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Transcutaneous Anti-Influenza Vaccination Promotes Both CD4 and CD8 T Cell Immune Responses in Humans

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Induction of T cell responses has become one of the major goals in therapeutic vaccination against viral diseases and cancer. The use of the skin as target organ for vaccine has been spurred by recent implication of epithelial dendritic cells in CD8 cell cross-priming and suggests that vaccination via the transcutaneous (TC) route may be relevant in the induction of cellular immune responses. We have previously shown that TC application of nanoparticles, on human skin explants, allows targeting of epidermal dendritic cells, possibly via hair follicles. In this study, we have investigated cellular immune responses against an influenza protein-based vaccine by TC vaccination, compared with i.m. vaccination in humans. In this study on 11 healthy volunteers, we found that a newly developed protocol based on cyanoacrylate skin surface stripping induced a significant increase in IFNγ-producing T cells specific for influenza vaccine by ELISPOT assays. Interestingly, TC vaccination induced both effector CD4 and CD8 T cell responses, whereas i.m. injection induced strong effector CD4 in the absence of CD8 T cells, as assessed by intracellular cytokine staining and tetramer analyses. This study proposes new perspectives for the development of vaccination strategies that trigger T cell immune responses in humans.

Materials and Methods

Study design

Eleven healthy volunteers were enrolled in the study regardless of previous vaccination against the influenza A vaccine Agrippal (Chiron). All volunteers provided written informed consent before enrollment. The objective of this pilot study was to determine safety and efficacy of influenza A vaccination by TC compared with i.m. route. Recruitment and vaccination were done far from seasonal influenza A vaccination. Seven subjects received TC vaccination using a commercially available Agrippal vaccine (0.5 ml) as provided by the manufacturer (three men, four women, age range: 25–52). Four subjects were vaccinated with the same dose of Agrippal vaccine via conventional i.m. injection (four men, age range: 25–52). CSSS was applied according to a standardized protocol approved by the ethic committee of the Charité-Universitätsmedizin Berlin, Germany for investigational studies on human volunteers. The procedure is described below. The vaccination sites were monitored for adverse events 1 h after vaccination and then on days 1, 3, 14, and 28 as described below. Fresh PBMCs were collected and processed immediately on day 0 before the application of vaccine and on days 14 and 28 after vaccination.

Anti-influenza vaccine

The anti-influenza vaccine Agrippal (season 2004–2005) was purchased from Chiron and contains hemagglutinin and neuraminidase surface antigens from the following strain: A/Fujian/411/2002 (H3N2) – strain X147 derived from de A/Wyoming/3/2003, an A/NewCaledonia/20/99 (H1N1) – like strain IVR-116 and B/Shanghai/361/2002/- like strain B/Chang/10/2003. Each 0.5-ml dose contained at least 15 μg of hemagglutinin Ag from each strain. Agrippal vaccine was purchased and used as provided by the manufacturer. The composition and volume of the product was not modified for study purposes.

TC vaccination

The 0.5 ml Agrippal vaccine was administered by TC route on the external part of the upper left arm: on either 16 cm² (four volunteers (TC No. 1, 4, 6, and 7), three women, one man, age range 25–52 years) or 32 cm² (three volunteers (TC No. 2, 3, and 5), one woman, two men, age range 25–50 years). The 0.5 ml Agrippal vaccine was administered by i.m. route (four volunteers, four men, age range 25–50 years). After an adjustment period of 30 min, one (four volunteers) or two (three volunteers) squares of 4 × 4 cm were delimited on the external part of the upper left arm using a permanent skin marker (Skin marker H7003 Falc). Shaving was performed on the investigational sites as well as on the surrounding skin (2 cm on the top and on the bottom of each investigational site and 1 cm on both sides) using a dry razor (Disposable razor, Art.-No. 182 H, Wilkinson Sword). After shaving, CSSS was performed as described elsewhere (15, 18). Briefly, a total amount of 190 mg cyanoacrylate glue (Superglue, UHU KG) was applied drop-wise from the original syringe as provided by the manufacturer. A microscope slide was used to spread the glue evenly on the skin surface, followed by the application of adhesive tape (6 × 5 cm, Art.-No. 571176–00000, Tesa Beiersdorf) and was massaged with a rubber roll to improve adherence (10 times). After hardening of the glue for 20 min, tape and glue were removed from the skin surface. A silicone barrier was formed by delimitation with Window-Colorpainter, Art.-No. 4469/ko, Max-Breuning, Wendelstein to avoid spreading of the vaccine. The vaccine was then applied in two subsequent aliquots of 0.25 ml vaccine on one square of 16 cm² or on two squares of 16 cm² each (total skin area of 32 cm²). Agrippal vaccine was applied drop-wise from the original syringe as provided by the manufacturer (16 drops of ~16 μl each) followed by a soft massage, which was performed by moving a gloved finger tip (care&serve, Wiros) saturated with Agrippal for 16 cm² each (total skin area of 32 cm²). After shaving, CSSS was performed as described elsewhere (15, 18).

Adverse events

Twenty-four hours after vaccine application, the hydrocolloid bandage was removed from the investigational sites. Medical examination of the investigational sites and performance of the addition of 100 μl tetramethylbenzidine substrate (Sigma-Aldrich) was used to quantify simultaneously TNF-α, IL-12p70, IL-10, IL-6, IL-1β, and IL-8 (Human Inflammation CBA kit; BD Biosciences) following manufacturer’s instruction. For data analysis, the Cytometric Bead Array (CBA) software programs (BD Biosciences) was used. During analysis, the mean fluorescence intensity value for the PE-associated fluorescence was recorded for each population of cytokine capture-beads. To allow quantification of the captured cytokines, standard curves were developed using cytokine standards. Standard curves were plotted (cytokine calibrator concentration vs mean PE fluorescence intensity) using a four-parameter logistic curve-fitting model (BD Biosciences CBA software). A total of 12 standards for each cytokine were used at concentrations ranging from 0 to 5000 pg/ml. Concentration of the cytokine in the plasma was considered to be directly proportional to the mean fluorescence intensity of the corresponding bead population measured in fluorescence channels (relative linear arbitrary units scaled from 0 to 10³). The specific concentration of each cytokine analyzed was determined through the comparison of the mean fluorescence intensity of unknown samples to the standard curve. Results are presented in pg/ml of each cytokine in plasma samples.

IFNγ-ELISPOT assays

The ELISPOT assay was run after careful determination of the optimal Ag doses and reproducibility of the assays in healthy donors (20). IFNγ-ELISPOT detection for human PBMC was performed as previously described (20). Briefly, 96-well ELISPOT plates (Millipore) were coated with anti-human IFNγ Ab (IgG1/B-B1, Dianclone). After blocking with 10% FCS, triplicate wells were filled with 100,000 PBMCs freshly isolated. Plates were incubated at 37°C for 20 h with Agrippal vaccine (1/1000 dilution of manufacturer’s solution). PHA (Abbott Laboratories) (0.5 μg/ml) and medium alone served as positive and negative controls, respectively. Wells were then washed and spots detected after the addition of anti-human IFNγ-biotin detection Ab (B-G1, Dianclone) (4 h, 37°C), followed by streptavidin alkaline phosphatase (1 h, 37°C) and substrate, 5-bromo-4-chloro-3-indolyl-phosphatase/4-nitroblue tetrazolium (Sigma-Aldrich). Plates were incubated at room temperature until spots appeared. Ag-specific spot-forming units (SFUs) were counted by two independent investigators, using an automated microscope (Zeiss). Samples were considered positive on detection of at least 50 SFU per million PBMC above background.

ELISA for detection of influenza-specific IgG and IgM plasma Abs

ELISA were performed using plasma samples of all volunteers. IMMUNOLIA LON 96-well plates (Dynatech Laboratories) were coated overnight at room temperature with 50 ng of recombinant hemagglutinin-Influenza A virus H1N1 (New cedonia 20099) or H3N2 (ProSpec-Transy Technogenie) in 0.1 μl sodium carbonate buffer (pH 9.6). The plates were washed with deionized H₂O containing 0.05% Tween 80.

Human plasma samples were diluted from 1:5, and a total of 100 μl of each dilution was incubated in the recombinant hemagglutinin-Influenza-coated plates for 1 h at 37°C. After washing, 100 μl of 1:10,000-diluted goat anti-human IgG Abs (Sigma-Aldrich) or 1:10,000-diluted goat anti-human IgM (Sigma-Aldrich) with HRP was added, and the mixture was incubated for 1 h at 37°C. Samples with detectable levels of IgG were further analyzed of isotype (IgG1 or IgG2 dosages). For detection of IgG1 and IgG2, 100 μl of 1:4,000-diluted mouse anti-human IgG Abs (Sigma-Aldrich) or 1:15,000-diluted mouse anti-human IgG (Sigma-Aldrich) with biotin was added and incubated for 1 h at 37°C. The plates were washed again and were incubated with 100 μl of 1:500-diluted Streptavidin-Peroxidase (Sigma-Aldrich) for 1 h at 37°C, followed by washing and the addition of 100 μl of tetramethylbenzidine substrate (Sigma-Aldrich) for 20 min. The reaction was stopped by adding 50 μl of 2 M H₂SO₄, and the OD at 450 nm was measured ( Molecular Devices).

Intracellular cytokine staining and cell differentiation assays

Frozen PBMCs were thawed in RPMI 1640 ( Invitrogen Life Technologies) containing 10% FCS (Seromed), 2 mMol/L L-glutamine (Invitrogen Life Technologies), and antibiotics (1000 IU/ml penicillin sodium, 1 mg/ml).
Table I. Monitoring of adverse events after TC and i.m. vaccination

<table>
<thead>
<tr>
<th>Mode of Administration</th>
<th>Local Vaccine Reaction</th>
<th>Systemic Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC 16 cm²</td>
<td>Erthema</td>
<td>Burning</td>
</tr>
<tr>
<td>TC 32 cm²</td>
<td>Pruritus</td>
<td>Desquamation</td>
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<tr>
<td>i.m.</td>
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</tbody>
</table>

a One volunteer experienced mild erythema, which occurred immediately after TC vaccination and persisted for 1 h.

b One volunteer experienced an episode of fever 24 h after i.m. injection (not reported). No significant differences between TC and i.m. group were observed.

Statistical analyses

Eleven individuals were included in the safety analysis and 10 individuals included in the cellular immune response analysis because one individual in the TC group failed to be present on day 28 of blood collection. Mann-Whitney tests were used to compare continuous variables between the groups. Data handling and graphical representation used Prism4.0 software (GraphPad). All statistical analyses were performed using SSSP11 for MacOS10 software. Statistical significance was set at p < 0.05.

Table II. Cytokine detection in plasma of vaccinated individuals

<table>
<thead>
<tr>
<th>Cytokines (pg/ml)</th>
<th>TC No. 1 D0</th>
<th>TC No. 1 D14</th>
<th>TC No. 1 D28</th>
<th>TC No. 4 D0</th>
<th>TC No. 4 D14</th>
<th>TC No. 4 D28</th>
<th>TC No. 5 D0</th>
<th>TC No. 5 D14</th>
<th>TC No. 5 D28</th>
<th>TC No. 6 D0</th>
<th>TC No. 6 D14</th>
<th>TC No. 6 D28</th>
<th>TC No. 7 D0</th>
<th>TC No. 7 D14</th>
<th>TC No. 7 D28</th>
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<td>IL-10</td>
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<td>IL-6</td>
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<td>IL-1β</td>
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<td>IL-8</td>
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<td>22</td>
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<thead>
<tr>
<th>Cytokines (pg/ml)</th>
<th>i.m. No. 8 D0</th>
<th>i.m. No. 8 D14</th>
<th>i.m. No. 8 D28</th>
<th>i.m. No. 9 D0</th>
<th>i.m. No. 9 D14</th>
<th>i.m. No. 9 D28</th>
<th>i.m. No. 10 D0</th>
<th>i.m. No. 10 D14</th>
<th>i.m. No. 10 D28</th>
<th>i.m. No. 11 D0</th>
<th>i.m. No. 11 D14</th>
<th>i.m. No. 11 D28</th>
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<td>TC vaccination</td>
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<td>—</td>
<td>—</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
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<td>TNF</td>
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<tr>
<td>IL-10</td>
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<td>IL-6</td>
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<td>IL-1β</td>
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<td>IL-8</td>
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</tbody>
</table>

a Cytokines were measured in the plasma of vaccinated individuals at different time points after administration by using a CBA inflammatory cytokine kit (BD Biosciences).

b Below detectable levels of CBA assays.
Results

TC vaccination based on CSSS is safe and well tolerated

TC administration of vaccines aims at targeting epidermal and dermal DCs as suggested by our previous work (17). CSSS removes cellular debris and sebum from hair follicle openings and, hereby, facilitates the penetration via the hair follicle infundibulum (18). Recent implication of DCs in T cell cross-priming and that TC vaccination may be relevant in the induction of cellular immune response suggest that targeting of skin LCs/DCs might be important in cross-priming of T cells (11–13). Based on our recent finding on the targeting of particles into the epidermal CD1a+ DC, we developed a protocol for TC vaccination using CSSS in humans and, therefore, mainly focused on safety and the induction of cellular immune responses.

In this study on 11 healthy volunteers, we assessed the safety and the induction of cellular immune response induction of this newly developed application protocol compared with conventional vaccination via i.m. injection using Agrippal vaccine, a commercially available anti-influenza vaccine.

The total amount of Agrippal vaccine (0.5 ml) was applied either TC on 16 cm² (n = 4) or 32 cm² (n = 3) skin surface on the external part of the upper arm or by i.m. injection into the deltoid muscle (n = 4) (see Materials and Methods section). All volunteers received the same amount of vaccine. They were followed for adverse events 1 h after vaccination and on days 1, 3, 14, and 28 (Table I). Overall, the TC vaccination was safe and well tolerated.

Only one volunteer exhibited a mild erythema with no other local reactions. No differences were observed between the two surfaces of vaccine application.

To measure inflammatory markers that might be up-regulated by these routes of vaccination, we performed cytometric bead array analyses of inflammatory cytokines and chemokines including IL-12p70, TNF-α, IL-10, IL-6, IL1β, and IL-8 (Table II). Low levels of IL-12p70 were detected in both TC and i.m. groups, while TNF-α, IL-10, IL-6, and IL1β were not detected in the plasma of both TC and i.m. vaccinated individuals. Interestingly, high levels of CXCL-8 (IL-8), a chemokine involved in the initiation and amplification of acute inflammatory reactions (21), were found in 5 of 6 TC vaccinated volunteers but not in i.m. group. However, no significant differences were observed in TC groups according to the size of skin surface treated.

TC vaccination after CSSS induced IFNγ-producing effector/memory T cell responses specific for influenza vaccine

IFNγ-ELISPOT assay is one of the most commonly used conventional method of rapid immuno-monitoring of cellular immune responses during viral infection or in new vaccination strategies against viruses. It allows us to detect rapidly mobilized IFNγ-producing effector T cells against Agrippal vaccine (Fig. 1A). As in previous studies, the positive response to the Agrippal vaccine compounds was defined as ≥50 IFNγ-SFU/million PBMC over background (20, 22). Five of six subjects in TC group (83%) and

Table III. Frequencies of influenza-specific effector T lymphocytes assessed by IFNγ-ELISPOT assays on PBMCs

<table>
<thead>
<tr>
<th>TC</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 28</th>
<th>i.m.</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Median</td>
<td>76.5</td>
<td>234.0</td>
<td>266.5</td>
<td>132.5</td>
<td>340.5</td>
<td>447.0</td>
<td></td>
</tr>
<tr>
<td>IQR*</td>
<td>160</td>
<td>130</td>
<td>280</td>
<td>290</td>
<td>88</td>
<td>486.5</td>
<td></td>
</tr>
<tr>
<td>Fold increase</td>
<td>—</td>
<td>3.0</td>
<td>2.6</td>
<td>—</td>
<td>3.0</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>

*IFNγ-ELISPOT assays were performed using Agrippal vaccine for stimulation of frozen PBMCs at days 0, 14, and 28 after vaccination. The evolution of variables between day 14 and baseline (or day 28 and baseline) were compared between the TC and i.m. groups using Mann-Whitney tests. Statistical significance was set at p value ≤0.05.

† The evolution of variables between day 14 and baseline (or day 28 and baseline) was similar between TC and i.m. vaccinated individuals (not significant p values). Statistical analyses were performed on six individuals in the TC group (data from one volunteer was not available on day 28) and four individuals in the i.m. group.

‡ IQR, Interquartile range.

Table IV. Frequencies of influenza-specific effector CD4 and CD8 lymphocytes assessed by intracellular cytokine staining

<table>
<thead>
<tr>
<th>TC</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 28</th>
<th>i.m.</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
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<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Range</td>
<td>0.00–0.10</td>
<td>0.07–0.13</td>
<td>0.08–0.17</td>
<td>0.00–0.24</td>
<td>0.16–0.44</td>
<td>0.00–0.50</td>
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</tr>
<tr>
<td>Median</td>
<td>0.03</td>
<td>0.10</td>
<td>0.12</td>
<td>0.00</td>
<td>0.36</td>
<td>0.16</td>
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<tr>
<td>IQR*</td>
<td>0.1</td>
<td>0.04</td>
<td>0.04</td>
<td>0.18</td>
<td>0.24</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Positive responders*</td>
<td>3/6</td>
<td>6/6</td>
<td>6/6</td>
<td>1/4</td>
<td>4/4</td>
<td>3/4</td>
<td></td>
</tr>
</tbody>
</table>

* IQR, Interquartile range.

§ Positive responders were defined as percentage of IFNγ-producing cells ≥0.05% after baseline subtractions. The evolution of variables between day 14 and baseline (or day 28 and baseline) were compared between the TC and i.m. groups using Mann-Whitney tests. Statistical analysis showed no significant differences between TC group and i.m. group at different time points. IFNγ-CD4+ was slightly higher at day 14 between TC and i.m. group p = 0.01.
3 of 4 subjects in i.m. group (75%) displayed heterogeneous levels of residual anti-influenza memory caused by pre-existing influenza immunity. However, all TC vaccinated individuals showed an increase in effector/memory T cell responses compared with baseline (Table III). No differences were observed between 16 or 32 cm² skin surface. Statistical analysis using the nonparametric Mann-Whitney U test showed that the evolution of variables between day 14 and baseline (or day 28 and baseline) was similar between TC and i.m. vaccinated individuals suggesting that TC application of Agrippal vaccine was immunogenic for the induction of cellular immune responses.

Because the study was first designed for safety and efficacy in cellular immune induction, we did not collect sera from all volunteers. However, plasma samples were used to measure total influenza-specific IgG or IgM by ELISA. Three of four individuals in i.m. group had detectable levels of anti-influenza IgG compared with two of seven individuals in the TC group. Even though i.m. might be more efficient to induce Ab responses compared with the TC group, statistical analysis showed no significant differences between these two groups (p = 0.1, Mann Whitney test at day 28) (Fig. 1B). Low level of anti-influenza IgM Abs was detected in one individual in the i.m. group only. We analyzed the level of neutralizing Ab responses in sera at day 28 of TC and i.m. individuals by hemagglutinin inhibition assay (HIA) using two strains (A/California and A/NewCaledonia influenza strain 2003/2004) (data not shown). Calculating the geometric mean titer of day 28 after vaccine administration showed no significant differences at that time point. This point will be further addressed in the ongoing Phase I study.

**Differential induction of influenza-specific CD4 and CD8 cell responses by TC and i.m. vaccination**

To study the quality of cellular immune responses with regard to CD4 and CD8 cell populations, we first performed intracellular cytokine staining of influenza-specific T cells by four color-fluorescence analysis (Table IV and Fig. 2). After stimulation of PBMCs with Agrippal vaccine for 16 h, we found an increased frequency of IFNγ-producing CD4 cells in both TC and, slightly higher in, i.m. vaccinated individuals (Table IV). At day 14, this increase from baseline was higher in the i.m. group. At day 28, however, the increase was equivalent in both groups. The absolute number of IFNγ-producing CD4 cells was also increased in both groups and remained slightly higher in the i.m. group than in the TC group (Fig. 2A, upper panel). Interestingly, we observed influenza-specific CD8⁺ IFNγ⁺ cells at day 14 in 6 of 6 individuals who had received TC vaccination (Table IV). In contrast, both frequency and absolute numbers of IFNγ-producing CD8 cells remained below the detectable level in all individuals of the i.m. group (0 of 4 responders at days 14 and 28) (Table IV and Fig. 2A, lower panel).

Because of the striking absence of CD8 responses after i.m. vaccination, we decided to perform an additional test by using HLA-A2-Flu epitope pentamer staining and flow cytometric analysis. HLA phenotyping revealed that one volunteer of each group expressed HLA-A201 Ags, which allowed us to perform pentamer A*0201/GILGFVFTL staining to identify GILGFVFTL-specific CD8 T cells restricted to HLA-A201 (Fig. 2B). Consistent with our flow cytometry findings, we found a high frequency of influenza-specific CD8 cells in volunteer No. 5 on day 14 (0.25% at day 14 compared with 0.1% at baseline) even though influenza-specific IFNγ production was not detectable in this individual. This method rises to five the number of TC vaccinated individuals that show influenza-specific CD8 responses. A lower frequency (0.15%) were observed at day 28 after TC vaccination, whereas no influenza-specific CD8 cells were observed at any time point in volunteer No. 9, who had received i.m. vaccination. Thus, TC vaccination with an inert vaccine may help to promote CD8 T cell responses in human.

We further analyzed the production of both IL-2 and IFNγ Type 1 cytokine production by influenza-specific CD4 and CD8 cells using multiparametric analysis (LSRII) (Fig. 3). For all individuals tested, increased double positive IFNγ/IL-2 in CD4 population was observed after influenza vaccine stimulation, suggesting a Th1 profile. However, only IFNγ⁺CD8⁺ cells were increased in TC (Fig. 3A) and not in i.m. vaccinated individuals (Fig. 3B).
Differentiation markers were analyzed on influenza-specific CD4 and CD8 cells producing IFNγ. We found a majority of influenza-specific CD3+CD4+ and CD3+CD8+ cells in four individuals by multiparametric analysis (LSRII; BD Biosciences). At least 1,000,000 live events according to forward- and side-scatter parameters, were accumulated and analyzed (see Materials and Methods section). Results are shown for two TC vaccinated volunteers (A) and two i.m. vaccinated volunteers (B) at indicated time points. Percent cytokine positive T cells are indicated in each plot.

**Discussion**

In this pilot study on 11 volunteers, we evaluated the safety and the immunogenicity of a newly developed TC vaccination protocol based on only one CSSS procedure before the vaccine application. We demonstrated that TC immunization with 0.5 ml influenza vaccine (Agrippal) was safe and immunogenic in healthy individuals on small skin surfaces (16 cm² and 32 cm²). These observations are in accordance with our previous experience with CSSS in percutaneous penetration studies, where CSSS was well tolerated by all vaccines (23, 24). We report the induction of T cell responses after TC vaccination of human volunteers, and we provide strong evidence that, in contrast to conventional i.m. injections, TC vaccination may help to induce not only CD4 but also CD8 T cell immune response. This is the first investigation on the application of a conventional vaccine by TC route that might be critical in the quality of cellular immune responses.

This skin pretreatment technique is not only another method to abrade the skin surface. Other than conventional adhesive tape stripplings or abrasion techniques (scotch pads or chemical abrasion etc.), which only increase the permeability of the interfollicular epidermis, CSSS also removes debris and sebum from the hair follicle openings (19) (Vogt et al., manuscript in preparation). As a result, topical applied compounds are deposited in the hair follicle openings for prolonged periods of time where they are protected from the regular shedding process, which occurs in the interfollicular epidermis as a result of the ongoing renewal of the epidermis (15). Because the density and dendricity of APCs is especially high around the upper part of the hair follicle and because the stratum corneum in the lower parts of the hair follicle openings getting increasingly permeable, we hypothesize that targeting of hair-follicle associated DCs may be especially relevant for TC vaccination strategies. Indeed, the stripping technique may not only enhance the permeability of antigenic peptides but may also induce LC/DC maturation. Immunostimulatory effects due to barrier disruption, which have been reported for conventional adhesive tape stripping, may also occur after CSSS, suggesting that the addition of CSSS to the TC protocol contributed significantly to the observed effects. CSSS may, hence, represent a valuable tool in TC vaccination, providing both enhanced penetration and immunostimulatory effects (25). Among these immunostimulatory effects, we found inconsistent low levels of IL-12p70 and high levels of IL-8 only in the sera of individuals vaccinated by TC route. The inflammatory chemokine CXCL-8 (IL-8) is important in the initiation and amplification of acute inflammatory reactions by recruiting neutrophils (21). Up-regulation of IL-8 and E-selectin gene expression has been reported even after minimal trauma of otherwise normal human skin, such as gentle rubbing (26). Our results further reinforce earlier studies on tape-stripping immunization in mouse models, which demonstrated that the application of immunogenic
compounds onto tape-striped skin could elicit a strong and protective immune response against pathogens and cancer (27, 28).

Furthermore, targeting of perifollicular skin DCs might improve in vivo loading of vaccine Ags into these DCs and subsequent induction of T cell responses, e.g., via cross-presentation. In our study, vaccine application of one or two 4 × 4 cm² skin surface areas pretreated with only one CSSS procedure was sufficient to induce cellular immune responses, respectively. Considering the fact that a significant portion of the vaccine was probably lost by unspecific binding to the skin surface, resulting in a reduced effective dose of vaccine in TC vaccinated compared with i.m. vaccinated volunteers, these results are especially encouraging. Similar to our findings, Kenney et al. (29) showed that the intradermal injection of only one fifth of the standard dose of influenza vaccine induced immune responses, which were similar or better to those induced by conventional i.m. injection.

The evaluation of effector/memory T cell frequency observed after TC vaccination was very similar to the one observed after i.m. vaccination and statistically not different. This result reinforces the fact that vaccination with Agrippal vaccine is immunogenic by both TC and i.m. route of vaccine administration. We found that TC vaccination with Agrippal vaccine induced a 3-fold increase of influenza-specific effector T lymphocytes at days 14 and 28 as measured by IFN-γ-ELISPOT assay. This is in accordance with previous studies in mouse models showing the privileged role of tissue DCs to increase cellular immune responses. In addition, studies have shown that tape-stripping induces activation of APCs in the skin (25, 30), which is an important step in the induction of efficient cellular responses. Our study shows that this pathway of immunization might be of importance in the induction of cellular immune responses in human.

We also showed that only TC vaccination induced both CD4 and CD8 cellular response, whereas i.m. vaccination induced stronger effector Th1 CD4 but not CD8 T cell responses. Indeed, the seasonal influenza vaccine preparation is selected for its high safety and efficacy in the induction of humoral responses by i.m. route. This could explain the high level of effector CD4 Th cells observed in the i.m. group. Influenza-specific CD4⁺ T cells function mainly to promote high-quality Ab responses but have not been shown to operate directly as chief effectors of virus control (31). We assessed the nature of the cellular immune responses by using IFN-γ-ELISPOT assays, cell surface marker staining against CD4 and CD8, specification of IFNγ and IL-2 production, as well as pentamer staining. Frequencies and absolute numbers of IFNγ-producing CD4 cells were increased in both TC and i.m. vaccinated individuals. In contrast, frequency and absolute numbers of IFNγ-producing CD8 cells remained below the detectable level in all individuals of the i.m. group (0 of 4 responders at days 14 and 28). We have also analyzed the production of both IL-2 and IFNγ Type 1 cytokines by influenza-specific CD4 and CD8 cells in four individuals by multiparametric analysis. Vaccine-induced Ag-specific T cells were producing both IFNγ and IL-2, confirming a Th1 profile. Again, influenza-specific CD8 response remained undetectable in the i.m. individuals. This is the first demonstration of such differences in quality of the cellular immune responses by different route of vaccination in humans and remains a crucial step in the development of vaccine strategies.

Because the study was first designed for safety and efficacy in cellular immune induction, we did not concentrate our efforts on Ab studies. However, we measured total IgG and IgM in the plasma of vaccinated individuals. Even though i.m. might be more efficient in inducing Ab responses (3 of 4 responders) compared with the TC group (2 of 7 responders), no significant differences were observed between these two groups. Neutralizing Ab responses were, however, analyzed at day 28 in both TC and i.m. groups tested for strain-specific hemagglutinin inhibition for two strains (A/California and A/NewCaledonia influenza strain 2003/2004). Calculating the geometric mean titers at day 28 after vaccine administration showed no significant differences at that time point. These results suggest that the i.m. route might be a better inducer of Ab production. Additional studies are necessary to conclude for the induction of Ab responses. This point will be analyzed in a future Phase I study.

The overall efficacy of different procedures for TC immunization has been demonstrated in numerous animal models (mice, rats, macaques, and others). Only few concepts have been translated into clinical applications, i.e., pilot studies and Phase I clinical trials. As described in the introduction section, Glenn and his group (9, 10) were the first group to immunize human volunteers via TC vaccination using patch systems. Those studies, however, focused on the induction of humoral immune responses.

Our work also differs from previous studies with regard to the vaccination protocol. In this pilot study, a commercially available anti-influenza vaccine, which does not contain any adjuvant, was applied once on healthy human volunteers on skin areas as small as 16 or 32 cm², respectively. In contrast to previous studies by other groups, we did not perform boost applications and no chemical or membrane permeabilization molecules were used as proposed by others (9, 10). During the preparation of this manuscript, Yagi et al. (32) demonstrated that five percutaneous immunizations using CTL epitopes allow induction of CTL responses specific for melanoma and HIV peptides. In this study, the authors performed subsequent CSSS procedures leading to a complete removal of the stratum corneum, followed by five percutaneous peptide immunizations on skin areas as large as 100 cm² (32) compared with 16 or 32 cm² in our study. Also, after such treatment, LCs have up-regulated HLA expression and costimulatory molecules. However, the surface used for immunization is extremely large compared with our studies. Lisziewicz et al. (33) recently showed that skin abrasion and vaccination using DNA induced an immune response to HIV virus in monkeys. However, this approach was partially invasive using large skin surface areas of four locations of 40 cm² in monkeys combined with several applications of large quantities of vaccine material (33).

In conclusion, we demonstrated that TC vaccination with 0.5 ml Agrippal vaccine, according to a newly developed application protocol based on one CSSS procedure, was safe and immunogenic in a limited number of healthy volunteers. The frequency of influenza-specific effector T lymphocytes was not different between the two groups, suggesting that both application protocols are effective. We provide strong evidence that in contrast to conventional i.m. injections, TC vaccination may induce not only CD4 but also CD8 cellular responses. This study raises important questions on the impact of the route of Ag entry on the quality of immune response. A confirmation of such results in future studies would suggest that TC vaccination may help to develop vaccination strategies in infectious diseases, such as HIV or hepatitis C, and cancer where cellular immune responses play a crucial role in the disease control. Therefore, the results encouraged us to evaluate the safety and the efficacy of this newly developed protocol for TC vaccination in a randomized comparative Phase I clinical trial on human volunteers and HIV-infected patients.

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