J Control Release. 2012 September 10; 162(2): 391–399. PMCID: PMC3462999

doi: 10.1016/j.jconrel.2012.06.031

# Transcutaneous vaccination via laser microporation

Richard Weiss,[a] Michael Hessenberger,[a] Sophie Kitzmüller,[a] Doris Bach,[a] Esther E. Weinberger,[a] Wolf D. Krautgartner,[b] Cornelia Hauser-Kronberger,[c] Bernard Malissen,[d] Christof Boehler,[e] Yogeshvar N. Kalia,[f] Josef Thalhamer,[a,*] and Sandra Scheiblhofer[a]

Author information ► Article notes ► Copyright and License information ►

## Abstract

Go to:

Driven by constantly increasing knowledge about skin immunology, vaccine delivery via the cutaneous route has recently gained renewed interest. Considering its richness in immunocompetent cells, targeting antigens to the skin is considered to be more effective than intramuscular or subcutaneous injections. However, circumvention of the superficial layer of the skin, the stratum corneum, represents the major challenge for cutaneous immunization. An optimal delivery method has to be effective and reliable, but also highly adaptable to specific demands, should avoid the use of hypodermic needles and the requirement of specially trained healthcare workers. The P.L.E.A.S.E.® (Precise Laser Epidermal System) device employed in this study for creation of aqueous micropores in the skin fulfills these prerequisites by combining the precision of its laser scanning technology with the flexibility to vary the number, density and the depth of the micropores in a user-friendly manner. We investigated the potential of transcutaneous immunization via laser-generated micropores for induction of specific immune responses and compared the outcomes to conventional subcutaneous injection. By targeting different layers of the skin we were able to bias polarization of T cells, which could be modulated by addition of adjuvants. The P.L.E.A.S.E.® device represents a highly effective and versatile platform for transcutaneous vaccination.

**Keywords:** Transcutaneous vaccination, Laser, Micropores, Targeting, Dendritic cells

## Graphical abstract

Go to:

## 1. Introduction

Go to:

Most vaccines are administered to muscle or subcutaneous tissue by injection using needle and syringe. Whereas the transcutaneous route was heavily employed during the smallpox eradication campaign by scarification with a bifurcated needle [1], currently only Bacille Calmette Guérin vaccine against tuberculosis and rabies vaccine in developing countries are delivered intradermally [2,3]. For both, the Mantoux method is used, which employs a conventional hypodermic needle inserted at a shallow angle into the skin. Invented by Mendel & Mantoux in the early 1900s [4], this technique requires well-trained healthcare personnel to achieve consistent delivery [5,6] and also involves the general disadvantages associated with the use of hypodermic needles, including pain and apprehension felt especially by children, needlestick injuries, and intentional re-use of needle and/or syringe. The latter two are responsible for more than half a million deaths annually due to transmission of HIV and hepatitis B and C [7].

In contrast to muscle, which cannot be considered a highly immunogenic organ [8], the skin represents an attractive target tissue for vaccination due to its high accessibility and its unique immunologic properties. The epidermis and dermis are rich in immunocompetent cells, which mediate the immune response following skin immunization [9–13]. Langerhans cells (LCs) and dermal dendritic cells (DCs) residing in the epidermal and the dermal compartment of the skin, respectively, are key players in skin immune responses. They capture, process, and present foreign antigen, and closely interplay with keratinocytes, mast cells and T lymphocytes [14,15]. Among the other cells in the epidermis and dermis, skin keratinocytes, in particular, have the potential to enhance and shape immune responses by producing cytokines and chemokines [12]. Compared to intramuscular injection, vaccination via the skin results in more efficient antigen trafficking into lymph nodes (LNs) [16–18].

The efficacy of skin vaccination has been recently demonstrated by several clinical studies [19], among them some showing comparable results at lower dosage [20–22], improved protection in elderly persons [23,24], or mucosal immunity [10,25,26].

Many vaccines could become candidates for delivery via the skin if an easily applicable, reliable, patient-friendly immunization method would be available. The major challenge for cutaneous immunization is to overcome the superficial layer of the skin, the stratum corneum, which acts as an almost impermeable barrier, preventing diffusion of molecules, particularly those of a molecular weight beyond 500 Da [27]. A variety of non- or minimally invasive methods to enhance transcutaneous antigen delivery have been developed, including liquid jet injection, epidermal powder immunization, tape stripping, skin abrasion, thermal ablation, ultrasound, tattooing, and the use of arrays of solid, coated, hollow or dissolvable microneedles, as reviewed by Bal et al. [28]. However, the skin is a heterogenous tissue and its properties highly depend on body location, age, skin type, hydration level, and body weight, and also vary between individuals [29]. Therefore, introduction of a methodology to circumvent the stratum corneum in a reproducible, but also highly adaptable manner would be desirable.

The P.L.E.A.S.E.® (Precise Laser Epidermal System) device developed by Pantec Biosolutions offers the opportunity to create aqueous micropores of approximately 150 μm diameter via controlled fractional

ablation of skin layers. This novel technique employs a diode-pumped Erbium:yttrium-aluminium-garnet (ER: YAG) laser, which emits energy at 2.94 μm — a major absorption wavelength of water molecules. Their excitation and evaporation leads to formation of pores filled with exudate, without causing thermal damage to adjacent skin tissue. The number of micropores to be created can be varied by the user as well as the laser fluence (energy per unit area) determining the depth of the micropores. This allows for selective targeting of different skin layers, rendering the P.L.E.A.S.E.® device adjustable to specific needs. The high pulse repetition rate of up to 1 kHz enables sequential creation of an array of several hundred micropores within a few seconds [30,31].

In most previous studies using ER: YAG lasers to circumvent the skin barrier, devices with a single laser beam and a fixed spot diameter of several millimeters have been employed [32–41]. Compared to fractional ablative systems, these devices remove a much larger amount of skin surface leading to a prolonged healing time. More recently, conventional fractional ER: YAG lasers have been employed, which create a micropore grid in the skin requiring manual adjustment to create additional pores [39,40], or which split the laser beam into separate beams using a grid to fractionate the energy [41]. In contrast, the P.L.E.A.S.E.® device uses a beam deflection unit that can generate any number of precise pores in an area with a diameter of up to 3 cm.

It has been demonstrated that laser microporation using the P.L.E.A.S.E.® device significantly increased in vitro transport of low molecular weight compounds [30,31,42], and also enabled delivery of functional antibodies through porcine, murine and human skin samples as well as through murine skin in vivo [43]. Moreover, in vivo laser microporation followed by delivery of follicle stimulating hormone for superovulation has been reported [44].

Very recently, delivery of drugs or ovalbumin as model antigen via microchannels generated by fractional ablation of the skin was demonstrated to be superior in comparison to tape stripping [45]. In our current paper we demonstrate for the first time the use of the P.L.E.A.S.E.® device for transcutaneous immunization (TCI) with protein antigens and provide an in-depth characterization of the resulting immune responses.

## 2. Materials and methods

Go to:

### 2.1. Mice and immunizations

BALB/c and C57BL/6 females aged 6–8 weeks were purchased from Charles River Laboratories (Sulzfeld, Germany), DO11.10, OT-II, OT-I, C57BL/6 and BALB/c expressing the CD45.1 alloantigen, and Lang-DTR-enhanced GFP (Lang-DTR) [46] backcrossed for more than 9 generations onto C57BL/6 or BALB/c backgrounds, respectively, were bred and maintained at the animal facility of the University of Salzburg and used at an age of 6–12 weeks. Ovalbumin (OVA) transgenic mice were crossed with CD45.1 BALB/c or C57BL/6 mice and the F1 generation was used as donors for transfer experiments. Animal experiments were conducted according to local guidelines approved by the Austrian Ministry of Science (Permit Number: GZ 66.012/0004-II/10b/2010). The day before laser microporation, animals were shaved on their back with a clipper and depilatory cream was used to remove residual hair. Laser microporation using the P.L.E.A.S.E.® device (Pantec Biosolutions) was performed by placing anesthetized mice with their back at the focal length of the laser. Poration parameters, i.e. number of pores/cm$^2$, number of pulses per pore, and fluence (energy applied per unit area) were pre-programmed using the device software and are indicated for each experiment. If not

stated otherwise, the respective antigens were applied as aqueous solution to the microporated skin area and left to completely dry.

### 2.2. Antigens

FITC-Dextran (MW: 2000 kDa), ovalbumin (OVA, grade V), beta-galactosidase (bGal), bovine serum albumin (BSA), and FITC were purchased from Sigma–Aldrich (Deisenhofen, Germany). FITC-BSA was prepared according to the protocol supplied by Sigma. Recombinant Phl p 5, a major grass pollen allergen with a MW of 38 kDa, was purchased from Biomay AG, Vienna, Austria.

### 2.3. Histological analysis

2 μm paraffin sections of skin samples were prepared and stained with haematoxylin/eosin using standard methods.

For fluorescence microscopy, microporated skin samples were embedded in Tissue-Tek® O.C.T. ™ compound in Tissue-Tek cryomolds (Sakura Fintek Europe B.V., Alphen aan den Rijn, Netherlands), gently frozen, and stored at − 70 °C until preparation of frozen sections (10 μm), which were mounted on microscopic slides, embedded in Mowiol and analyzed on a fluorescence microscope (Olympus IX70).

For scanning electron microscopy, samples were fixed for 2 h with Karnovsky [47], postfixation was performed with 1% osmium tetroxide ( buffered at pH 6.5 with 0.1 M sodium cacodylate ) for further 2 h. The postfixed samples were dehydrated in an ascending series of ethyl alcohol, critical-point-dried, and subsequently sputtered with gold (~ 5 nm) and analyzed in an environmental scanning electron microscope, ESEM XL30 (FEI, Philips, Eindhoven, the Netherlands), operating at 20 kV.

### 2.4. Transcutaneous delivery through porcine skin

Dermatomed porcine ear skin (thickness: 0.75 mm) was porated at 900 pores/30 pulses per pore. Following poration, the skin samples were mounted in Franz diffusion cells with a cross-sectional area of 2.9 $cm^2$. 1 mL of unbuffered FITC-BSA (MW ~ 70 kDa) solution (10 mg/mL) was placed in the donor compartment. Tris buffer (pH 7.4) was used as receptor solution and samples of 3 mL thereof were taken after 24 h to determine permeation via measurement of fluorescence at $\lambda_{ex}$496 nm and $\lambda_{em}$500–650 nm. By soaking the skin samples in 6 mL of a suitable extraction medium for 4 h followed by filtration through PVDF membranes (0.45 μm), the amount of FITC-BSA deposited in the skin was determined.

### 2.5. Flow cytometric analysis of DLN-DCs

BALB/c mice (n = 4) were laser microporated at a fluence of 0.76 J/$cm^2$ per pulse, generating an array of 1600 pores with a diameter of 2 cm applying either 1 or 8 pulses per pore. 100 μl of FITC-dextran in PBS [10 mg/ml] was topically administered, or was s.c. injected. After 72 h, cells from axillary and inguinal LNs were prepared [48], pooled for each group, and surface stained with anti-MHC-II (clone M5/114, BD Pharmingen), anti-CD11b (clone M1/70, BioLegend), anti-CD103 (clone M290, BD Pharmingen), followed by intracellular staining using anti-CD207 mAb (clone 929F3; Dendritics-AbCys). Cells were recorded on a FACSCanto II flow cytometer and analyzed using FACSDiva Software (BD Biosciences).

### 2.6. In vivo proliferation and characterization of OVA transgenic T cells

For in vivo proliferation assays, on day 0, $2 \times 10^6$ splenocytes from OT-II, OT-I, or DO.11.10 donors (CD45.1 background) were labelled with a Vybrant CFDA SE Cell Tracer Kit (CFSE, Life Technologies) and adoptively transferred to naive recipient mice as described [48]. OVA was applied to laser microporated skin (900 pores, 1.5 cm diameter, 6 pulses delivered at 1.9 J/cm$^2$ per pulse) on day 1 and was completely absorbed within 10–20 min. 6 days later, DLN cells were prepared and proliferation was assessed by gating on CD45.1 + CD4 + (CD8 + in case of OT-I) cells and calculating the proliferation index (proliferated/non-proliferated cells). In some groups, Langerin + cells were ablated by i.p. injection of 1 μg diphtheria toxin (DT) on days − 1, 2, and 5.

Proliferated vs. non-proliferated DO11.10 T cells were further analyzed by surface staining with anti-CD134 (clone OX-86, eBioscience), anti-CD62L (clone MEL-14, eBioscience), anti-CD45RB (clone C363.16A, eBioscience), anti-Tim-1 (RMT1-4, eBioscience), anti-Tim-3 (RMT3-23, eBioscience), anti-CD25 (clone PC61, BD Pahrmingen), anti-CD152 (clone UC10-4B9, eBioscience), and intracellular staining with anti-T-bet (clone eBio4B10, eBioscience), anti-Gata-3 (clone TWAJ, eBioscience), and anti-FoxP3 (clone FJK-16 s, eBioscience), after application of 100 μg OVA distributed between two non-overlapping microporated areas (400 pores, 1 cm diameter, 1–6 pulses delivered at 1.9 J/cm$^2$ per pulse), or a respective s.c. injection. 10,000 splenocytes per mouse from these groups were cultured in vitro together with 100,000 X-ray irradiated at a dose of 60 Gy (80 kV, 16 mA) syngeneic feeder cells in the presence of 20 μg/mL OVA for 3 days and culture supernatants were analyzed for cytokine secretion using FlowCytomix Multiplex kits (eBioscience) according to the manufacturers protocol.

### 2.7. Characterization of endogenous immune responses

BALB/c LangDTR mice were immunized by applying 200 μg OVA distributed between two non-overlapping microporated areas (400 pores, 1 cm diameter, 6 pulses delivered at 0.76 J/cm$^2$ per pulse) on days 0 and 7. One group was injected i.p. with 1 μg DT on day − 1. Mice were sacrificed on day 14, when blood samples were drawn and splenocytes were harvested. Antibody levels in sera were measured by a luminometric ELISA assay at indicated serum dilutions lying within the linear range of the assay. Biologically functional IgE was assessed by an in vitro basophil release assay. Splenocytes were cultured without feeder cells and cytokines were measured as described above. A more detailed description of these assays can be found elsewhere [49].

Immunization of BALB/c mice (n = 3) with beta-galactosidase was performed by applying 5 μg of protein on days 0 and 11, and 10 μg of protein on day 31 on microporated skin (1500 pores, 2.5 cm diameter, 8 pulses delivered at 1.9 J/cm$^2$ per pulse) with or without TLR agonists poly I:C (Sigma), R848 (Alexis), or phosophothioate CpG ODN1826 (Biomers) at the indicated doses applied in PBS or PBS/36% DMSO (only R848 groups). Sera were analyzed for IgE via in vitro basophil release assay on day 44 and splenocytes were analyzed for IL-4 and IFN-γ secretion by ELISPOT assay as described [48].

To assess immune responses against Phl p 5, BALB/c mice (n = 5) were immunized on days 0 and 10 with 5 μg Phl p 5 applied to microporated skin (400 pores, 1 cm diameter, 4 pulses delivered at 1.9 J/cm$^2$ per pulse) with or without 100 μg ODN1826 or via s.c. injection of the same dose. Sera taken at day 28 were analyzed by luminometric ELISA and in vitro basophil release assay.

### 2.8. Statistical analysis

Statistical significance between groups was assessed by unpaired Students *T*-Test (alpha = .05) using

SigmaStat 2.0. Groups that failed normality test were compared using Mann–Whitney rank sum test.

## 3. Results

Go to:

### 3.1. Targeting of different skin layers

Transcutaneous application of macromolecules via laser-generated micropores using the P.L.E.A.S.E® device has been previously demonstrated in vitro for porcine as well as human skin [43]. To evaluate the immune responses generated by transcutaneous vaccination we first established the optimal laser parameters for mouse skin. Fig. 1A shows an array of 400 laser-generated micropores per $cm^2$ in the skin of a BALB/c mouse. By modulating the number of pulses per pore, different skin depths can be targeted. At low pulse numbers, the stratum corneum is weakened and partly removed, while the epidermis stays mainly intact (Fig. 1B). By increasing the pulse number, the epidermis is removed, exposing the underlying dermis (Fig. 1C), which can be further ablated to increase pore depth (Fig. 1D). At the applied fluence of 1.9 $J/cm^2$ per pulse, no carbonization takes place, enabling high diffusion rates via aqueous micropores (Fig. 1E).

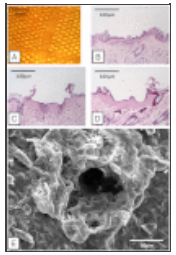

Fig. 1

Histological analysis of laser-generated micropores in mouse skin. A) Top view of skin after laserporation using 2 pulses (F = 1.9 $J/cm^2$ per pulse), 400 pores/$cm^2$. Panels B–D show representative HE-stained paraffin skin **...**

When applying FITC-labeled BSA to microporated skin, the antigen selectively attaches to the surface of the micropores from where it slowly diffuses into deeper layers of the skin (Fig. 2). In vitro experiments with porcine skin samples have shown that FITC-BSA (10 mg/mL, unbuffered aqueous solution) permeated 0.75 mm dermatomed skin at a rate of 222.1 ± 21.4 μg/$cm^2$ per 24 h while 51.2 ± 8.6 μg/$cm^2$ remained deposited on the skin. Overall, 8.2% of the applied dose was delivered within 24 h.

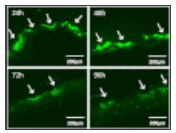

Fig. 2

In vivo uptake of FITC-BSA via laser-generated micropores in mouse skin. A 5 mm × 5 mm piece of gauze was soaked with 80 μL of FITC-labeled BSA

(10 mg/mL), applied to laser-generated micropores ...

### 3.2. Antigen uptake is mainly mediated by Langerin negative dendritic cells

As a next step, we investigated antigen uptake and transport to skin draining lymph nodes (DLN) by flow cytometric analysis of DLN DCs, 72 h after application of FITC labeled dextran to micropores. DCs in DLNs were gated according to their high expression of MHC class II, further separated into Langerin (CD207) positive and negative cells and analyzed for uptake of FITC-dextran (Fig. 3A). With increasing pulse numbers (corresponding to pore depth) we found increasing numbers of FITC + MHC $II^{high}$ dendritic cells (Fig. 3B). Laserporation induced higher levels of CD207 + CD11b − CD103 − as well as CD207 − CD11b + DCs compared to subcutaneous injection which may represent migrating LCs and dermal DCs, respectively that have just entered the DLNs (Fig. 3C). However, when we investigated which cell types had actually taken up antigen, we found that predominantly CD207 − cells were positive for FITC(− dextran). Interestingly, antigen applied by s.c. injection was taken up mainly by CD207 − CD11b − DCs while laserporation also induced antigen uptake by CD207 − CD11b + DCs, indicating that laserporation facilitates antigen uptake (and probably presentation) by CD11b-like or inflammatory DCs (Fig. 3D).

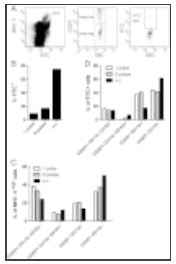

Fig. 3

Analysis of DCs in skin DLNs 72 h after application of FITC-dextran to laser porated mouse skin. A) Gating of MHC-IIhigh DCs (left), separation into Langerin positive and negative DCs (middle), and quantitation of FITC-dextran positive cells (right). ...

### 3.3. Antigen application via P.L.E.A.S.E.® activates antigen specific T cells in DLNs

To assess antigen specific T cell activation via transcutaneous vaccination, OVA specific transgenic T cells (DO11.10 or OT-II for class II and OT-I for class I) were labeled with CFSE and adoptively transferred to syngeneic recipient mice. To distinguish donor cells from recipient cells, donor mice were of the CD45.1 haplotype while WT recipients were CD45.2+. Application of OVA to laser-generated micropores resulted in a dose-dependent proliferation of OVA-specific T cells (Fig. 4A, top panel). Proliferation increased with the number of laser pulses and maximal T-cell stimulation was usually achieved with 4–8 laser pulses (supplementary Fig. 1). As expected, application of proteins to micropores mainly activated CD4 + DO11.10 or OT-II T cells and to a much lesser extent CD8 + OT-I cells (Fig. 4A). Although we cannot expect that 100% of the epicutaneously applied antigen is taken up, TCI via micropores induced significantly higher T cell proliferation compared to s.c. injection (Fig. 4A middle panel) and similar levels compared to i.d. injection of the same amount of antigen (supplementary Fig. 3). By selectively ablating Langerin positive cells using Lang-DTR mice, we could further show that activation of CD4 + OVA-transgenic T cells is, at least in part, dependent on the

presence of epidermal and/or dermal Langerin + DCs (Fig. 4A middle panel).

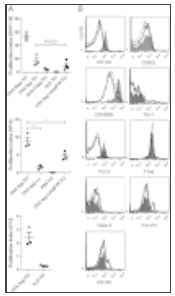

Fig. 4

T cell responses in skin draining lymph nodes. CD45.1 + OVA transgenic DO11.10, OT-II, or OT-I T cells were transferred to syngeneic mice and T cell activation was assessed by CFSE in vivo proliferation assay. A) Proliferation indices after application ...

To further investigate the immune profile of antigen-specific T cells after TCI vs. s.c immunization, we compared the expression profile of proliferating vs. non-proliferating CD45.1 cells via flow cytometry ( Fig. 4B). Applying OVA to pores of different depth (1, 4 and 6 pulses) did result in a very similar expression pattern of surface and intracellular markers (only the results for 6 pulses are shown). S.c. injection and TCI induced nearly identical activation patterns. In all groups proliferating T cells significantly ($P < 0.001$) downregulated CD45RB (and partly also CD62L), indicative of a memory phenotype. However, downregulation of CD134 (OX-40, $P < 0.001$) at the same time suggests that these T cells were not fully activated. Regarding Th1/Th2 differentiation markers, we found no changes in the expression levels of the key transcription factors T-bet (Th1) and Gata-3 (Th2). However, surface expression of Tim-3 (Th1/Th17) was significantly downregulated in proliferating T cells ($P < 0.001$), while Tim-1 (Th2) was unaffected. With respect to regulatory T cell-associated markers we found no induction of CD152 + (CTLA4), CD25, or FoxP3 in proliferating T cells.

### 3.4. Cytokine profile of activated DO11.10 cells after TCI vs. s.c. injection

Splenocytes of vaccinated recipients were also re-stimulated in vitro and the cytokine profile was evaluated in cell culture supernatants using a Th1/Th2/Th17 10-plex bead assay. Compared to s.c. injection, TCI showed a trend towards increased Th2 polarization indicated by elevated levels of IL-4 and also enhanced IL-5 but also significantly promoted IFN-γ secretion (Th1) and even more prominently IL-17 and GM-CSF, both cytokines that can be attributed to Th17 cell differentiation (Fig. 5 ). In general, Th1/Th2/Th17 cytokine secretion increased with pore depth and reached a maximum at 4 pulses; however, only IL-17 and GM-CSF significantly differed when comparing 1 pulse to 4 pulse application. While these data implicate that TCI via laser-generated micropores can induce profoundly different T cell polarization profiles compared to s.c. injection, the Th17 phenotype found in adoptively transferred DO11.10 cells could not be confirmed in the following in vivo vaccination experiments.

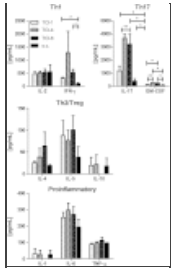

Fig. 5

Cytokine profiles of adoptively transferred DO11.10 cells. OVA-specific DO11.10 cells were transferred to syngeneic mice and 100 μg OVA was applied by TCI or s.c. injection. Subsequently, splenocytes were prepared and re-stimulated in ...

### 3.5. TCI primes Th2-polarized immune responses that can be converted to Th1 responses by adjuvantation

In contrast to the previous experiments employing injected transgenic T cells, analysis of the animal's own T cells, which had expanded after TCI, elicited Th2 polarized immune responses. In these experiments, we immunized LangDTR mice with OVA applied to micropores generated with 6 pulses delivered at 1.9 J/cm$^2$ per pulse. One group had been depleted of Langerin + DCs by a single injection of diphtheria toxin (DT) and a non-depleted group served as control. Independent of DT administration, both groups clearly mounted Th2 responses as indicated by high IgG1 to IgG2a ratios ( Fig. 6A), presence of IgE (Fig. 6B), as well as lack of IFN-γ, and induction of IL-4 ELISPOTs. Of the 13 cytokines measured by Th1/Th2/Th17/Th22 multiplex bead assay, only IL-13, IL-22, and IL-6 were significantly elevated compared to naïve mice. No Th1 or Th17 cytokines were detectable (Fig. 6C).

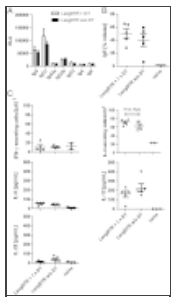

Fig. 6

Immune responses against OVA in BALB/C LangDTR mice with or without depletion of Langerin + cells. One group was depleted of Langerin + DCs by a single injection of DT (1 × DT) one day before the first TCI. Immune ...

The results obtained after immunization with OVA were confirmed using two further antigens (bGal, Phl p 5), different doses and various pulse numbers. In all cases, clear Th2 responses were elicited, as indicated by high IgG1 to IgG2a ratios. In the absence of the adjuvant CpG, the same dose of Phl p 5 applied via TCI proved to be more immunogenic compared to s.c. injection. Only TCI, but not s.c. injection induced antigen specific IgE. However, these Th2 responses could be converted to Th1

responses by co-application of TLR agonists. Of the tested agonists, CpG were most promising in suppressing TCI induced IgE responses and inducing Th1 biased cellular responses, therefore further experiments with two different antigens, namely bGal and Phl p 5, were performed with CpG as adjuvant (Fig. 7).

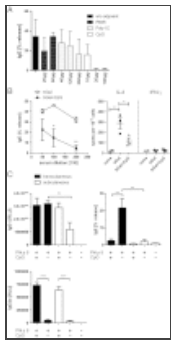

Fig. 7

Endogenous immune responses in BALB/c mice. A) Influence of different TLR agonists on TCI induced IgE responses against bGal (n = 3, serum dilution 1:100). B) IgE and ELISPOT responses in mice immunized with bGal ± 10 μg ...

## 4. Discussion

Go to:

First described in 1998 as a new method to induce robust immune responses via topical application of a vaccine antigen together with an adjuvant [50], TCI has been a topic of intense investigation. Combining the advantages of needle free administration with targeting a tissue rich in immunocompetent cells, this method promises an attractive alternative to conventional vaccination methods.

In our current work, we investigated the applicability of laser microporation using the P.L.E.A.S.E.® device for TCI. By sequential fractional ablation, this device offers the unique opportunity to target different layers of the skin, thereby addressing different cell types such as LCs or dermal DCs. We could demonstrate that even in the mouse the stratum corneum can be precisely removed without substantial damage to the underlying epidermis (Fig. 1A). Alternatively deeper skin layers can be targeted (Fig. 1B–D). In contrast to the study of Chen et al. [45], no signs of thermal damage to the tissue surrounding the pores could be detected (Fig. 1) and complete skin re-epithelialization was achieved within 2 days (supplementary Fig. 1). Similar results were observed in human volunteers [51] and during a clinical in vitro fertilization study, demonstrating safety and clinical applicability of the device [44]. The amount of antigen uptake and concomitant activation of T cells strongly correlated with pore number and depth (Fig. 3B and supplementary Fig. 2). Although targeting the epidermis (1 pulse) showed a trend towards increasing recruitment of CD207 + DCs, compared to targeting the dermal compartment (8 pulses) ( Fig. 3C), this was not reflected by the distribution of DC subpopulations that actually had acquired antigen (Fig. 3D). However, compared to s.c. injection, TCI via laser microporation induced a different pattern of antigen presenting DCs. While both modalities predominantly induced antigen presentation by CD207 − DCs, TCI favored the CD207 − CD11b + subset (Fig. 3D) specialized in CD4 T cell activation and humoral immunity [52]. Although our analysis focused on MHC-II$^{high}$ DCs, which

presumably represent migrating DCs from the periphery, the high percentage of FITC + MHC-II$^{high}$ DCs after s.c. injection of FITC-dextran suggests that some, if not the majority of the antigen had been taken up by LN resident DCs that subsequently upregulated MHC-II [53]. This is supported by the observation that the subcapsular sinus of the skin draining LNs was filled with high amounts of FITC-dextran shortly after s.c. injection, but not after TCI (data not shown).

It has been previously demonstrated that physical disruption of the outermost skin layers results in secretion of pro-inflammatory cytokines by skin keratinocytes and subsequent activation and migration of DCs [12,54]. To investigate the immunogenicity of protein antigen without adjuvant after TCI vs. s.c. injection, we first used adoptively transferred OVA specific T cells as a readout system, as this would facilitate detection of activated T cells and characterization of their polarization. Additionally, due to the short schedule of these experiments, complete ablation of both, dermal as well as epidermal Langerin + cells using LangDTR transgenic mice was possible [55]. Our data shows dose dependent activation of CD4 T cells that was partly reliant on the presence of CD207 + DCs, and also weak activation of OT-I (CD8 +) cells (Fig. 4A). Although this indicates some level of cross-presentation of exogenously acquired antigen by DCs, similar experiments with bGal showed no induction of in vivo CTLs (not shown). TCI induced superior CD4 activation compared to s.c. injection of the same amount of antigen, although less antigen can be expected to actually reach the draining LNs (compare Fig. 3B). This indicates that either more potent subtypes of antigen-presenting DCs (elevated levels of CD11b + cells) were induced and/or that TCI elicited an enhanced activation status of DCs migrating from the skin compared to LN resident DCs that had taken up the antigen in the absence of a local inflammatory stimulus.

Analysis of extracellular activation markers and intracellular polarization markers on proliferating T cells revealed no major differences between TCI and s.c. injection (Fig. 4). TCI induced a more pronounced induction of a broad panel of cytokines in DO11.10 cells than s.c. injection, most notably IFN-γ (Th1) and IL-17 and GM-CSF, which has recently been identified as a novel cytokine in the repertoire of Th17 cells [56]. On the cytokine level, we found a difference between targeting the epidermis (1 pulse) and targeting dermal layers of the skin (4 or 6 pulses) in that only targeting deeper layers of the skin resulted in enhanced Th1/Th17 responses. These findings are in line with other groups who propose functional specializations of DC subsets with regards to immune polarization and activation of T cell subsets [57–59].

Surprisingly, the T helper polarization profile of transferred and activated transgenic cells differed from that of the animal's own expanding T cells after immunization. For the antigens tested, TCI resulted in clear Th2 biased immune responses, indicated by high IgG1 to IgG2a ratios, induction of IL-4 and IL-13, and lack of Th1 and Th17 cytokines (Fig. 6A, C and Fig. 7 B/C). This discrepancy can be explained by the artificially high transgenic T cell precursor frequencies in the recipients, which potentially lead to T cell competition for antigen presenting cells resulting in weak or abnormal responses [60,61]. TCI but not s.c. immunization induced antigen-specific IgE (Fig. 6B and Fig. 7 A, B, and C). Also, no effect of Langerhans cell ablation could be observed. However, due to the longer schedule of these studies, we could not ablate Langerin + dermal DCs over the course of the experiment, due to unacceptable weight loss of mice repeatedly receiving DT. Therefore, in this setting, only epidermal Langerhans cells were absent over the entire course of the experiment, whereas dermal Langerin + DCs quickly repopulate the skin [62]. Together this suggests that epidermal Langerhans cells are dispensable for TCI-induced Th2 responses, while dermal Langerin + DCs contribute at least partly to TCI-induced immunogenicity.

Again, in the absence of adjuvant, TCI proved to be more potent than s.c. injection (Fig. 6C). It has been previously shown that barrier disruption of skin leads to promotion of Th2 type immune responses [63], and uptake of allergen via barrier-disrupted skin is discussed as a major source of allergic sensitization [64,65]. However, by application of suitable adjuvants, such as CpG ODN, this Th2 phenotype could be successfully converted towards a balanced Th1/Th2 response, with concomitant suppression of TCI induced IgE (Fig. 7B and C). This is in line with data showing that CpG ODN prevented Th2 mediated skin inflammation in a model of AD and also could reverse established eosinophilic inflammation [66].

In summary, we demonstrated for the first time that TCI via laser microporation is a potent method for inducing immune responses via the skin. The formation of precise micropores of defined number and depth allow for high levels of antigen uptake and generation of immune responses equal or higher, compared to those induced by s.c. injection of antigen. Our data indicate that targeting different layers of the skin has the potential to bias different T cell polarization patterns. Through adjuvantation, the immune phenotype can be further modulated, making laser microporation with the P.L.E.A.S.E. ® device a versatile platform for vaccine delivery.

## Acknowledgments

Go to:

This work was supported by grants from the Austrian Science Fund, project # P21125, and the Christian Doppler Research Association, and by Biomay AG, Vienna, Austria. None of the funding institutions were involved in study design, the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the article for publication.

Go to:

## Appendix A. Supplementary data

**Supplementary material:**

Supplementary Fig. S1 Skin recovery after laser microporation. Representative skin sections taken at different time points after laserporation using 4 or 8 pulses per micropore.

Supplementary Fig. S2 A Pulse (pore-depth)-dependent proliferation of DO11.10 T cells 6 days after application of 20µg OVA to laser-microporated skin. B) Beginning thermal damage (indicated by carbonization) on edges of micropores after delivery of 8 pulses.

Supplementary Fig. S3 Comparison of transcutaneous vaccination via laser generated micropores to i.d. injection. 20µg of OVA or PBS (vehicle control) was applied via skin patch to microporated skin (TCI) or epicutaneously applied on intact skin (EPI), or intradermally injected (i.d.). Proliferation of adoptively transferred DO11.10 cells was measured 3 days later in skin draining lymph nodes.

Click here to view.(1.3M, docx)

## References

Go to:

1. Baxby D. Smallpox vaccination techniques; from knives and forks to needles and pins. Vaccine. 2002;20:2140–2149. [PubMed]

2. Rabies vaccines: WHO position paper — recommendations. Vaccine. 2010;28:7140–7142. [PubMed]

3. Andersen P., Doherty T.M. The success and failure of BCG — implications for a novel tuberculosis

vaccine. Nat. Rev. Microbiol. 2005;3:656–662. [PubMed]

4. Weniger B.G., Papania M.J. Alternative vaccine delivery methods. In: Plotkin S.A., Orenstein P.A., editors. Vaccines. Elsevier; Amsterdam: 2008. pp. 1357–1392.

5. Mitragotri S. Immunization without needles. Nat. Rev. Immunol. 2005;5:905–916. [PubMed]

6. Hawkridge A., Hatherill M., Little F., Goetz M.A., Barker L., Mahomed H., Sadoff J., Hanekom W., Geiter L., Hussey G. Efficacy of percutaneous versus intradermal BCG in the prevention of tuberculosis in South African infants: randomised trial. BMJ. 2008;337:a2052. [PMC free article] [PubMed]

7. WHO World Health Organization; Geneva: 2004. Safety of Injections: Global Facts and Figures (WHO/EHT 04/04)

8. Hohlfeld R., Engel A.G. The immunobiology of muscle. Immunol. Today. 1994;15:269–274. [PubMed]

9. Debenedictis C., Joubeh S., Zhang G., Barria M., Ghohestani R.F. Immune functions of the skin. Clin. Dermatol. 2001;19:573–585. [PubMed]

10. Enioutina E.Y., Visic D., Daynes R.A. The induction of systemic and mucosal immune responses to antigen-adjuvant compositions administered into the skin: alterations in the migratory properties of dendritic cells appears to be important for stimulating mucosal immunity. Vaccine. 2000;18:2753–2767. [PubMed]

11. Flacher V., Bouschbacher M., Verronese E., Massacrier C., Sisirak V., Berthier-Vergnes O., de Saint-Vis B., Caux C., Dezutter-Dambuyant C., Lebecque S., Valladeau J. Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria. J. Immunol. 2006;177:7959–7967. [PubMed]

12. Gutowska-Owsiak D., Ogg G.S. The Epidermis as an Adjuvant. J. Invest. Dermatol. 2012;132:940–948. [PubMed]

13. Hon H., Jacob J. Tracking dendritic cells in vivo: insights into DC biology and function. Immunol. Res. 2004;29:69–80. [PubMed]

14. Akbari O., Panjwani N., Garcia S., Tascon R., Lowrie D., Stockinger B. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. J. Exp. Med. 1999;189:169–178. [PMC free article] [PubMed]

15. Kupper T.S., Fuhlbrigge R.C. Immune surveillance in the skin: mechanisms and clinical consequences. Nat. Rev. Immunol. 2004;4:211–222. [PubMed]

16. Steinman R.M., Banchereau J. Taking dendritic cells into medicine. Nature. 2007;449:419–426. [PubMed]

17. Sugita K., Kabashima K., Atarashi K., Shimauchi T., Kobayashi M., Tokura Y. Innate immunity mediated by epidermal keratinocytes promotes acquired immunity involving Langerhans cells and T cells in the skin. Clin. Exp. Immunol. 2007;147:176–183. [PMC free article] [PubMed]

18. Valladeau J., Saeland S. Cutaneous dendritic cells. Semin. Immunol. 2005;17:273–283. [PubMed]

19. PATH Program for appropriate technology in health, Seattle. 2009. Intradermal delivery of vaccines:

A review of the literature and potential for development for use in low- and middle-income countries.

20. Auewarakul P., Kositanont U., Sornsathapornkul P., Tothong P., Kanyok R., Thongcharoen P. Antibody responses after dose-sparing intradermal influenza vaccination. Vaccine. 2007;25:659–663. [PubMed]

21. Kenney R.T., Frech S.A., Muenz L.R., Villar C.P., Glenn G.M. Dose sparing with intradermal injection of influenza vaccine. N. Engl. J. Med. 2004;351:2295–2301. [PubMed]

22. Khanlou H., Sanchez S., Babaie M., Chien C., Hamwi G., Ricaurte J.C., Stein T., Bhatti L., Denouden P., Farthing C. The safety and efficacy of dose-sparing intradermal administration of influenza vaccine in human immunodeficiency virus-positive patients. Arch. Intern. Med. 2006;166:1417. [PubMed]

23. Arnou R., Icardi G., De Decker M., Ambrozaitis A., Kazek M.P., Weber F., Van Damme P. Intradermal influenza vaccine for older adults: a randomized controlled multicenter phase III study. Vaccine. 2009;27:7304–7312. [PubMed]

24. Van Damme P., Oosterhuis-Kafeja F., Van der Wielen M., Almagor Y., Sharon O., Levin Y. Safety and efficacy of a novel microneedle device for dose sparing intradermal influenza vaccination in healthy adults. Vaccine. 2009;27:454–459. [PubMed]

25. Ghose C., Kalsy A., Sheikh A., Rollenhagen J., John M., Young J., Rollins S.M., Qadri F., Calderwood S.B., Kelly C.P., Ryan E.T. Transcutaneous immunization with Clostridium difficile toxoid A induces systemic and mucosal immune responses and toxin A-neutralizing antibodies in mice. Infect. Immun. 2007;75:2826–2832. [PMC free article] [PubMed]

26. Hickey D.K., Aldwell F.E., Tan Z.Y., Bao S., Beagley K.W. Transcutaneous immunization with novel lipid-based adjuvants induces protection against gastric *Helicobacter pylori* infection. Vaccine. 2009;27:6983–6990. [PubMed]

27. Bos J.D., Meinardi M.M. The 500 Dalton rule for the skin penetration of chemical compounds and drugs. Exp. Dermatol. 2000;9:165–169. [PubMed]

28. Bal S.M., Ding Z., van Riet E., Jiskoot W., Bouwstra J.A. Advances in transcutaneous vaccine delivery: do all ways lead to Rome? J. Control. Release. 2010;148:266–282. [PubMed]

29. Kleesz P., Darlenski R., Fluhr J.W. Full-body skin mapping for six biophysical parameters: baseline values at 16 anatomical sites in 125 human subjects. Skin Pharmacol. Physiol. 2012;25:25–33. [PubMed]

30. Bachhav Y.G., Summer S., Heinrich A., Bragagna T., Bohler C., Kalia Y.N. Effect of controlled laser microporation on drug transport kinetics into and across the skin. J. Control. Release. 2010;146:31–36. [PubMed]

31. Yu J., Bachhav Y.G., Summer S., Heinrich A., Bragagna T., Bohler C., Kalia Y.N. Using controlled laser-microporation to increase transdermal delivery of prednisone. J. Control. Release. 2010;148:e71–e73. [PubMed]

32. Wang K.H., Fang J.Y., Hu C.H., Lee W.R. Erbium:YAG laser pretreatment accelerates the response of Bowen's disease treated by topical 5-fluorouracil. Dermatol. Surg. 2004;30:441–445. [PubMed]

33. Fang J.Y., Lee W.R., Shen S.C., Wang H.Y., Fang C.L., Hu C.H. Transdermal delivery of

macromolecules by erbium:YAG laser. J. Control. Release. 2004;100:75–85. [PubMed]

34. Shen S.C., Lee W.R., Fang Y.P., Hu C.H., Fang J.Y. In vitro percutaneous absorption and in vivo protoporphyrin IX accumulation in skin and tumors after topical 5-aminolevulinic acid application with enhancement using an erbium:YAG laser. J. Pharm. Sci. 2006;95:929–938. [PubMed]

35. Lee W.R., Shen S.C., Liu C.R., Fang C.L., Hu C.H., Fang J.Y. Erbium:YAG laser-mediated oligonucleotide and DNA delivery via the skin: an animal study. J. Control. Release. 2006;115:344–353. [PubMed]

36. Lee W.R., Pan T.L., Wang P.W., Zhuo R.Z., Huang C.M., Fang J.Y. Erbium:YAG laser enhances transdermal peptide delivery and skin vaccination. J. Control. Release. 2008;128:200–208. [PubMed]

37. Lee W.R., Shen S.C., Fang C.L., Zhuo R.Z., Fang J.Y. Topical delivery of methotrexate via skin pretreated with physical enhancement techniques: low-fluence erbium:YAG laser and electroporation. Lasers Surg. Med. 2008;40:468–476. [PubMed]

38. Lee W.R., Shen S.C., Zhuo R.Z., Wang K.C., Fang J.Y. Enhancement of topical small interfering RNA delivery and expression by low-fluence erbium:YAG laser pretreatment of skin. Hum. Gene Ther. 2009;20:580–588. [PubMed]

39. Lee W.R., Shen S.C., Pai M.H., Yang H.H., Yuan C.Y., Fang J.Y. Fractional laser as a tool to enhance the skin permeation of 5-aminolevulinic acid with minimal skin disruption: a comparison with conventional erbium:YAG laser. J. Control. Release. 2010;145:124–133. [PubMed]

40. Lee W.R., Shen S.C., Al-Suwayeh S.A., Yang H.H., Yuan C.Y., Fang J.Y. Laser-assisted topical drug delivery by using a low-fluence fractional laser: imiquimod and macromolecules. J. Control. Release. 2011;153:240–248. [PubMed]

41. Forster B., Klein A., Szeimies R.M., Maisch T. Penetration enhancement of two topical 5-aminolaevulinic acid formulations for photodynamic therapy by erbium:YAG laser ablation of the stratum corneum: continuous versus fractional ablation. Exp. Dermatol. 2010;19:806–812. [PubMed]

42. Bachhav Y.G., Heinrich A., Kalia Y.N. Using laser microporation to improve transdermal delivery of diclofenac: increasing bioavailability and the range of therapeutic applications. Eur. J. Pharm. Biopharm. 2011;78:408–414. [PubMed]

43. Yu J., Kalaria D.R., Kalia Y.N. Erbium:YAG fractional laser ablation for the percutaneous delivery of intact functional therapeutic antibodies. J. Control. Release. 2011;156:53–59. [PubMed]

44. Zech N.H., Murtinger M., Uher P. Pregnancy after ovarian superovulation by transdermal delivery of follicle-stimulating hormone. Fertil. Steril. 2011;95:2784–2785. [PubMed]

45. Chen X., Shah D., Kositratna G., Manstein D., Anderson R.R., Wu M.X. Facilitation of transcutaneous drug delivery and vaccine immunization by a safe laser technology. J. Control. Release. 2012;159:43–51. [PMC free article] [PubMed]

46. Kissenpfennig A., Henri S., Dubois B., Laplace-Builhe C., Perrin P., Romani N., Tripp C.H., Douillard P., Leserman L., Kaiserlian D., Saeland S., Davoust J., Malissen B. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity. 2005;22:643–654. [PubMed]

47. Karnovsiky M.J. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 1965;27:137A–138A.

48. Stoecklinger A., Grieshuber I., Scheiblhofer S., Weiss R., Ritter U., Kissenpfennig A., Malissen B., Romani N., Koch F., Ferreira F., Thalhamer J., Hammerl P. Epidermal langerhans cells are dispensable for humoral and cell-mediated immunity elicited by gene gun immunization. J. Immunol. 2007;179:886–893. [PubMed]

49. Hartl A., Weiss R., Hochreiter R., Scheiblhofer S., Thalhamer J. DNA vaccines for allergy treatment. Methods. 2004;32:328–339. [PubMed]

50. Glenn G.M., Rao M., Matyas G.R., Alving C.R. Skin immunization made possible by cholera toxin. Nature. 1998;391:851. [PubMed]

51. Kalia Y.N., Bachhav Y.G., Bragagna T., Bohler C. P.L.E.A.S.E.® (Painless Laser Epidermal System): A New Laser Microporation Technology. Drug Deliv. Technol. 2008;8:26–31.

52. Guilliams M., Henri S., Tamoutounour S., Ardouin L., Schwartz-Cornil I., Dalod M., Malissen B. From skin dendritic cells to a simplified classification of human and mouse dendritic cell subsets. Eur. J. Immunol. 2010;40:2089–2094. [PubMed]

53. Henri S., Guilliams M., Poulin L.F., Tamoutounour S., Ardouin L., Dalod M., Malissen B. Disentangling the complexity of the skin dendritic cell network. Immunol. Cell Biol. 2010;88:366–375. [PubMed]

54. Nickoloff B.J. Keratinocytes regain momentum as instigators of cutaneous inflammation. Trends Mol. Med. 2006;12:102–106. [PubMed]

55. Poulin L.F., Henri S., de Bovis B., Devilard E., Kissenpfennig A., Malissen B. The dermis contains langerin + dendritic cells that develop and function independently of epidermal Langerhans cells. J. Exp. Med. 2007;204:3119–3131. [PMC free article] [PubMed]

56. McGeachy M.J. GM-CSF: the secret weapon in the T(H)17 arsenal. Nat. Immunol. 2011;12:521–522. [PubMed]

57. Klechevsky E., Morita R., Liu M., Cao Y., Coquery S., Thompson-Snipes L., Briere F., Chaussabel D., Zurawski G., Palucka A.K., Reiter Y., Banchereau J., Ueno H. Functional specializations of human epidermal Langerhans cells and CD14 + dermal dendritic cells. Immunity. 2008;29:497–510. [PMC free article] [PubMed]

58. He R., Oyoshi M.K., Jin H., Geha R.S. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. Proc. Natl. Acad. Sci. U. S. A. 2007;104:15817–15822. [PMC free article] [PubMed]

59. Mathers A.R., Janelsins B.M., Rubin J.P., Tkacheva O.A., Shufesky W.J., Watkins S.C., Morelli A.E., Larregina A.T. Differential capability of human cutaneous dendritic cell subsets to initiate Th17 responses. J. Immunol. 2009;182:921–933. [PubMed]

60. Zinkernagel R.M., Hengartner H. Regulation of the immune response by antigen. Science. 2001;293:251–253. [PubMed]

61. Smith A.L., Wikstrom M.E., Fazekas de St Groth B. Visualizing T cell competition for peptide/MHC

complexes: a specific mechanism to minimize the effect of precursor frequency. Immunity. 2000;13:783–794. [PubMed]

62. Schwarz A., Noordegraaf M., Maeda A., Torii K., Clausen B.E., Schwarz T. Langerhans cells are required for UVR-induced immunosuppression. J. Invest. Dermatol. 2010;130:1419–1427. [PubMed]

63. Strid J., Hourihane J., Kimber I., Callard R., Strobel S. Disruption of the stratum corneum allows potent epicutaneous immunization with protein antigens resulting in a dominant systemic Th2 response. Eur. J. Immunol. 2004;34:2100–2109. [PubMed]

64. Boralevi F., Hubiche T., Leaute-Labreze C., Saubusse E., Fayon M., Roul S., Maurice-Tison S., Taieb A. Epicutaneous aeroallergen sensitization in atopic dermatitis infants — determining the role of epidermal barrier impairment. Allergy. 2008;63:205–210. [PubMed]

65. Strid J., Strobel S. Skin barrier dysfunction and systemic sensitization to allergens through the skin. Curr. Drug Targets Inflamm. Allergy. 2005;4:531–541. [PubMed]

66. Kootiratrakarn T., Fujimura T., Sano K., Okuyama R., Aiba S., Tagami H., Terui T. Development of a novel Ag-specific immunotherapy using CpG oligodeoxynucleotides in a new, unique mouse cutaneous eosinophilic inflammation model. Eur. J. Immunol. 2005;35:3277–3286. [PubMed]