Vaccination with human papillomavirus type 16-derived peptides using a tattoo device

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A bstract

Tattooing has been shown to be very efficient at inducing immunity by vaccination with DNA vaccines. In this study, we examined the usability of tattooing for delivery of peptide vaccines. We compared tattooing with subcutaneous (s.c.) needle injection using peptides derived from human papillomavirus type 16 (HPV16) proteins. We observed that higher peptide-specific immune responses were elicited after vaccination with the simple peptides (E744–62, and E749–57) and keyhole limpet hemocyanin-(KLH)-conjugated peptides (E749–57, L218–38, and L2108–120) with a tattoo device compared to s.c. inoculation. The administration of the synthetic oligonucleotide containing immunostimulatory CpG motifs (ODN1826) enhanced the immune responses developed after s.c. injection of some peptides (E744–62, KLH-conjugated L218–38 and L2108–120) to levels close to or even comparable to those after tattoo delivery of identical peptides with ODN1826. The highest efficacy of tattooing was observed in combination with ODN1826 for the vaccination with the less immunogenic E648–57 peptide and KLH-conjugated and non-conjugated E749–57 peptides which form the visible aggregates that could negatively influence the development of immune responses after s.c. injection but probably not after tattooing. In summary, we first evidenced that tattoo administration of peptide vaccines that might be useful in some cases efficiently induced both humoral and cell-mediated immune responses.

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1. Introduction

Peptide-based vaccines are being developed for the treatment of infections [1], allergic and autoimmune diseases [2–4] and cancer [5,6]. The synthesis of peptides in clinical grade is relatively easy and peptides are free of any oncogenic potential, in contrast to DNA vaccines directed against oncoproteins [5]. Moreover, no major toxicity has been reported after peptide vaccination. To design peptide-based vaccines, the identification of specific antigens is initially required followed by immunogenic epitope(s) identification [5]. The major disadvantage of peptide-vaccine therapy is the restriction of one peptide to a single MHC molecule. However, this problem may be overcome by administration of combinations of various peptides, so-called peptide cocktail vaccines [6]. In some clinical trials, partial or complete tumor regression has been observed in approximately 10–30% of patients after therapeutic peptide vaccinations [7].

To enhance the immunogenicity of peptide-based vaccines, different strategies are used. The natural epitopes may be modified to improve the affinity to the MHC molecules, the stability of MHC complexes, or proteolytic stability and bioavailability [5]. Peptides are typically administered with an adjuvant such as incomplete Freund adjuvant (FA), aluminium salts and immunomodulatory molecules [6,8] or conjugated with helper proteins [5] or lipids [9]. A number of adjuvants have been described and may be used in animals, yet in many cases adjuvants induce untoward reactions that limit their broad applicability, e.g. they provoke high inflammation, irritation, ulceration, etc. Currently, only few adjuvants are approved for use in humans [10]. Therefore, delivery methods that allow efficient immunization without adjuvants are highly desirable.

For DNA vaccination, tattooing has been shown to induce higher cellular and humoral immune responses than intramuscular needle injection [11,12]. The tattoo procedure causes many minor mechanical injuries followed by hemorrhage, necrosis, inflammation, and regeneration of the skin and thus non-specifically stimulates the immune system [13]. Therefore, tattooing may partially substitute for the function of adjuvants [12]. To the best of our knowledge, tattooing has not yet been tested for administration of peptide vaccines. However, tattoo delivery of a modified amino acid, bleomycin, has been reported as a promising therapeutic modality in large keloids and hypertrophic scars [14].
In this study, we examined the implication of tattoo delivery on peptide vaccination. As model peptide antigens, we used human papillomavirus type 16 (HPV16)-derived peptides containing either a CTL epitope (E749–57 and E648–57), a B-cell epitope (L218–38 and L208–120) or combined CTL, Thelper- and B-cell epitopes (E744–62). Our results indicate that tattoo delivery is a more efficient method of peptide immunization than s.c. needle injection.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice aged 6–8 weeks (H-2b; Charles River, Sulzfeld, Germany) were used in the immunization experiments. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals at the Center for Experimental Biomodels, Charles University, Prague.

2.2. Cell lines

The efficacy of HPV16-derived peptide vaccines was evaluated using TC-1 tumor cells [15] prepared by transformation of primary C57BL/6 mouse lung cells with HPV16 E6/E7 oncoproteins and activated H-ras (kindly provided by T.-C. Wu, Johns Hopkins University, Baltimore, MD). Cells were grown in Dulbecco’s Modified Eagle’s Medium (D-MEM; PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (PAA Laboratories), 2 mM l-glutamine, and 100 µg/ml penicillin.

2.3. Plasmid

The construction of the plasmid pBSC/E7GGG.GUS has been described previously [16]. Briefly, the fusion gene E7GGG.GUS coding for the mutated (D21G, C24G and E26G) HPV16 E7 protein denoted E7GGG and Escherichia coli beta-glucuronidase was inserted downstream of the CMV promoter into the EcoRI site of the mammalian expression plasmid pBSC [17].

2.4. Synthetic peptides

The HPV16-derived peptides, E749–57 (RAHYNIVTF), E744–62 (QAEPPRDAHYVINFTCCCKD, acetylated on the first amino acid), E648–57 (EVYDFADFRL), L218–38 (LYKTCKQAGTCDPDIIKVEG) and L208–120 (LVEETSFIDAGAP), were used for immunization and detection of induced immune responses. In addition, the peptide L2108–120 (LVEETSFIDAGAP) was utilized in the ELISA detection assays against the L2108–120 peptide. The peptides were synthesized by the Fmoc solid-phase method (Clonestar Peptide Services, Brno, Czech Republic). Peptide purity was determined to be >90% by high-pressure liquid chromatography (HPLC) and peptide sequences were validated by mass spectrometry (MALDI-TOF). The L218–38, L208–120 and L2108–120 were conjugated with the carrier KLH protein by the glutaraldehyde method (Clonestar Peptide Services). Lyophilized peptides were dissolved in water (E749–57, E744–62 and L218–38) or a small amount of DMSO followed by addition of water (E648–57, L208–120 and L207–122). For immunizations, 10× concentrated PBS was added to the dissolved peptides to obtain the final dilutions of peptides in 1× PBS. The E749–57 peptide (both simple and KLH-conjugated) tended to aggregate in PBS.

2.5. Immunizations

Mice were immunized two or three times at 2-week interval. One immunization dose contained 50 or 100 µg of a peptide with or without 50 µg of CpG adjuvant (ODN1826: TCCATGACGTTCCT- GAGCTT; GENERI BIOTECH, Hradec Kralove, Czech Republic), 50 µg of a KLH-peptide conjugate or 1 µg of DNA. For needle delivery, peptides were s.c. injected into the dorsum of mice in a final volume of 200 µl PBS using a 29G needle (Chirana, Stara Tura, Slovak Republic) hold in horizontal orientation and inserted about 5 mm subcutaneously. The KLH-conjugated peptides in PBS were delivered either without FA or mixed 1:1 (v/v) with complete FA (Gibco) for the first s.c. needle injection and with incomplete FA (Sigma) for the following immunizations. For tattoo delivery, peptides were administered in 20 µl PBS on the shaved skin at the dorsum and then a commercial tattoo machine (Rotary 12000 AL, Bortech Tattoogroßhandel, Wuppertal, Germany) was used for delivery. The tattoo device was adjusted to allow exposure of the needle tip 1–2 mm beyond the barrel guide. This depth of tattooing into the mouse skin has been shown to result in the immediate location of tattooed inks mainly in the dermis and to a lower extent in the epidermis [13]. A skin surface area of approximately 2 cm × 1 cm was tattooed by 50 one-second treatments with a five-needle unit (5-linear tattoo needle, Bortech Tattoogroßhandel) oscillating at a voltage of 17.4 V set on the power supply (DC POWER SUPPLY, DF 1730 SB3A, Bortech Tattoogroßhandel) corresponding to a frequency of 145 Hz (145 punctures per second). Thus, every tattooed mouse received during one immunization a total number of 36 250 (5 × 50 × 145 = 36 250) solid needle pricks. The tattoo procedure was well tolerated. The preparation of cartridges for DNA vaccination and gene gun immunization were performed as described previously [16]. Briefly, 1 µg of the pBSC/E7GGG.GUS plasmid was coated onto 0.5 mg of 1 µm gold particles and was delivered by the gene gun at a discharge pressure of 400 psi into the shaven skin of the abdomen as a single immunization.

2.6. Preparation of splenocytes for assays of E7-specific T-cell responses

For in vitro assays, pools of splenocytes from each vaccinated group (3 mice per group) were prepared 2 weeks after the last vaccination and used either fresh (ex vivo) or after restimulation with peptides for 5–7 days. Splenocytes were stimulated with the HPV16-derived E648–57 [18], E749–57 [20] or E744–62 [21] peptides carrying the H-2b CTL epitope or cultured in medium without the peptides (negative control). All samples were cultivated in two parallel wells.

2.7. ELISPOT assay

The IFN-γ-ELISPOT assay was performed using both fresh splenocytes (1 × 10^6/well) and splenocytes after 6-day restimulation. The 96-well filtration plates (Millipore Corp., Bedford, MA) were coated with 10 µg/ml rat anti-mouse IFN-γ antibody (BD Biosciences Pharmingen, San Diego, CA) in 50 µl of PBS per well. After overnight incubation at 4 °C, the wells were washed and then blocked with culture medium containing 10% fetal calf serum. Different concentrations of either non-stimulated or stimulated splenocytes from each vaccinated group of mice, starting from 1 × 10^6/well, were added to the wells. Cells were incubated at 37 °C for 20 h either with or without different concentrations (from 0.001 to 10 µg/ml) of the E749–57, E744–62 or E648–57 peptide. The plates were washed and incubated with 5 µg/ml biotinylated anti-IFN-γ antibody (BD Biosciences Pharmingen) in 50 µl of PBS per well at 4 °C overnight. After washing, the avidin-horseradish peroxidase conjugate (BD Biosciences Pharmingen) was added and the plates were incubated for 2 h at room temperature. After washing, spots were developed by adding 50 µl of 0.5 mg/ml aminoethylcarbazole solution (Fermentas, Hanover, MD) and 0.03% H_2O_2 and incubated at room temperature for 1 h. Spots were
evaluated by the Eli.Scan ELISPOT Scanner (A.ELVIS, Hannover, Germany).

2.8. Intracellular cytokine staining (ICS)

The intracellular cytokine staining of IFN-γ in CD8+ cells was performed as described recently [22]. In brief, the splenocytes were incubated with the peptides for 20 h (ex vivo assay) or 6 days with one exchange of media (assay after restimulation). Twelve hours before staining, GolgiStop (BD Biosciences Pharmingen) was added to the culture medium according to the manufacturer’s instructions. Surface CD8 molecules were stained with PE-conjugated rat anti-mouse CD8a monoclonal antibody (BD Biosciences Pharmingen). Then, cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences Pharmingen) and intracellular IFN-γ was stained with FITC-conjugated rat anti-mouse IFN-γ monoclonal antibody (BD Biosciences Pharmingen). The stained cells were measured on a Coulter Epics XL flow cytometer (Coulter, Miami, FL) and analyzed by Flowjo 7.1.2. software (TreeStar, Ashland, OR).

2.9. Tetramer staining

In the tetramer-staining assay, the E749–57-specific CD8+ cells were detected in the splenocytes restimulated with the E749–57 peptide for 5 or 7 days as described previously [23]. After restimulation, splenocytes were centrifuged and resuspended in 50 μl RIA buffer (PBS supplemented with 1% BSA and 0.09% sodium azide) containing 5 μg/ml rat anti-mouse CD16/CD32 (Fc-block; BD Biosciences Pharmingen) and incubated on ice for 15 min. Washed cells were stained in 20 μl with 0.5 μl the PE-labeled H-2Db/E749–57 tetramer reagent (PeliMer H-2Db/E7, Sanquin, Amsterdam, Netherlands) for 20 min at room temperature. The 5 μl of RIA buffer with 50 μg/ml of FITC-conjugated rat anti-mouse CD8a monoclonal antibody (BD Pharmingen) was added and splenocytes were incubated for another 20 min at room temperature. After washing, the stained cells were measured on a Coulter Epics XL flow cytometer (Coulter) and analyzed by Flowjo 7.1.2. software (TreeStar).

2.10. Detection of peptide-specific antibodies by ELISA

Sera of immunized mice were collected either 10 days after each immunization or 14 days after the final immunization, and analyzed in enzyme-linked immunosorbent assay (ELISA). The 96-well plates were coated overnight at 4 °C with 50 μl PBS containing 10 μg/ml of the E744–62, L2108–120 or L2107–122 peptides or KLH protein (Merck Biosciences, Nottingham, UK). Antibodies against the L2108–120 peptide were detected using the L2107–122 peptide because ELISAs with other coating substances like the simple or BSA-conjugated L2108–120 peptide were less sensitive. Plates were washed three times in washing buffer (PBS with 0.3% Tween 20) and blocked with 100 μl 3% milk in washing buffer for 1 h at 37 °C. Mouse sera diluted in 50 μl of 1.5% milk in washing buffer were added to the plate either at a single dilution of 1:50 or at a dilution of 1:10 followed by twofold dilutions starting at 1:50 and ending at 1:104,857,600 and incubated for 1 h at 37 °C. Non-specific binding was determined using the dilution of 1:10 or 1:50 of the mouse sera on plates coated with PBS only. Then, the plates were washed and incubated with 50 μl well of sheep anti-mouse IgG antibodies conjugated to horseradish peroxidase (HRP; Amersham Biosciences, Buckinghamshire, UK) diluted 1:2000 or rat anti-mouse IgG1 or IgG2a antibodies conjugated to HRP (BD Pharmingen) diluted 1:500 in 1.5% milk in washing buffer for 1 h at 37 °C. After final washings, the plates were stained with 100 μl of 0.1% 3,3′-diaminobenzidine (Sigma) and 0.003% H2O2 in 0.1 M acetic acid (pH 6) for 20–30 min. The reaction was stopped by addition of 50 μl of 1 M sulfuric acid and the absorbance was measured at 450 nm. Titres were expressed as reciprocals of the final serum dilution giving an absorbance higher than 0.2. Sera with a titre less than 10 were considered negative and a value of 1 was assigned to them for computational purposes.

2.11. The HPV16-neutralization assay

The neutralization assay was performed as described previously [24]. Briefly, the 293TT cells were incubated with a mixture of HPV16 L1/L2 pseudovirions encapsidating the gene coding for secreted alkaline phosphatase (SEAP) and sera diluted 1:50 and 1:200 in DMEM (Sigma). Polyclonal neutralizing rabbit antiserum specific for HPV16 L1 and L2 were used as positive controls. Detection of SEAP in cell culture supernatant was performed with the chemiluminescent SEAP Reporter Gene Assay (Roche). All sera were tested in duplicates.

2.12. Tumor protection experiment

Mice (5 per group) were vaccinated three times at 2-week interval. Two weeks after the last vaccination, mice were s.c. challenged into the back with 3 × 106 TC-1 cells suspended in 0.15 ml PBS. Tumor cells were administered under anaesthesia with etomidate (0.5 mg i.p.; Janssen Pharmaceutica, Beerse, Belgium). Tumor growth was monitored twice a week. Tumor size was calculated from three perpendicular measurements using the formula (a × b × c)π/6.

2.13. Statistical analysis

Tumor formation was analyzed by the log-rank test. Tumor growth was evaluated by two-way analysis of variance. Data of endpoint ELISAs were analyzed by the Mann–Whitney test (Wilcoxon Rank sum test). A difference between groups was considered significant at P < 0.05. Calculations were performed using Prism software, version 4.0 (Graph-Pad Software, San Diego, CA).

3. Results

3.1. Tattoo delivery of the E749–57 peptide with CpG motifs elicited cellular immune responses comparable to those after gene-gun DNA vaccination with pBSC/E7GGG.GUS

To evaluate the efficacy of peptide immunization by tattooing, we compared the peptide vaccine based on the HPV16 E7 immunodominant CTL epitope and our most efficient DNA vaccine against the E7 oncoprotein [16]. We immunized mice three times with 100 or 50 μg of the E749–57 peptide in combination with 50 μg of CpG motifs by tattooing and with 1 μg of the pBSC/E7GGG.GUS plasmid administered by the gene gun. Then, we performed the ELISPOT assay to detect IFN-γ-secreting CD8+ T cells in splenocytes from vaccinated mice. The results of a representative experiment, in which splenocytes were restimulated with the E749–57 peptide for 6 days, are shown in Fig. 1A. The immune response induced by tattooing was comparable with that after gene-gun DNA immunization.

The in vitro assays, i.e. ELISPOT (Fig. 1A), ICS and tetramer staining (data not shown), revealed that after vaccination with 50 μg of the E749–57 peptide combined with 50 μg of CpG motifs, the counts of E7-specific CD8+ T lymphocytes reached 60–100% of those observed after vaccination with 100 μg of the E749–57 Peptide with 50 μg of CpG motifs.

The potential of tattooing with the E749–57 peptide vaccine was also assessed by the preventive immunization against TC-1 cells (Fig. 1B). In contrast to naive mice that developed tumors within 19 days after inoculation of tumor cells, all tattooed mice remained
were detected after 6-day restimulation with the E7 49–57 peptide by an ELISPOT assay. Cytokine cultures were prepared from pools of splenocytes, and IFN-γ producing cells were also found after immunization with the E744–62 peptide and CpG motifs (about 0.4% versus 0.2% IFN-γ+ CD8+ T cells), the E749–57 peptide (0.04% versus 0.01%) and the E744–62 peptide (0.02% versus 0.01%).

For the tetramer assay, splenocytes were restimulated by a 7-day incubation with the E749–57 peptide (Fig. 2B). Higher rates of CD8+ lymphocytes were tetramer positive in splenocytes from tattooed mice than from animals after s.c. administration of the E749–57 peptide with CpG motifs (about 9% versus 0.1%), the E744–62 peptide with CpG motifs (about 6% versus 4%), the E749–57 peptide alone (about 0.7% versus 0.4%) and the E744–62 peptide alone (about 0.9% versus 0.5%). The stimulation of CD8+ T cells was so high in groups of mice tattooed with the E749–57 peptide or the E744–62 peptide with CpG motifs that the numbers of tetramer+/CD8+ T cells were enhanced not only after incubation with the E749–57 peptide but also after cultivation in a medium without the peptide (about 1% or 0.3%, respectively, versus 0.02%, compared with the negative control naive mice). No E7-specific splenocytes were revealed in the control groups of naive mice and those treated with CpG motifs by tattooing.

Humoral responses induced by peptide vaccination were determined by ELISA using the E744–62 peptide. All mice immunized with the E744–62 peptide (12/12) developed E7-specific antibodies, while all animals vaccinated with the E749–57 peptide (12/12) or CpG motifs alone (3/3) and naive controls (3/3) did not produce E7-specific antibodies (Fig. 2C). The end-point titration of sera from mice immunized with the E744–62 peptide revealed that the geometric mean titre of E7-specific IgG antibodies produced after tattoo vaccination with the peptide in combination with CpG motifs was about 60-fold higher than after subcutaneous immunization with the identical vaccine. Similarly, an about 10-fold higher geometric mean titre was found in mice tattooed with the peptide alone than in those immunized subcutaneously. The addition of CpG motifs enhanced the levels of E7-specific antibodies after immunization by both s.c. needle injection and tattooing. Furthermore, we determined subclasses of E7-specific IgG antibodies, IgG1 and IgG2a, produced in mice after the vaccination with the E744–62 peptide (Fig. 2D). In correlation with the previous detection of total IgG antibodies (Fig. 2C), the summation of IgG1 and IgG2a antibodies was higher in mice vaccinated with the peptide by tattoo and/or in combination with CpG motifs than that in mice immunized subcutaneously and/or in the absence of CpG motifs. The production of IgG1 antibodies was slightly decreased in the presence of CpG motifs. While IgG2a antibodies were not found in mice vaccinated with the E744–62 peptide in the absence of CpG motifs, all 3 mice and 2 out of 3 mice vaccinated with the peptide in combination with CpG motifs by tattooing or s.c. needle injection, respectively, produced IgG2a antibodies.

To determine whether tattooing with the E749–57 or E744–62 peptide is able to protect mice against E7-expressing tumors, animals were immunized three times and then challenged with TC-1 cells (Fig. 2E). The efficacy of this antitumor immunization corresponded with the activation of CD8+ T cells found in the in vitro approach.
Fig. 2. Comparison of tattooing with s.c. needle injection using E7-derived peptides. Mice (n = 8) were immunized three times at 2-week interval with 100 µg of the E7\textsubscript{49–57} or E7\textsubscript{44–62} peptide with or without 50 µg CpG motifs by either tattooing or s.c. injection. Fourteen days after the last vaccination, three mice were sacrificed and their blood sera and splenocytes were used in in vitro assays (A–D), and five mice were inoculated with 3 × 10\textsuperscript{4} TC-1 tumor cells. The development of tumors was monitored twice a week (E). Naive mice and mice tattooed with CpG motifs were used as controls. (A) Ex vivo intracellular staining of IFN-γ produced by CD8\textsuperscript{+} cells in lymphocyte cultures prepared from pools of splenocytes and (B) tetramer staining of CD8\textsuperscript{+} cells after 7-day restimulation of lymphocyte cultures prepared from pools of splenocytes. (A and B) Control lymphocytes were cultivated without the peptide. Columns, mean of two samples; bars, S.D. (C) E7-specific IgG antibodies in individual sera. Columns, geometric mean titres. (D) E7-specific IgG1 and IgG2a antibodies in individual sera. Columns, mean absorbances. (E) Formation of TC-1-induced tumors. No. of mice with tumors/no. of mice per group is indicated. Asterisks, statistical significance (* P < 0.05, ** P < 0.01) in comparison with the group of naive mice; right square brackets followed by asterisks, statistical comparison of the indicated groups of mice.

assays. All naive mice and mice tattooed with CpG motifs developed tumors within 19 days. In contrast, all groups of mice immunized with the E7\textsubscript{49–57} or E7\textsubscript{44–62} peptides were significantly protected against the formation of TC-1-induced tumors (P < 0.05, compared to naive mice). Surprisingly, while vaccination with the E7\textsubscript{49–57} peptide alone given either subcutaneously or using the tattoo device protected 2 or 3 out of 5 mice, respectively (non-significant, tattoo versus s.c. delivery), and all mice tattoo vaccinated with the E7\textsubscript{49–57} peptide in combination with CpG motifs remained tumor-free for the whole observation period of 61 days, all mice s.c. immunized with the E7\textsubscript{49–57} peptide in combination with CpG motifs developed tumors within 30 days (P < 0.01, tattoo versus s.c. delivery). All animals immunized with the E7\textsubscript{44–62} peptide in combination with CpG motifs either by tattooing or subcutaneously or with the E7\textsubscript{44–62} peptide alone delivered by tattooing remained tumor-free, while 3 out of 5 mice immunized subcutaneously with the E7\textsubscript{44–62} peptide alone developed tumors (P < 0.05, tattoo versus s.c. delivery).
Altogether, our data indicate that in comparison with s.c. injection, tattoo delivery of peptide vaccines induced higher both cell-mediated and humoral immune responses. Addition of CpG motifs further enhanced the efficacy of vaccination by the tattoo device.

3.3. Tattoo delivery of the E6_{48–57} Peptide with CpG motifs induced higher CTL responses than s.c. injection

To examine whether tattooing is a more efficient method than subcutaneous needle injection for peptide vaccination carrying different CTL epitopes, we performed an immunization experiment with the E6_{48–57} peptide, using the E7_{49–57} peptide as a positive control.

Mice were immunized three times with 100 μg of peptides combined with 50 μg of CpG motifs and challenged with TC-1 cells (Fig. 3A and B). Naive mice developed tumors in 26 days after challenge, while all animals tattoo vaccinated with the E7_{49–57} peptide in combination with CpG motifs were protected against tumor formation ($P < 0.01$, compared to naive mice) and 3 out of 5 mice immunized subcutaneously with the E7_{49–57} peptide in combination with CpG motifs remained tumor-free for 61 days after challenge ($P < 0.01$, compared to naive mice). The data indicated that the conditions in the experiment shown in Fig. 3A and B were less stringent than those in the preceding experiment (Fig. 2E), allowing to notice the antitumor effect of less efficient vaccines. All mice tattoo immunized with the E6_{48–57} peptide with CpG motifs developed tumors within 47 days; however, the tumors formed later and grew much more slowly than in mice (3/5) immunized s.c. with the identical vaccine during 61 days of screening (non-significant, tattoo versus s.c. delivery, Fig. 3A and B). The tattoo delivery of the E6_{48–57} peptide together with the CpG motifs elicited significant protection against TC-1-tumor formation ($P < 0.05$, compared to naive mice), while the antitumor effects observed after s.c. injection of the identical vaccine were not significant in comparison with naive mice. Moreover, higher tumor formation and tumor growth were observed after injection of TC-1 cells in mice vaccinated with the E6_{48–57} peptide in comparison with the E7_{49–57} peptide ($P < 0.05$, comparison of tattoo delivery of peptides with CpG motifs; non-significant, comparison of s.c. delivery).

The intracellular IFN-γ staining of CD8+ splenocytes revealed enhancement of E6-specific CD8+ T cells in splenocytes isolated from mice tattooed with the E6_{48–57} peptide with CpG motifs in comparison with those from naive animals or mice vaccinated with the identical vaccine subcutaneously (0.2% versus 0.01%, Fig. 3C). Similar results were obtained in the ELISPOT assay (data not shown). The immunization with the E7_{49–57} peptide in combination with CpG motifs by tattooing induced E7-specific CTLs detected by ELISPOT and ICS (data not shown) in concordance with the previously obtained results (Fig. 2A and B).

3.4. Tattooing with the KLH-conjugated E7_{49–57} peptide induced higher CTL responses than s.c. injection

To evaluate the effects of tattooing on the induction of specific cellular responses after vaccination with KLH-conjugated peptides, we immunized mice with the KLH-conjugated HPV16 E7_{49–57} peptide using the non-conjugated E7_{49–57} peptide as a positive control.
Naive mice were used as a negative control, mice tattooed with 100 μg of the KLH-conjugated E749–57 peptide were sacrificed and their blood sera and splenocytes were used in ELISA using the KLH protein (Fig. 4B). Three immunizations with the KLH-conjugated E749–57 peptide induced KLH-specific antibodies in 17 out of 18 mice, while all mice vaccinated with the non-conjugated E749–57 peptide with CpG motifs (3/3) and naive mice (3/3) did not produce KLH-specific antibodies. The end-point titration of sera revealed that the geometric mean titre (GMT) of KLH-specific IgG antibodies produced in mice tattooed with the KLH-conjugated peptide in combination with CpG motifs was about 20-fold lower than that in mice immunized with the identical vaccine subcutaneously (about 250 versus 5000). Similarly, an about 40-fold lower GMT was found in mice tattooed with the KLH-conjugated peptide alone than in those immunized subcutaneously (about 50 versus 2000). The addition of CpG motifs enhanced the levels of KLH-specific antibodies after immunization by both s.c. needle injection and tattooing. Furthermore, the mixture with FA slightly enhanced the GMT of KLH-specific antibodies after s.c. delivery.

3.5. The production of L2-specific antibodies after immunization with KLH-conjugated peptides L218–38 and L2108–120 using a tattoo device was higher than that after s.c. injection

To reveal the effects of tattooing on the induction of specific humoral responses after vaccination with the KLH-conjugated peptides, we immunized mice three times with the KLH-conjugated HPV16-derived L218–38 and L2108–120 peptides either with or without CpG motifs using a tattoo device or s.c. injection. The follow-up of the production of L2-specific antibodies after individual immunizations showed that the first vaccination with the KLH-conjugated peptides induced antibodies in 37 out of 60 mice and that the number of positively reacting mice increased to 58 out of 60 mice after the second vaccination. After three immunizations, mice (10/10) vaccinated with the KLH-conjugated L218–38 and L2108–120 peptides subcutaneously without CpG motifs developed low levels of L2-specific antibodies (GMT of about 300), while mice (10/10) immunized with identical vaccines by tattooing produced high titres of L2-specific antibodies (GMT about 10 000, P < 0.01, tattoo versus s.c. delivery) and comparable differences in titres of L2-specific antibodies were also found in mice immunized with the KLH-conjugated L218–38 or L2108–120 peptides in combination with CpG motifs (P < 0.05 and non-significant, respectively, tattoo versus s.c. delivery, Fig. 5A and B). Addition of CpG motifs enhanced the antibody production both after s.c. injection and tattoo delivery. Antibody production was also increased by FA for the s.c. delivered KLH-conjugated peptides. The effect of FA was higher than that of CpG motifs (P < 0.05 for the L218–38 peptide) and was not further augmented by the combination of FA with CpG motifs. The levels of L2-specific antibodies observed after tattoo delivery of the KLH-conjugated L2 peptides in combination with CpG motifs were comparable with the humoral responses after s.c. injection of the vaccines mixed with FA. However, none of these L2-specific antibodies showed any neutralization activity in the HPV16-neutralization assay (titre <1:200; data not shown).
Fig. 5. Comparison of humoral responses after vaccination with the KLH-conjugated L2 peptides. Mice (n = 5) were immunized three times at 2-week interval with 50 μg of the KLH-conjugated L218–38 or L2108–120 peptide with or without 50 μg of CpG motifs using a tattoo device or s.c. needle injection. Ten days after each immunization, sera from mice were collected and L218–38-specific (A), L2108–120-specific (B) or KLH-specific (C and D) antibodies were detected in ELISA. Columns, geometric mean titres after the first (white), the second (light grey), and the third (dark grey) immunization dose; square brackets followed by asterisks, statistical comparison (*P < 0.05, **P < 0.01) of indicated groups. After the third vaccination, the L218–38-specific (E), L2108–120-specific (F) or KLH-specific (G and H) IgG1 and IgG2a antibodies were assessed in individual sera. Columns, mean absorbances.
production of L2-specific antibodies was not found in the control group of naïve mice (0/5).

Production of KLH-specific antibodies was found in all mice (60/60) immunized with KLH-conjugated peptides after the first immunization (GMT of about 500) and further increased after the second and the third immunizations (to a GMT of about 10 000 and 100 000, respectively; Fig. 5C and D). The levels of KLH-specific antibodies shown in Fig. 5C and D mostly corresponded to the production of L2-specific antibodies (Fig. 5A and B). Higher levels of KLH-specific antibodies were found in mice immunized with the KLH-conjugated L2 \text{18–38} or L2 \text{108–120} peptides by tattooing in comparison with subcutaneous immunization (non-significant and \(P<0.01\), respectively, tattoo versus s.c. delivery). Similarly, mice tattooed with the KLH-conjugated L2 \text{18–38} or L2 \text{108–120} peptides combined with CpG motifs developed higher titres of KLH-specific antibodies than those immunized subcutaneously (\(P<0.01\) and non-significant, respectively, Fig. 5C and D). There was no relevant difference in the production of KLH-specific antibodies between groups of mice immunized with the KLH-conjugated L2 peptides either in the mixture with FA by s.c. needle injection or in combination with CpG motifs by tattoo. However mice immunized with the KLH-conjugated L2 \text{18–38} or L2 \text{108–120} peptides combined with CpG motifs in mixture with FA developed higher titres of KLH-specific antibodies than mice immunized with identical peptides in combination with CpG motifs by tattoo (\(P<0.01\) and \(P<0.001\), respectively) or with FA by s.c. application (\(P<0.01\) for the L2 \text{108–120} peptide). No KLH-specific antibodies were found in the control group of naïve mice (0/5).

The analysis of the production of IgG1 and IgG2a antibodies revealed higher levels of both L2- and KLH-specific IgG1 antibodies in groups of mice immunized without CpG motifs, while the IgG2a antibodies were enhanced in groups of mice immunized with vaccines containing CpG motifs (Fig. 5E–H). We did not find any difference in the IgG1 to IgG2a ratio in association with peptide delivery method.

In summary, our results indicate that s.c. injection of the KLH-conjugated L2 peptides without any adjuvant induced lower levels of specific antibodies in comparison with tattoo delivery. However, the addition of adjuvancing CpG motifs substantially enhanced antibody production elicited after s.c. delivery to the extent comparable to that after tattooing.

4. Discussion

In this study we compared various methods of immunization with peptides and peptide-conjugates and observed that peptide vaccines delivered by tattooing consistently elicited higher specific both cellular and humoral immune responses than s.c. needle injection. Mostly, the co-administration of CpG motifs (ODN1826) markedly enhanced the immune responses.

The E749–57 epitope is considered to be the immunodominant H–2\text{b} CTL epitope of the HPV16 E7 protein [25,19]. For vaccination purposes, the E749–57 peptide is usually administered with an adjuvant, e.g. FA [25–27]. We found that three s.c. immunizations with 100 \(\mu\)g of the E749–57 peptide or the longer E744–62 peptide in PBS solution in the absence of any adjuvant induced the E7-specific CTL response that was demonstrated in the tetramer assay (Fig. 2B) and also protected a portion of mice against the tumor formation after challenge with 3 \(\times\) 10\text{4} TC-1 cells (Fig. 2E). Previously, two s.c. injections with 20 or 50 \(\mu\)g of the E749–57 peptide in PBS failed to induce CTL responses detectable in the chromium-release cytoxicity test [28,29] and two doses of 20 \(\mu\)g of this peptide did not protect mice against development of tumors after injection of 5 \(\times\) 10\text{5} TC-1 cells [28]. Moreover, two intraperitoneal injections of 100 \(\mu\)g of the E749–57 peptide were not able to protect mice against the challenge with 5 \(\times\) 10\text{4} TC-1 cells and did not elicit the E7-specific CTL response detectable in the tetramer assay [30]. The higher dose and number of immunizations used for peptide vaccination and also lower dose of challenging TC-1-tumor cells administered in this work could explain the breaking through the detection limit.

Surprisingly, the addition of the CpG motifs to the s.c. injection of the E749–57 peptide substantially decreased induced CTL responses and protection against the challenge with TC-1 cells (Fig. 2A, B and E). We observed that addition of PBS to the water-dissolved E749–57 peptide caused mild precipitation that was further increased after the addition of CpG motifs, which could influence the presentation of the E749–57 peptide by antigen-presenting cells (APC) and result in the observed decrease in cellular immune responses [31]. Similarly, the KLH-conjugated E749–57 peptide was fully aggregated in PBS buffer in both the presence and absence of CpG motifs, which could contribute to poor immunogenicity induced after s.c. delivery of the KLH-conjugated E749–57 peptide (Fig. 4A). In contrast, the tattoo delivery of either the KLH-conjugated or non-conjugated E749–57 peptide in combination with CpG motifs induced a very high activation of E7-specific CD8\text{+} T cells (Figs. 2 and 4A). As the peptide aggregates can be solubilized by ultrasound [32], the tattoo procedure might also mechanically disrupt the aggregates of the peptide and the CpG motifs and thus result in their higher accessibility for APC uptake. Alternatively, different APCs presenting peptides after s.c. and tattoo delivery might have different ability to present a precipitated peptide. The experiment comparing the immunization effects of the aggregated and solubilized E749–57 peptide was not performed because we did not find convenient conditions (including sonication of the solution, utilization of a physiological solution, and decreasing a dose of the peptide and CpG motifs) for stable solubilization of peptides and CpG motifs at concentrations inducing immune responses above detection limits. Precipitation was not visible in any immunization solution of the E744–62 peptide, E648–57 peptide or KLH-conjugated L2 \text{18–38} and L2 \text{108–120} peptides.

The addition of the CpG motifs in the immunization with the E744–62 peptide and KLH-conjugated E749–57, L2 \text{18–38} and L2 \text{108–120} peptides enhanced humoral and/or cell-mediated immune responses both after the tattoo administration and s.c. injection (Figs. 2, 4 and 5). In both peptide delivery methods, the adjuvant also modulated the type of immune response as it shifted E7-, L2- and KLH-specific antibody production from IgG1 to IgG2a antibodies (Figs. 2D and 5D–F).

The CpG motifs alone have been shown to activate both CTL and NK cells and the treatment by intratumoral or s.c. injections of CpG motifs has reduced the growth of TC-1-induced tumors [33,34]. However, we did not observe any protection against TC-1 challenge or non-specific activation of the immune system in mice tattooed with CpG motifs in a preventive immunization setting (Fig. 2), which is in concordance with the published results showing that preventive administration of CpG motifs (ODN1826) has no obvious antitumor effects [35].

To evaluate the potency of tattooing for the delivery of peptide vaccines, we also compared this immunization strategy with highly efficient DNA vaccination against the E7 oncoprotein, the E7GGG.GUS fusion gene administered intradermally by the gene gun [16]. We found that three tattoo immunizations with 100 \(\mu\)g of the E749–57 peptide in mixture with 50 \(\mu\)g of CpG motifs stimulated immune responses comparably to three immunizations with 1 \(\mu\)g of pBSC/E7GGG.GUS plasmid delivered by the gene gun (Fig. 1).

The H–2\text{b} E648–57 minimal CTL epitope has been shown to bind MHC class I \(\alpha\) molecules and also to be naturally presented by E6-expressing TC-1 cells [18], but two immunizations with 150 \(\mu\)g of this peptide and 20 \(\mu\)g of saponin Quil A into both hind foot pads were not able to prime mice in vivo [36,37]. In this work, we used the longer E648–57 peptide described previously to be optimal for

\[ <0.01, \text{respectively, tattoo versus s.c. delivery.} \]

\[ P<0.01, \text{respectively, tattoo versus s.c. delivery.} \]

\[ P<0.001, \text{respectively, tattoo versus s.c. delivery.} \]

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\[ P<0.01, \text{respectively, tattoo versus s.c. delivery.} \]
activation of the E6-specific CD8+ T cells [18]. Three immunizations with 100 μg of the E6GA–57 peptide combined with the 50 μg of CpG motifs inhibited the growth of TC-1 tumor cells in mice, but only the tattoo delivery induced detectable levels of the E6-specific CTLs, while subcutaneous injection of the identical vaccine failed to prime the E6-specific CTLs (Fig. 3). For efficient induction of CTL responses in H-2b mice by the E6 peptide, some improvements of the E6 vaccine have been achieved, e.g., by the D51I mutation of the E6 epitope leading to the higher stability of the peptide [37]. The tattoo delivery of peptides in combination with CpG motifs described in this work is another improvement.

The E744–62 peptide alone injected in PBS induced E7-specific antibodies (Fig. 2C) but both the L218–38 and the L2108–120 peptides needed to be conjugated with KLH to elicit detectable humoral response (data not shown). This can be explained by the need for T-cell help in the induction of efficient antibody production. While the E744–62 peptide contains both the B-cell and Th-cell epitope [27,38], the L218–38 and L2108–120 peptides lack a Th-cell epitope and so activation of Th lymphocytes is probably mediated only by the conjugated KLH protein [39–41]. The induction of the L2-specific antibodies after s.c. injection of the KLH-conjugated L2108–120 peptide observed in this work is in concordance with the previous findings of Slupetzky et al. [40]. Antibodies against the L218–38 peptide have been previously elicited in rabbits [39]. Comparable levels of L2-specific antibodies were elicited by tattooing with the KLH-coupled L2 peptides and s.c. injection of the vaccines in the mixture with CpG motifs or FA (Fig. 5A and B), which indicates that adjuvanting effects of tattoo delivery could support humoral responses. Similarly, tattoo delivery of the E744–62 peptide with or without CpG motifs induced more E7-specific antibodies than s.c. injection of the identical vaccines (Fig. 2C), suggesting that multiple puncturing of the skin was advantageous for the induction of immune responses. Surprisingly, tattoo delivery of the KLH-conjugated E749–57 peptide induced lower titres of KLH-specific antibodies than s.c. injection (Fig. 4B). We suggest that the observed effect might be caused by aggregation of the KLH-conjugated E749–57 peptide which could have been less detrimental to the induction of humoral responses after s.c. delivery.

Tattooing with peptides in combination with CpG motifs might be a method of choice for the induction of peptide-specific immune responses. The utility of tattoo delivery is above all for vaccination with peptides of low immunogenicity or with a tendency to aggregate. Moreover, a major advantage of tattooing lies in the possible elimination of adjuvants that are necessary for vaccination by s.c. needle injection and may cause severe adverse effects. Furthermore, peptide tattooing allows the replacement of the less safe DNA vaccines. Tattooing is well tolerated by animals and thus could be applied in laboratory conditions or veterinary practices. However, the usage of tattooing for vaccination of humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in humans is less acceptable since it is associated with local damage of skin.


