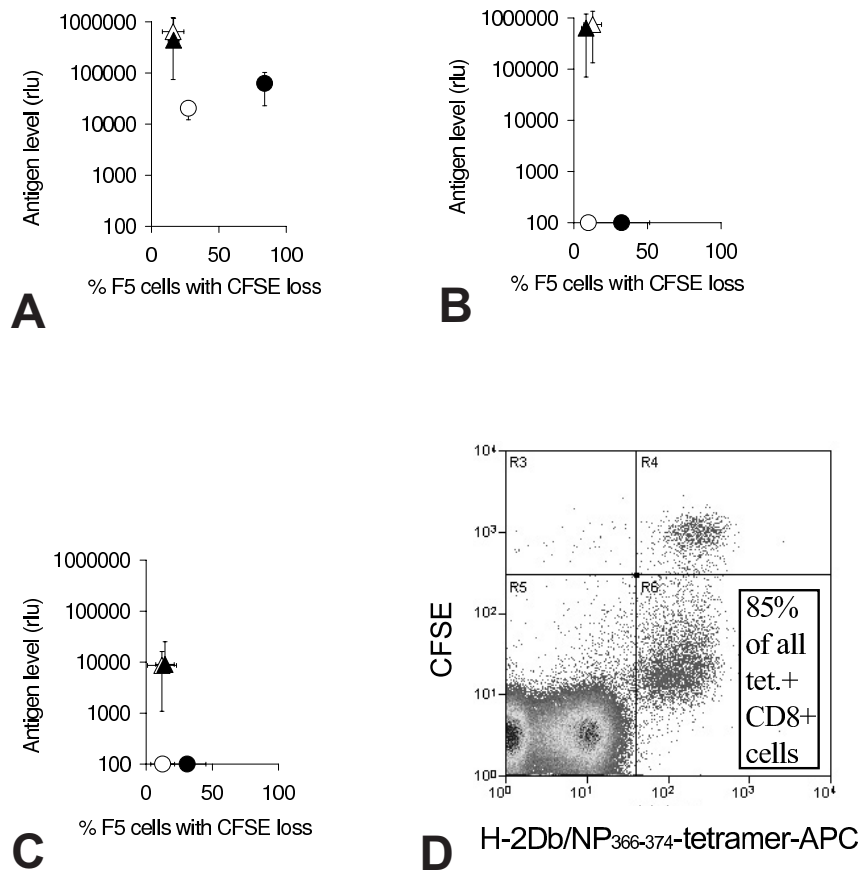
Purified influenza A/HK/2/68 virus was provided by G. Rimmelzwaan (Department of Virology, Erasmus University). We infected mice intranasally with 20 hemagglutinating units (HAU) of virus in PBS, under general anesthesia. Influenza

A/HK/2/68-specific antibodies were detected in a hemagglutination inhibition (HAI) assay. Serum samples (50  $\mu$ l) were serially 1:2 diluted with PBS (1% BSA) in round-bottomed polystyrene microtiter plates, in duplicate, and 50  $\mu$ l of A/HK/2/68 influenza virus corresponding to 4 HAU was added to each well. After 45 min incubation at room temperature, we added 50  $\mu$ l of 0.5% chicken red blood cells to each well. After 1 h, we determined the maximal serum dilution that fully inhibited the hemagglutination caused by the virus in the duplicate wells. The HAI titer was expressed as the reciprocal of this serum dilution.



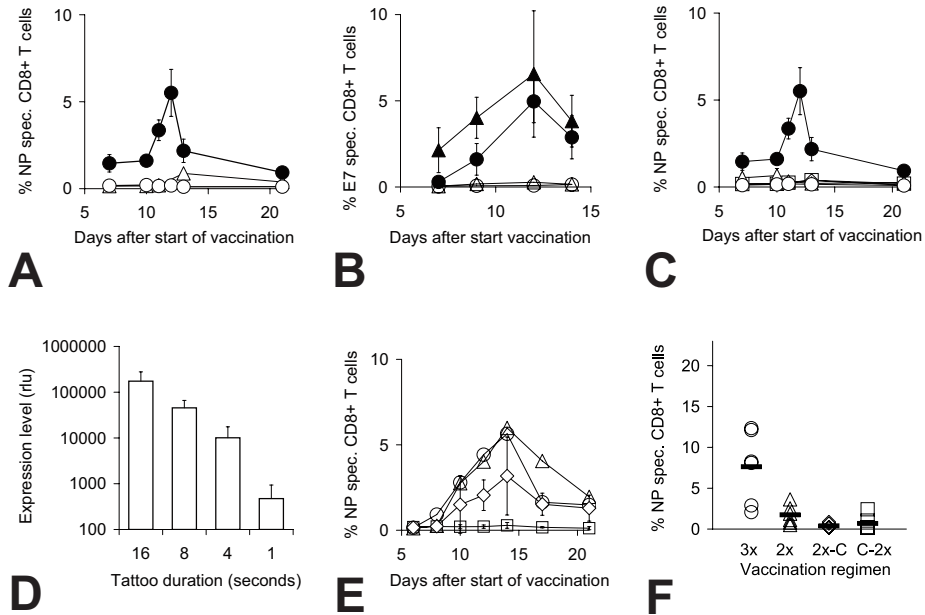
**Figure 1.** DNA tattooing results in transfection of epidermal and dermal cells and leads to efficient T-cell induction.

(a) The abdominal skin of a mouse was tattooed with a  $\beta$ -galactosidase (*lacZ*)-encoding construct. Six hours after treatment, transfected cells were shown in a skin biopsy, using the X-gal substrate to generate a blue precipitate in cells expressing the transgene (arrows). (b) The abdominal skin of a mouse tattooed with empty vector and processed as in a. (c) NP<sub>366</sub>-specific CD8<sup>+</sup> T-cell responses induced by tattoo (tat., circles, n = 5) or intramuscular (i.m., triangles, n = 5) DNA vaccination. The d1TTFC-NP DNA vaccine was administered three times at 2-week intervals. T-cell responses were measured 7 d after the last DNA administration, by staining peripheral blood lymphocytes with H-2D<sup>b</sup>/NP<sub>366-374</sub> tetramers. Horizontal bars depict averages. (d) NP<sub>366</sub>-specific T-cell responses induced by tattoo DNA vaccination (circles, n = 5) and i.m. DNA vaccination (triangles, n = 5) with the Luc-NP DNA vaccine. (e) Kinetics of Luc-NP antigen expression after a single intradermal DNA tattoo (circle) and after a single intramuscular DNA injection (triangle).



**Figure 2.** Analysis of antigen production and presentation after intradermal and intramuscular DNA vaccination.

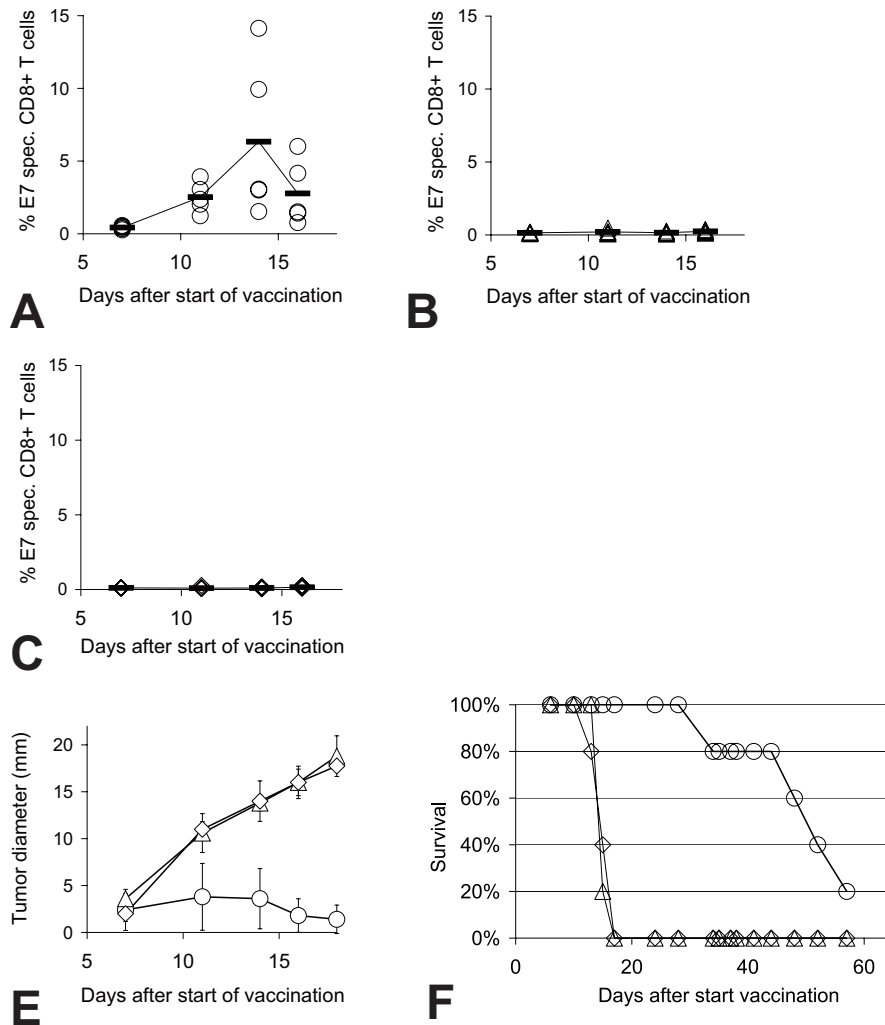
(a–c) Cohorts of mice ( $n = 7$ ) were vaccinated once with the Luc-NP vaccine, either by intradermal DNA tattooing (filled circles) or by intramuscular DNA injection (filled triangles). Control groups ( $n = 3$ ) received a plasmid encoding luciferase only (intradermal, open circles; intramuscular, open triangles). CFSE-labeled naive F5 T cells were transferred on day 1 (a), day 8 (b) or day 22 (c) after DNA administration, and were recovered from the draining lymph nodes 3 d after transfer. Proliferation was assessed by analysis of CFSE loss of H-2Db/NP<sub>366-374</sub> tetramer-positive CD8<sup>+</sup> cells. (d) Representative draining lymph node sample from the tattooed cohort on day 4.



**Figure 3.** Features of tattoo DNA vaccination.

(a) NP<sub>366</sub>-specific T-cell responses in cohorts of mice (n = 5) upon vaccination with d1TTFC-NP on day 0, 3 and 6 either by tattoo (filled circles) or intramuscular injection (open triangles). Control mice (open circles) were tattooed with a TTFC mock vaccine. NP<sub>366</sub>-specific T-cell responses were determined at indicated time points by tetramer staining of peripheral blood lymphocytes (n = 5). (b) The E7<sub>49</sub>-specific T-cell response after intradermal application of the sigE7hsp DNA vaccine at day 0, 3 and 6 in cohorts of mice (n = 6), either by DNA tattooing on the left leg (filled circles) or by gene gun on both flanks of the abdomen (filled triangles). Control groups of mice (n = 3) were vaccinated with empty vector (open triangles and open circles). (c) NP<sub>366</sub>-specific T-cell responses in cohorts of mice (n = 5) upon subcutaneous vaccination with the NP<sub>366</sub> peptide in incomplete Freund adjuvant at day 0 (open triangles), subcutaneous injection of NP<sub>366</sub> peptide with CpG in PBS at day 0 (open circles) or at day 0, 3 and 6 (open squares), NP<sub>366</sub> peptide with CpG in PBS tattoo vaccination at day 0, 3 and 6 (open diamonds) and DNA tattoo vaccination with d1TTFC-NP on day 0, 3 and 6 (filled circles). (d) Antigen expression level after single tattoos of 16, 8, 4 and 1 s, as determined by a light-sensitive camera 1 d after tattooing with the Luc-NP vaccine. (e) NP<sub>366</sub>-specific T-cell responses (n = 5) induced by 16 s (open circles), 8 s (open triangles) and 4 s (open diamonds) tattoos on day 0, 3 and 6, or a single 16 s tattoo on day 0 (open squares). (f) In mice vaccinated with the Luc-NP DNA vaccine at day 0, 3 and 6, the replacement of Luc-NP with a mock DNA vaccine on day 0 (open squares; C-2x) or day 6 (open diamonds; 2x-C) reduces T-cell responses from levels induced by three consecutive tattoos (open circles; 3x), to levels induced by two consecutive tattoos (open triangles; 2x).

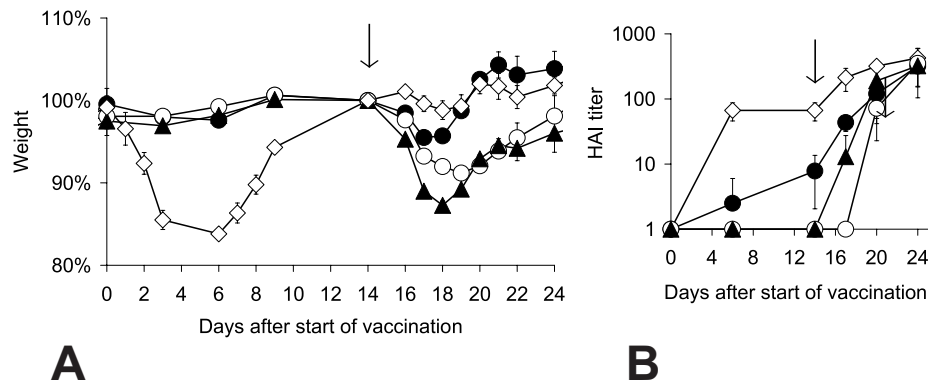




**Figure 4.** Tattoo DNA vaccination leads to rapid induction of functional tumor-specific T cells.

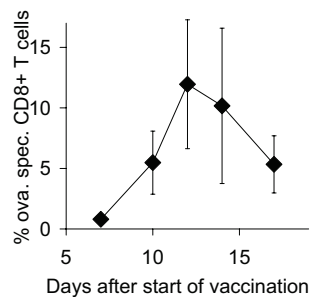
(a-c) Mice were injected subcutaneously with  $10^5$  TC-1 cells. Three days later, mice were vaccinated three times at 3-d intervals either by GFP-E7 tattooing (open circles; a), by intramuscular injection (open triangles; b), or by tattooing with a mock vaccine (open diamonds; c). E7<sub>49</sub>-specific CD8<sup>+</sup> T-cell responses were determined by major histocompatibility complex tetramer staining of peripheral blood cells at indicated time points. Horizontal bars depict averages. (d) Tumor outgrowth in the cohorts of mice depicted in a-c; symbols are as in a-c. (e) Long-term survival of the mice depicted in a-c; symbols are as in a-c. Values represent mean  $\pm$  s.d.





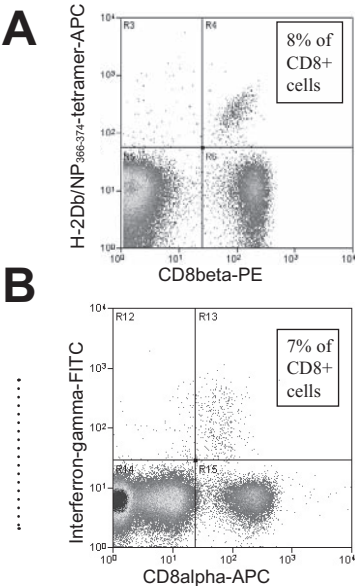
**Figure 5.** Tattoo DNA vaccination induces B-cell immunity against influenza A within 2 weeks.

(a) Cohorts of Balb/c mice ( $n = 6$ ) were vaccinated at day 0, 3 and 6 either by intramuscular injection (filled triangles) or by tattooing (filled circles) with a construct encoding hemagglutinin. A positive control group was infected intranasally with influenza (open diamonds) and a negative control group tattooed using a mock vaccine (open circles). All mice were challenged with a sublethal dose of the influenza A virus at day 14 (indicated by the arrow), and weighed daily. The hemagglutinin-tattooed mice showed a significantly smaller drop in weight than the mock-tattooed mice (Student t-test,  $P = 0.039$ ). Both in the mock-vaccinated and the intramuscularly vaccinated group, one mouse died from influenza-induced pneumonia. These animals were not included in the analysis. Values represent the weight relative to the weight at time of challenge. Error bars, s.e.m. (b) Influenza A/HK/2/68-specific antibodies in the sera of the mice depicted in a, at various time points after the start of vaccination and the influenza A challenge (symbols as in a). The presence of antibodies was assessed in an HAI assay. Values represent mean  $\pm$  s.d.

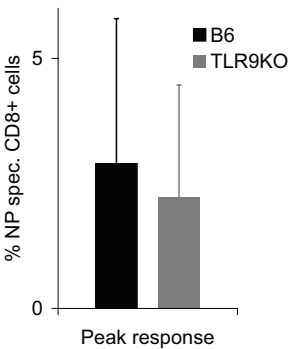
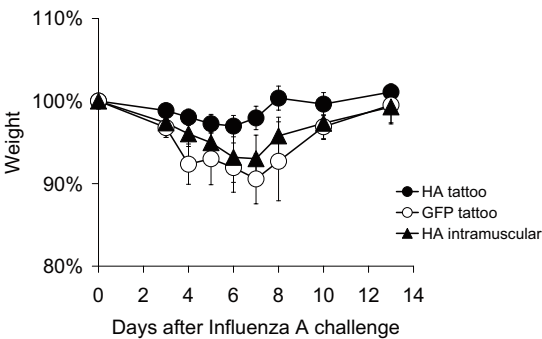


**Supplementary Figure 1.** DNA tattoo vaccination efficiently induces T cells against internal epitopes. Responses specific for the Chicken Ovalbumin<sub>257-264</sub> epitope, in mice ( $n=5$ ) that were short-interval tattoo vaccinated with a construct encoding whole chicken ovalbumin. Values represent the mean  $\pm$  standard deviation.

**Supplementary Figure 2.** T cells induced by DNA tattoo vaccination are capable of direct effector function. (a) Tetramer staining of PBLs of a representative mouse, at day 11 after vaccination with the Luc-NP DNA vaccine. (b) Intracellular interferon- $\gamma$  staining of the same blood sample as in (a).



**Supplementary Figure 3.** Tattoo DNA vaccination induces long-term immunity against influenza A. Cohorts of Balb/c mice (n=6) were vaccinated with a construct encoding HA, either at day 0, 14 and 28 by i.m. injection (▲) or at day 0, 3 and 6 by tattooing (●). A negative control group was tattooed using a mock vaccine (○). All mice were intranasally infected with a sublethal dose of the influenza A virus 109 days after the last DNA administration and weighed daily after the challenge. The HA tattooed mice showed a significantly smaller drop in weight than the mock tattooed mice (student t-test: P=0.038). Values represent the weight relative to the weight at time of challenge. Error bars depict the standard error of the mean.



**Supplementary Figure 4.** Absence of TLR9 does not measurably affect the immunogenicity of DNA tattoo vaccination. Peak NP366-specific T cell response induced by Luc-NP short interval tattoo DNA vaccination, in wildtype C57BL/6 mice (black bar) compared to TLR9<sup>-/-</sup> mice (gray bar). Values represent the mean +/- standard deviation.

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