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Transcutaneous Anti-Influenza Vaccination Promotes Both CD4 and CD8 T Cell Immune Responses in Humans[1]

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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 cyanocrylate 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. *The Journal of Immunology,* **2008, 180: 1482–1489.**

Induction of T cell responses has become a major goal in therapeutic vaccination against chronic viral diseases, such as HIV or hepatitis C, as well as in antitumor vaccination (1, 2). Currently, however, only live attenuated vaccines, e.g., vaccines against tuberculosis, measles, and rubeola, have been shown to induce strong CD8 T cells, but their applicability in immunosuppressed individuals is limited by the risk of uncontrolled virus dissemination. Thus, efforts have to be directed toward an improvement of T cell immune responses also in immunosuppressed, e.g., HIV-infected, individuals (3, 4). The use of the skin as target organ for vaccine design has been spurred by the fact that immature dendritic cells (DCs)[4] can be found at high densities in the epidermis and the dermis of human skin (5, 6). Successful noninvasive vaccination by topical application of protein, peptides, and DNA has been shown in many mouse models where intradermal or transcutaneous (TC) vaccination induced both protective humoral and cellular immune responses (7, 8). In a first attempt to immunize humans via TC vaccination, Glenn et al. (9) induced robust immune responses against heat-labile enterotoxin from *Escherichia coli* in human volunteers using a patch system and demonstrated that improved Ab responses can be obtained in elderly individuals vaccinated against influenza by intradermal injection and additional application of an immunostimulatory patch containing heat-labile enterotoxin from *E. coli* (10). These studies, however, focused on the induction of humoral responses. Recent implications of epithelial DCs in CD8 cell cross-priming suggest that vaccination via the TC route may be relevant in the induction of cellular immune responses (11–13).

One major obstacle to TC vaccine delivery is the stratum corneum, which is a major constituent of the skin barrier. Different procedures, including gene gun technology, electroporation, and stripping, have been proposed to facilitate the percutaneous penetration of vaccine preparations (14). Recent work by our group and others supports the importance of hair follicles in percutaneous penetration processes (15–17). In contrast to the interfollicular epidermis, the hair follicle infundibulum has to be considered as highly permeable, suggesting that DCs, which reside in and around the hair follicle, are more accessible for topically applied vaccines than DCs in the interfollicular epidermis. Cyanoacrylate skin surface stripping (CSSS) facilitates follicular penetration by removing cellular debris and sebum from the hair follicle openings (18). In fact, we found that the use of CSSS increased the penetration of topically applied nanoparticles into human-terminal hair follicles (19) and that topically administered nanoparticles, after application of CSSS on human skin explants, entered epidermal Langerhans cells (LCs), possibly via hair follicles (17). In this study, we have focused on the relevance of this administration route on the induction of effector T cell responses in humans.

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[4] Abbreviations used in this paper: DC, dendritic cell; TC, transcutaneous; CSSS, cyanoacrylate skin surface stripping; SFU, spot-forming unit; RT, room temperature; LC, Langerhans cell.

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 Ags from following strain an 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/Shangai/361/2002/- like strain B/jiangsu/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 cm^2 (four volunteers (TC No. 1, 4, 6, and 7), three women, one man, age range 25–52 years) or 32 cm^2 (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 $\times$ 4 cm were delimitated 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 cyanacrylate glue (Superglue, UHU KG) was applied drop-wise on each investigational site. A microscope slide was used to spread the glue evenly on the skin surface, followed by the application of adhesive tape (6 $\times$ 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-Colorpaste, Art.-No. 4469/ko, Max-Bringmann, 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 cm^2 or on two squares of 16 cm^2 each (total skin area of 32 cm^2). Agrippal vaccine was applied drop-wise from the original syringe as provided by the manufacturer (16 drops of $\sim$16 μl each) followed by a soft massage, which was performed by moving a gloved finger tip (care&serve, Wiros) saturated with Agrippal vaccine for 1 min in circles on each investigational site.

This application procedure was followed by an incubation time of 20 min and by the application of a hydrocolloid bandage (Comfeel Plus Transparent 9 $\times$ 14 cm, Art.-No. 3542, Coloplast A/S) for 24 h. The volunteers were instructed not to take a shower or bath and to avoid any activity that causes sweating or mechanical stress on the investigational sites, e.g., physical exercise, during these 24 h.

Intramuscular injection of Agrippal vaccine

Agrippal vaccine (0.5 ml) as provided by the manufacturer was injected i.m. into the Musculus deltoideus of the left arm after careful disinfection, according to Good Clinical Practice.

Adverse events

Twenty-four hours after vaccine application, the hydrocolloid bandage was removed from the investigational sites. Medical examination of the investigational sites was performed and local tolerance was assessed immediately on days 1, 3, 14, and 28 after vaccination by evaluation for erythema, pruritus, burning, and desquamation (graded 0–3, nonsevere to severe). On each visit, the volunteers were interviewed for systemic reactions including rashes, pain, fever, headaches, shivering, diarrhea, and malaise.

Analysis of cytokines

For the quantitative evaluation of cytokine secretion into the plasma, 50 μl of plasma were collected from fresh PBMCs at each time point after vaccination. The Cytometric Bead Array immunoassay system (CBA; BD Biosciences) 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 10^4). 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, Diaclone). 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, Diaclone) (4 h, 37°C), followed by streptavidin alkaline phosphatase (1 h, 37° C) and substrate, 5-bromo-4-chloro-3-indolyl-phosohate/4-nitrobluetetrazolium (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. IMMU LON 96-well plates (Dynatech Laboratories) were coated overnight at room temperature with 50 ng of recombinant hemagglutinin-Influenza A virus H1N1 (New caledonia 20/99) or H3N2 (ProSpec-Trany Technogene) in 100 μl of 0.1 M sodium carbonate buffer (pH 9.6). The plates were washed with demineralized H_2O 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 IgG1 Abs (Sigma-Aldrich) or 1:15,000-diluted mouse anti-human IgG2 (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 H_2SO_4, 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 UI/ml penicillin sodium, 1 mg/ml

Table I. *Monitoring of adverse events after TC and i.m. vaccination*

Mode of Administration	Local Vaccine Reaction				Systemic Reactions
	Erythema	Pruritus	Burning	Desquamation	
TC 16 cm^2	1/4[a]	0/4	0/4	0/4	0/4
TC 32 cm^2	0/3	0/3	0/3	0/3	0/3
i.m.	0/4	0/4	0/4	0/4	1/4[b]

[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.

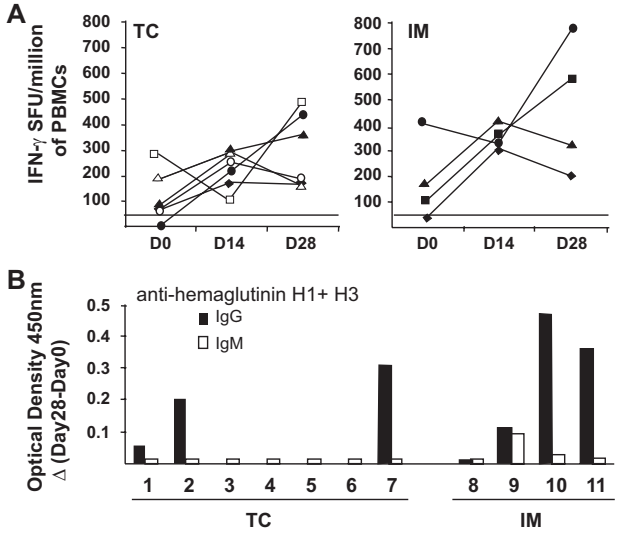

FIGURE 1. IFNγ-producing effector/memory T cell response after TC and i.m. vaccination. *A*, We examined fresh PBMC from 11 healthy individuals, including seven vaccinated by TC route and four vaccinated by i.m. route after informed consent. Four volunteers received 0.5 ml of Agrippal vaccine (Chiron) on 16 cm^2 (closed symbols) and three volunteers received 2×0.25 ml of Agrippal vaccine TC on 2×16 cm^2 (32 cm^2) (opened symbols). Four volunteers received 0.5 ml of Agrippal vaccine by i.m. injection. IFNγ ELISPOT assays were performed on 100,000 PBMCs stimulated with Agrippal vaccine (1/1000 dilution). Background unstimulated cells were retracted, and positive responders were defined by ≥50 SFU/million PBMC as shown by dashed lines. *B*, Hemagglutinin H1 plus H3-specific Abs were measured by ELISA using plasma samples of all volunteers. Samples from TC (No. 2 and 7) and i.m. (No. 10 and 11) vaccinated individuals were mainly composed of IgG1 as measured by ELISA. OD measurements at 450 nm are shown between days 0 and 28.

streptomycin sulfate, and 250 ng/ml amphotericin B). Cells were stimulated with either the influenza vaccine solution (1:1000 dilution) or 1 μg/ml PHA for 16 h at 37°C. Brefeldin A (5 μg/ml) (Sigma Chemical) was added to the well 4 h before harvesting to detect intracellular cytokines. First, IFNγ production was detected by intracellular staining as follows: samples were stained in PBS-1X with either CD4-PC7 or CD8-PC7 for 20 min at room temperature (RT). Cells were fixed with Paraformaldehyde-4% at 4°C for 20 min. After washing and permeabilizing with saponin 0.1%, cells were stained with allophycocyanin-conjugated anti-IFNγ Abs. Flow cytometric analyses were done using FACSCalibur flow cytometer (BD Biosciences). Second, both IFNγ and IL-2 detection were performed by multiparameter fluorescence analysis as follow: samples were stained in PBS-1X for 10 min at RT with CD4-allophycocyanin Cy7, CD3-PerCp-Cy5.5, or CD8-Pacific Blue (BD Biosciences) for 20 min at 4°C. Then, 100 μl of Fix and Perm Medium A (Caltag Laboratories) was added to each sample for 10 min at RT. Washed cells were resuspended with 100 μl of Fix and Perm Medium B (Caltag Laboratories) and incubated with intracellular Abs against IFNγ (Alexa700) and IL-2 (FITC) for 20 min at RT. Flow cytometric analyses were performed using the LSR2 flow cytometer (BD Immunocytometry Systems). At least 1,000,000 live events, according to forward- and side-scatter parameters, were accumulated and analyzed. Data files were then exported and analyzed using FlowJo software (Tree Star). The lymphocyte gate was set based on forward and side scatter for further analysis.

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 graphic representation used Prism4.0 software (GraphPad). All statistical analyses were performed using SSPS11 for MacOS10 software. Statistical significance was set at $p \leq 0.05$.

Table II. *Cytokine detection in plasma of vaccinated individuals*

	TC No. 1			TC No. 3			TC No. 4			TC No. 5			TC No. 6			TC No. 7		
Cytokines (pg/ml)[a]	D0	D14	D28	D0	D14	D28	D0	D14	D28	D0	D14	D28	D0	D14	D28	D0	D14	D28
TC vaccination																		
IL-12p70	—[b]	—	—	—	—	4	—	—	—	—	—	—	—	5	—	—	—	—
TNF	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IL-10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IL-6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IL-1b	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IL-8	—	46	—	169	435	262	—	277	70	29	329	122	—	—	—	22	258	181

	i.m. No. 8			i.m. No. 9			i.m. No. 10			i.m. No. 11		
Cytokines (pg/ml)[a]	D0	D14	D28	D0	D14	D28	D0	D14	D28	D0	D14	D28
i.m. vaccination												
IL-12p70	—	—	—	4	5	—	—	—	3	—	—	—
TNF	—	—	—	—	—	—	—	—	—	—	—	—
IL-10	—	—	—	—	—	—	—	—	—	—	—	—
IL-6	—	—	—	—	—	—	—	—	—	—	—	—
IL-1b	—	—	—	—	—	—	—	—	—	—	—	—
IL-8	—	—	—	—	—	—	—	—	—	—	—	—

[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.

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

	IFNγ SFU/million of PBMCs[a]					
	TC			i.m.		
	Day 0	Day 14[b]	Day 28[b]	Day 0	Day 14[b]	Day 28[b]
n	6	6	6	4	4	4
Range	4–281	99–290	160–472	35–400	307–413	200–764
Median	76.5	234.0	266.5	132.5	340.5	447.0
IQR[c]	160	130	280	290	88	486.5
Fold increase	—	3.0	2.6	—	3.0	3.8

[a] IFN-γ 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.
[b] 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.
[c] IQR, Interquantile range.

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 Aggripal vaccine, a commercially available anti-influenza vaccine.

The total amount of Agrippal vaccine (0.5 ml) was applied either TC on 16 cm^2 ($n = 4$) or 32 cm^2 ($n = 3$) 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. 1*A*). 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 IV. *Frequencies of influenza-specific effector CD4 and CD8 lymphocytes assessed by intracellular cytokine staining*

	TC			i.m.		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
$IFN\gamma^+$ among CD4 cells						
n	6	6	6	4	4	4
Range	0.00–0.10	0.07–0.13	0.08–0.17	0.00–0.24	0.16–0.44	0.00–0.50
Median	0.03	0.10	0.12	0.00	0.36	0.16
IQR[a]	0.1	0.04	0.04	0.18	0.24	0.38
Positive responders[b]	3/6	6/6	6/6	1/4	4/4	3/4
$IFN\gamma^+$ among CD8 cells						
n	6	6	6	4	4	4
Range	0.00–0.58	0.00–0.46	0–0.52	0	0	0
Median	0.00	0.06	0.00	0	0	0
IQR[a]	0.15	0.19	0.17	0	0	0
Positive responders[b]	1/6	4/6	2/6	0/4	0/4	0/4

[a] IQR, Interquantile range.
[b] 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. $IFN\gamma^+CD4^+$ 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-exiting 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 cm^2 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. 1*B*). 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 titers at day 28 after vaccine administration showed no significant differences at that time point. This point will be further addressed in the on going 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. 2*A*, *upper panel*). Interestingly, we observed influenza-specific $CD8^+IFN\gamma^+$ cells at day 14 in 4 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. 2*A*, *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. 2*B*). 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.

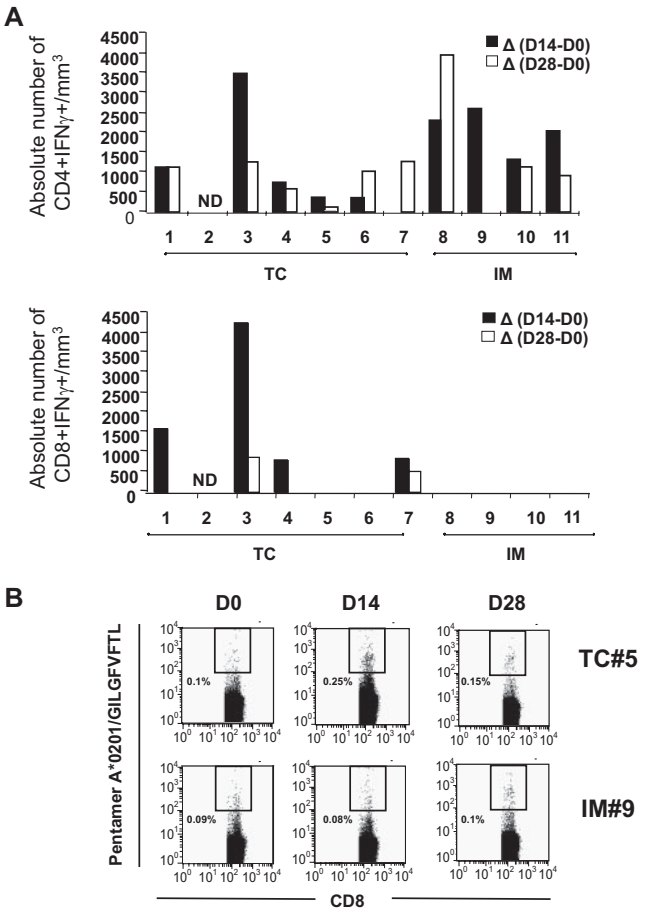

FIGURE 2. Distinct T cell immune responses by TC route compared with i.m. route of immunization. *A*, We performed intracellular IFNγ staining of influenza-specific T cells by flow cytometric analysis. PBMCs from vaccinated individuals were stimulated with the Agrippal vaccine (1/1000 dilution) for 16 h. Brefeldin A (5 μg/ml) was added 4 h before harvesting. ICS was performed for IFNγ by flow-cytometric assay (allophycocyanin-conjugated anti-IFNγ) and gated on $CD4^+$ and $CD8^+$ T cells. Absolute number of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ in PBMCs of vaccinated individuals were calculated for each group. At least 1,000,000 live events, according to forward- and side-scatter parameters, were accumulated and analyzed (see *Materials and Methods* section). *B*, Detection of HLA-A201 restricted Ag-specific CD8 cells in a TC vaccinated individual. HLA-A2 phenotype was observed in only 1/6 TC vaccinated and 1/4 i.m. vaccinated individuals. PBMCs were analyzed to influenza-specific T cells by flow cytometric analysis. GILGFVFTL-specific CD8 T cells restricted to HLA-A201 were detected by PE-associated pentamer A*0201/GILGFVFTL and CD8 staining. Results represent the flow cytometric analysis of GILG FVFTL-specific CD8 T lymphocytes in one TC vaccinated subject at days 0, 14, and 28 (volunteer TC No. 5) and one i.m. vaccinated subject at the same time points (volunteer i.m. No. 9).

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 $IFN\gamma^+CD8^+$ cells were increased in TC (Fig. 3*A*) and not in i.m. vaccinated individuals (Fig. 3*B*).

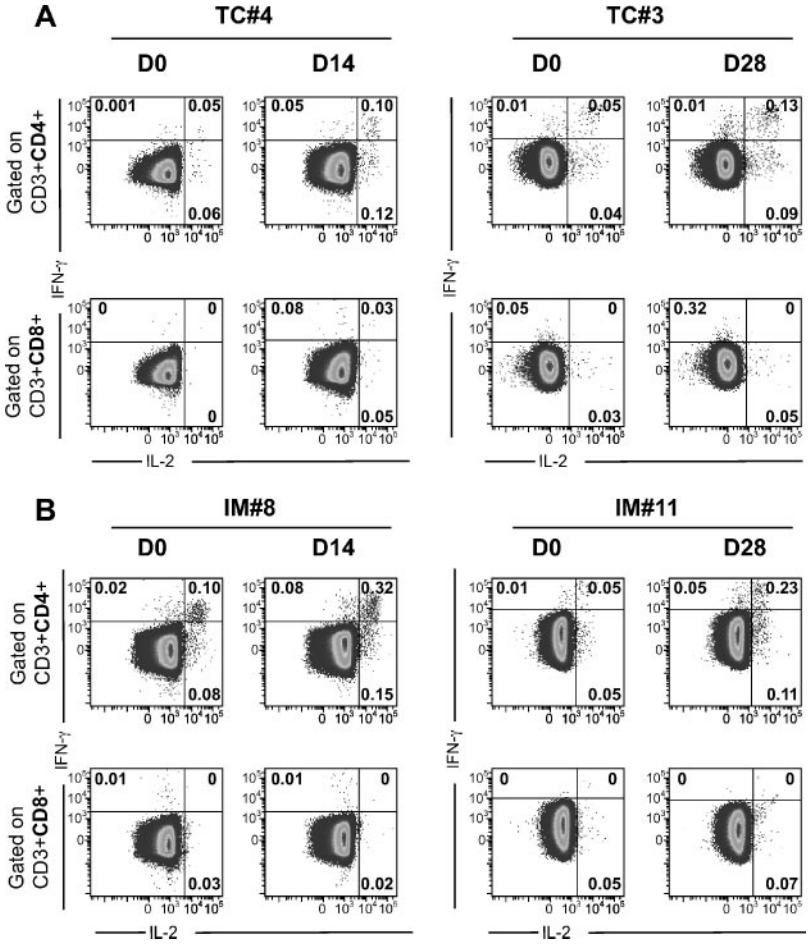

FIGURE 3. IL-2 and IFNγ production by influenza-specific effector CD4 and CD8 responses after TC and i.m. vaccination. PBMCs from vaccinated individuals were stimulated with the Agrippal vaccine (1/1000 dilution) for 16 h. Brefeldin A (5 mg/ml) was added 4 h before harvesting. We analyzed the production of both IL-2 (FITC-conjugated Abs) and IFNγ (Alexa700-conjugated Abs) Type 1 cytokines by 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.

Differentiation markers were analyzed on influenza-specific CD4 and CD8 cells producing IFNγ. We found a majority of influenza-specific $CD3^+CD4^+$ T cells expressed CD27 (85%) and variably expressed CD45RA (37% $CD45RA^+$) and a limited proportion expressed CCR7 (35%). In addition, a majority of influenza-specific $CD3^+CD8^+$ T cells expressed CD27 (77%) and CD45RA with a limited expression of CCR7 (5%) (data not shown).

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 cm^2 and 32 cm^2). 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 strippings 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, topically 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, which was also present in some i.m. vaccinated volunteers and, more importantly, high levels of IL-8 only in the sera of individuals vaccinated by TC route. CXCL-8 (IL-8) is an inflammatory chemokine that has an important role 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 cm^2 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 IFNγ-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 IFNγ-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 cm^2, 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 percutanous 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 percutanous peptide immunizations on skin areas as large as 100 cm^2 (32) compared with 16 or 32 cm^2 in our study. Also, after such treatment, LCs have upregulated 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 cm^2 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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Disclosures

The authors have no financial conflict of interest.

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