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Synergistic effects of dendritic cell targeting and laser-microporation on enhancing epicutaneous skin vaccination efficacy

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Abstract: Due to its unique immunological properties, the skin is an attractive target tissue for allergen-specific immunotherapy. In our current work, we combined a dendritic cell targeting approach with epicutaneous immunization using an ablative fractional laser to generate defined micropores in the upper layers of the skin. By coupling the major birch pollen allergen Bet v 1 to mannan from *S. cerevisiae* via mild periodate oxidation we generated hypoallergenic Bet-mannan neoglycoconjugates, which efficiently targeted CD14⁺ dendritic cells and Langerhans cells in human skin explants. Mannan conjugation resulted in sustained release from the skin and retention in secondary lymphoid organs, whereas unconjugated antigen showed fast renal clearance. In a mouse model, Bet-mannan neoglycoconjugates applied via laser-microporated skin synergistically elicited potent humoral and cellular immune responses, superior to intradermal injection. The induced antibody responses displayed IgE-blocking capacity, highlighting the therapeutic potential of the approach. Moreover, application via micropores, but not by intradermal injection, resulted in a mixed TH1/TH17-biased immune response. Our data clearly show that applying mannan-neoglycoconjugates to an organ rich in dendritic cells using laser-microporation is superior to intradermal injection. Due to their low IgE binding capacity and biodegradability, mannan neoglycoconjugates therefore represent an attractive formulation for allergen-specific epicutaneous immunotherapy.

Keywords: dendritic cell targeting; C type lectin receptors; mannan; skin vaccination; laser microporation; epicutaneous immunization; hypoallergen; specific immunotherapy

Introduction

In the past decade the skin has gained renewed attention as target for vaccinations due to its unique features such as accessibility, high abundance of skin-resident professional antigen presenting cells (APCs), and efficient drainage through lymphatics [1]. The main dendritic cell (DC) populations found in human skin are epidermal Langerhans cells as well CD1a⁺, CD14⁺ and CD141⁺ DCs in the dermal compartment [2]. These APC populations differ in their capacity to sense pathogens by utilizing differential expression of pattern recognition receptors such as Toll-like receptors (TLRs), NOD-like receptors, and C-type lectin receptors (CLRs) [2, 3]. The latter are immune receptors that recognize carbohydrate epitopes and thus mediate antigen uptake and cross-presentation, and modulate DC immune polarization [4]. Different populations of skin-resident APCs express specific sets of CLRs depending on the cell lineage, maturation status and function [3]. Based on these findings, several CLR targeting approaches aiming to deliver antigen to specific APC subsets have been published recently [3, 5].

The concept of targeting antigens to skin-resident APCs enables attractive approaches for the rational design of novel allergy vaccines [5]. It allows for proper modulation of the immune response based on the targeted receptors. Moreover, regarding allergy vaccines, delivery of the antigen to the upper layers of the skin minimizes the risk of systemic adverse reactions due to the relatively poor vasculature of the skin. However, disrupting the *stratum corneum* barrier in a precise and controlled way poses a major challenge for efficient antigen delivery into the skin [6]. To date, the best options for overcoming the skin barrier are the use of microneedles and laser ablation [7, 8]. In line with this, we have shown in a mouse model of allergen specific immunotherapy, that epicutaneous immunization (ECi) via laser microporation is equally efficient compared to subcutaneous immunotherapy, but avoids a therapy induced boost of TH2 cytokines [9]. However, application of allergen to the skin of naïve mice can also result in TH2 biased immune responses [10, 11]. This is a potential risk for allergy vaccination, as it may induce new sensitizations, especially when using allergen extracts [12, 13]. Addition of adjuvants has been demonstrated to overcome this unwanted effect and divert the response towards a TH1 or Treg phenotype [10, 14]. These data suggest that epicutaneous immunotherapy may greatly benefit from using immunomodulatory molecules that polarize the immune response towards a non-TH2 phenotype. In this context, allergen-carbohydrate neoglycoconjugates are ideal candidates for skin vaccination since they are able to target the allergen to APCs via specific CLRs while promoting a specific type of immune response [3-5, 15]. We and others have previously shown that allergen-mannan neoglycoconjugates are efficiently targeted to

APCs, and are potent inducers of humoral and cellular immune responses [16-18]. In addition, carbohydrate conjugation renders vaccines hypoallergenic (i.e. they no longer bind IgE) [18], thus avoiding potential side effects as observed in the clinic with epicutaneous delivery of allergen extracts [19].

In the current work, we explore how the combination of epicutaneous immunization via laser-microporation with a skin-resident DC targeting vaccine formulation shapes and potentiates anti-allergic immune responses.

Materials and methods

Materials

Mannan (MN) from *Saccharomyces cerevisiae* (average molecular weight approx. 46 kDa), Concanavalin A (ConA), sodium cyanoborohydride (NaCNBH_3), sodium borohydride (NaBH_4), sodium periodate (NaIO_4), sodium ascorbate, aminoguanidine hydrochloride, Tris(3-hydroxypropyltriazolylmethyl)amine (THTPA), Cytochalasin B, Amiloride hydrochloride, Filipin III from *Streptomyces filipinensis*, and Chlorpromazine were purchased from Sigma Aldrich. Anthrone was purchased from Fluka, pHrodo was obtained from Invitrogen. CF647 was purchased from Biotium, 6-FAM alkyne, Cy5.5 Hydrazide, and Cy5.5-NHS from Lumiprobe, and NHS-PEG-5-Azido from BaseClick.

Expression and purification of recombinant allergens

The major allergens Bet v 1.0101 and Phl p 5.0101 were expressed from pET28b constructs in *Escherichia coli* strain BL21 Star (DE3) (Invitrogen). Proteins were purified according to published protocols [20, 21]. Endotoxin removal was performed by using EndoTrap® red (Hyglos). Protein purity was assessed by SDS-PAGE electrophoresis followed by Coomassie staining as described elsewhere. Endotoxin content was evaluated by Limulus amoebocyte lysate assay (Cape Cod) according to the manufacturer's instructions. Proteins used for all experiments had more than 95% purity and the endotoxin content was lower than 0.3 pg/μg. Finally, proteins were dialyzed against 2mmol/L sodium phosphate buffer pH 7.4 and stored at -20°C.

Generation of allergen-mannan Glycoconjugates

Conjugates were generated by mild oxidation of the mannan moiety followed by reductive amination as described previously [18]. Briefly, 10 mg of mannan were dissolved in 300 μL of an aqueous

solution of 8 mg/mL NaIO_4 and vigorously stirred in the dark at room temperature (RT) for 1 h. Excess of NaIO_4 was then removed from the reaction mixture by dialysis against 50 mM sodium phosphate buffer pH 7 at 4 °C in the dark using Spectra/Por dialysis tubing, MWCO 6000–8000. Subsequently, 2 mg of lyophilized protein were reconstituted in 300 μL of the dialyzed oxidized mannan and 100 μL of a freshly prepared NaCNBH_3 solution (10 mg/mL in 50 mM sodium phosphate buffer, pH 7) were added and the reaction mixture was kept in the dark and stirred at RT for 24 h. To stop the coupling reaction, 40 μL of a NaBH_4 solution (5 g/L in 50 mM borate buffer, pH 9.5) were added to reduce any remaining aldehyde groups. The reaction was stirred in the dark for 6 h at 4°C. Afterwards, samples were subjected to size exclusion chromatography using a 26/60 Sephacryl S-300 column (GE Healthcare) in an ÄKTA-Explorer chromatographic system (GE-Healthcare). Fractions corresponding to high molecular weight of Bet v 1-mannan neoglycoconjugates (Bet-MN) were used for subsequent experiments. Coupling efficiency was monitored by 10% reducing SDS-PAGE using colloidal Coomassie staining for protein visualization.

The hydrodynamic radius of Bet-MN was analyzed by dynamic light scattering (DLS802; Viscotek) at 1 mg/mL in PBS, pH 7. Prior to measurements, samples were centrifuged 5 min at 14,000 g. Data were accumulated for 15 $\times$ 5 s and the correlation function was automatically fitted into the combined data curve. Intensity distribution was calculated using the solvent settings for water. The calculated intensity distribution was weighted statistically by mass using the mass model for proteins (OmniSize v 3.0).

Characterization of Bet-MN by Fourier-Transformed Infrared Spectroscopy (FTIR)

Protein and carbohydrate structure of Bet-MN was studied by attenuated total reflectance (Bio-ATR) Fourier transform infrared spectroscopy (FTIR) using a Tensor 27 instrument (Bruker). Samples were deposited onto the Bio-ATR cell, 20 μL , and spectra were obtained from the average of 120 scans collected over 4000-900 cm^{-1} . Absorption spectra were shown after atmospheric compensation. Comparison of secondary structure elements in Bet v 1 and Bet-MN was performed by analyzing the amide I band (1710-1600 cm^{-1}) after correcting for carbohydrate contribution. Mannan structure was monitored in the carbohydrate fingerprint region 1200-900 cm^{-1} . Second derivative spectra were obtained following the Savitsky-Golay method (9 smoothing points). All data were acquired and processed using the OPUS software v 6.5 (Bruker).

Protein and carbohydrate concentration within the glycoconjugates was estimated by the IR absorption of the Amide II and mannan fingerprint by using standard curves of mannan and Bet v 1 as a reference.

Assessing mannan integrity via Concanavalin A binding

Mannan integrity in the Bet-MN neoglycoconjugates was analyzed via a precipitation assay using the mannan-binding lectin Concanavalin A (ConA) as described by Mislovicová et al [22]. In short, 500 μ L of increasing amounts of free mannan or Bet-MN [5–300 μ g/mL] in assay buffer (50 mM sodium phosphate, 0.1 M NaCl, 0.1 mM CaCl_2 , 0.1 mM $\text{MnCl}_2 \times 4\text{H}_2\text{O}$, pH 7) were added to 500 μ L ConA [150 μ g/mL] in assay buffer. The reaction mixtures were stirred at 37 °C for 2 h. Afterwards, samples were centrifuged for 10 min at 8400 g and washed twice with 1 mL 1 M NaCl, and the pellet was dissolved in 100 μ L of 50 mM sodium phosphate pH 10.5. The amount of precipitated mannan was measured by the anthrone method [23]. In order to confirm the specificity of the ConA/mannan interaction, ConA agonist methyl α , D mannopyranoside was added to a final concentration of 25 mM.

Allergenicity of Bet-MN neoglycoconjugates

The allergenic potential of Bet-MN neoglycoconjugates was assessed by IgE-dependent degranulation of rat basophil leukaemia RBL-2H3 cells (RBL) as described previously [24]. RBL-2H3 cells were incubated with IgE-containing sera from Bet v 1-sensitized BALB/c mice. Degranulation was triggered by the addition of serial dilutions of Bet-MN, or Bet v 1 as a reference. Beta-hexosaminidase release was measured by enzymatic cleavage of the fluorogenic substrate 4-methylumbelliferyl-N-acetyl-b-D-glucosaminide (4-MUG). Data is expressed as percentage of total release after Triton X-100 treatment.

In vitro uptake, dendritic cell and complement activation

Bone marrow derived dendritic cells (BMDCs) were generated as described previously [18]. BMDCs were thawed and placed in complete medium in 96-well polystyrene V-bottom plates (Greiner) for 2 h. 10^5 cells per well were treated with 30 μ g/mL pHrodo-labeled Bet v 1 or Bet-MN and uptake was analyzed 60 min later by flow cytometry. BMDCs were additionally surface-stained with anti-CD11c Brilliant violet 421 (Clone N418, Biolegend), MHC-II APC (Clone M5/114.15.2, Biolegend) and CD86 APC-Cy7 (Clone GL-1, Biolegend) in order to determine DC activation.

Bet-MN uptake mechanisms were studied by preincubating freshly generated BMDCs for 2 hours with the following inhibitors of endocytosis: Cytochalasin B, an inhibitor of actin polymerization, which prevents phagocytosis [25], Amiloride, which inhibits macropinocytosis by blocking Na/H^+ exchange in macropinosomes [26], Chlorpromazine, inhibiting Clathrin-mediated endocytosis [27], and Filipin, an inhibitor of Caveolae-dependent endocytosis [28, 29]. In order to assess mannan dependent endocytosis we used free mannan as a competitive inhibitor at the indicated

concentrations. Then, 100 µg/mL of CF-647 labeled Bet v 1, or Bet-MN were added. After 4 h uptake was assessed by flow cytometry. Data is presented as percentage of total uptake in cell culture medium without inhibitors. As a negative control, BMDCs were metabolically inactivated by keeping them at 4 °C, and antigen was added under these conditions to assess the unspecific binding.

To assess complement activation by Bet-MN, equal volumes (10 µL) of Veronal buffer (Sigma), human plasma, and various concentrations of Bet-MN in PBS were mixed and incubated for 1 h at 37°C. The reaction was stopped by adding one volume of SDS-sample buffer followed by 5 min incubation at 95 °C. Samples were separated by SDS-PAGE in 7.5-20% gradient gels and C3a formation was measured by western blotting using a biotinylated anti-human C3 antibody (clone D17/1, Biolegend, 1:1000) and Streptavidin-HRP (Biolegend, 1:2000). Purified human C3a des Arg (Hycult Biotech) was used as a positive control.

Ethics Statement

Experiments involving human material were conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. Human skin explants were obtained with informed consent following abdominal resection after cosmetic surgery from healthy donors (Bergman Clinics, Bilthoven, The Netherlands or University Hospital Salzburg, Austria). Experiments were approved by the respective Institutional Review Boards).

Animal experiments were approved by the Austrian Ministry of Science (permit number: BMWFW-66.012/0003-WF/V/3b/2015).

Targeting of skin-resident APCs in human skin explants

For tracking antigen internalization in human skin explants, 6FAM-labeled Bet v 1 and Bet-MN were generated via click chemistry. For this, Bet-MN was amino-functionalized by adding ethylenediamine prior to the reduction with NaBH₄. Then Bet v 1 and Bet-MN were treated with NHS-PEG-5-Azide and subjected to click chemistry as described [30].

Human skin explants were obtained within 24 h after cosmetic surgery from healthy donors. An area of approximately 10 cm² was treated using the P.L.E.A.S.E. Professional[®] (Pantec Biosolutions) laser microporation system with the following settings: fluency of 22.5 kJ/cm², 4 pulses/pore, repetition rate of 200 Hz, pulse duration of 75 µs, and a pore density of 10%. Then, 100 µg (1 mg/mL) of 6-FAM labelled Bet-MN or Bet v 1 were topically distributed and allowed to dry. A similar amount of antigen,

100 µg (0.5 mg/mL), was distributed on a 10 cm² area by ten equidistant intradermal injections of 20 µL each. Biopsies of 0.6 cm² (10 per condition) were collected and cultured with the epidermal side upwards in 48 well plates with 1mL of IMDM supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10 µg/ml gentamycin. Biopsies were cultured for 48 hours at 37°C, 5% CO₂ and cellular crawl-outs were harvested and pooled per experimental condition. Analysis was performed by flow cytometry using an LSRFortessa X-20 (BD) and live APC populations were determined based on the expression of surface markers HLA-DR BV510 (clone G46-6, BD Biosciences), CD14 AF700 (clone M5E2, Biolegend), CD1a APC (clone HI149, BD Biosciences), CD141 BV711 (clone 1A4, BD Biosciences) and EpCAM BV421 (clone EBA-1, BD Biosciences).

In order to visualize the depth and shape of laser micropores in human skin, 6 mm² biopsies from the area of immunization were embedded in OCT compound (TissueTek) and frozen in the gas phase of liquid nitrogen. Tissue sections of 6 µm were deposited on gelatin-coated slides, stained with Haematoxylin-Eosin for close examination of micropores and search for signs of laser-induced thermal stress.

Mice immunizations and histologic analysis of laser microporated skin

Female, 6–10 week-old BALB/c mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained at the animal facility of the University of Salzburg according to local guidelines for animal care. Mice (n = 5) were immunized 2 times with 20 µg of Bet v 1 or Bet-MN in PBS on days 0 and 14. Immunizations were applied at two sites on the back (40 µL each) either intradermally, or via laser microporation using the P.L.E.A.S.E. Professional[®] laser microporation device. For epicutaneous immunizations (ECi) mice were depilated the day before using depilatory cream. On the day of immunization, mice were anesthetized and two non-overlapping 1 cm² areas on the back were laser-microporated using the following settings: 4 pulses/pore, repetition rate of 500 Hz, pulse duration of 50 µs, and a pore-density of 5%. Then, 40 µL of antigen were applied on top of each laser-microporated area and allowed to dry. Immunization sites were covered with a plastic adhesive patch (OPsite Flexifix). On day 28, mice were sacrificed and blood was collected for measuring Bet v 1 specific IgG antibodies using a luminometric ELISA (see Supplementary Methods). IgE blocking capacity of sera was tested using RBL-2H3 cells and high titered sera from allergic donors as described in the online supplement. Splenocytes from treated mice were re-stimulated *in vitro* with Bet v 1 for 72 h and secreted cytokines were measured using the Flowcytomix mouse TH1/TH2/TH17/TH22 13plex kit (eBioscience) according to the manufacturer's protocol. See Methods section in the online supplement for more details.

Langerin-DTR-EGFP (langDTR) mice [31] were backcrossed onto BALB/c background for 12 generations. Mice were immunized on days 0 and 14 with 20 µg of Bet v 1 or Bet-MN either via laser-microporated skin or intradermally as described above. Langerin⁺ cells were conditionally depleted by intraperitoneal injection of 1 µg diphtheria toxin (DT) on the day before starting the experiment and 400 ng twice a week during the whole experiment. Weight and behavior of the mice were regularly controlled to detect any adverse effects due to the DT administration. Mice were sacrificed on day 28 and Bet v 1-specific antibody levels in sera were measured.

Histological analysis of the skin morphology and the cellular infiltrate upon laser microporation was assessed by fluorescent microscopy. Depilated mice were immunized epicutaneously or intradermally on the back with 20 µg of Bet v 1, Bet-MN, or PBS. Mice were sacrificed after 4 h, 1 day or 3 days later and skin from the immunization area was collected, immediately embedded in OCT compound (TissueTek) and frozen in the gas phase of liquid nitrogen. Tissue sections of 6 µm were prepared and deposited on poly-L-Lysine covered microscopy slides. Sections were fixed with cold acetone, rehydrated in PBS, blocked with naive mouse serum, stained with anti-mouse CD11b Alexa Fluor 488 (clone M1/70, eBioscience) for 30 min and counterstained with DAPI. A rat-IgG2b Alexa Fluor 488 (clone eB149/10H5, Biolegend) was used as isotype control. After washing, sections were mounted with PermaFluor (Thermo Fisher Scientific) and observed under an immunofluorescence microscope (Zeiss). Image processing was performed using ImageJ [32].

In vitro antigen permeation

To determine the skin permeation capacity of Bet v 1 or Bet-MN when immunized via intradermal injections or laser micropores, the molecules were labeled with Cy5.5 NHS ester and Cy5.5 hydrazide, respectively. Modification of Bet v 1 was performed as recommended by the manufacturer. The modification of Bet-MN with Cy5.5 hydrazide was performed by adding a 10-fold molar excess of hydrazide reagent after coupling Bet v 1 to mannan by aminative reduction. Resulting labeled neoglycoconjugates were purified by size exclusion chromatography as described above.

Skin permeation capacity of Bet v 1 and Bet-MN was evaluated *in vitro* using pig skin as a model. Permeation rates were determined using a Franz diffusion cell as described by Dodou et al <http://dx.doi.org/10.3109/10837450.2014.915569>. Briefly, skin sections from the shoulder of freshly slaughtered pigs were collected and shaved. After removal of fatty tissue, full thickness skins (2-3mm) were treated with 20 µg of Cy5.5 labeled Bet-MN or Bet v 1 either via ID or ECI and mounted in a static Franz diffusion cell with an exchange area of 1 cm². The receptor chambers were filled with 8 mL of PBS and kept at 37 °C under stirring. Permeation to the lower chamber was monitored by

collecting 100 μ L of the lower chamber at 1 h, 3 h, 6 h and 24 h and measuring the amount of antigen. Volume collected at each time was replaced with PBS. The amount of antigen was determined by adding 100 μ L of 6 M guanidinium hydrochloride to 20 μ L the tissue homogenate and measuring the fluorescence of Cy5.5 (675/710 Ex/Em) using a fluorescent plate reader (Infinite M200 Pro plate reader, Tecan). Standard curves of Cy5.5 labeled Bet-MN and Bet v 1 were used to calculate the amount of antigen in the lower chamber.

In vivo antigen distribution

To determine the amount of Bet v 1 or Bet-MN delivered to different organs *in vivo*, mice (n=3) were immunized with 20 μ g Bet v 1-Cy5.5 or Bet-MN-Cy5.5 intradermally or epicutaneously as described above. After 0.5, 6, 14 and 48 hours, mice were euthanized and the organs were harvested. A skin area of 1 cm² was excised per immunization site. Then skin draining lymph nodes (i.e. axillary, brachial and inguinal) were harvested. In addition, the spleen, kidneys and liver were collected. All organs were homogenized in 0.5 mL of PBS, except for the liver (1 mL). The amount of antigen was determined by adding 100 μ L of guanidinium hydrochloride to 20 μ L the tissue homogenate and measuring the fluorescence of Cy5.5 (675/710 Ex/Em) using a fluorescent plate reader (Infinite M200 Pro plate reader, Tecan). Standard curves were constructed using Cy5.5 labelled Bet v 1 and Bet-MN. Tissue homogenates from naïve mice were used to determine the background fluorescence for each organ as well as matrix interferences with Cy5.5 fluorescence.

Results

Generation and Characterization of Bet-MN neoglycoconjugates

Bet-Mannan neoglycoconjugates (Bet-MN) were prepared by mild oxidation of mannan polysaccharide followed by reductive amination as previously described [18]. Conjugation efficacy was nearly 100% as revealed by SDS-PAGE (Supplementary Fig. 1). Resulting Bet-MN was fractionated over size exclusion chromatography in order to generate neoglycoconjugates with a more defined size distribution. For subsequent studies we used the high molecular weight fraction with a mean hydrodynamic radius of 10 nm which showed the highest uptake efficacy and activation of BMDCs (Supplementary Fig. 2).

We explored the composition and structure of the Bet-MN neoglycoconjugates by Fourier-Transform Infrared spectroscopy (FTIR). The IR spectrum of the Bet-MN neoglycoconjugates showed the typical amide I and amide II bands corresponding to the peptide backbone in the region of 1700-1450 cm^{-1} and the mannan fingerprint in the region 1200-900 cm^{-1} (Fig. 1A). Analysis of the second derivative of the amide I band (1710-1600 cm^{-1}) [33, 34] revealed typical strong beta sheet signals (1625-1635 cm^{-1} and 1695-1685 cm^{-1}) corresponding to the natural Bet v 1 fold, which remained unaltered within Bet-MN (Fig. 1B). Only minor changes consistent with a slight increase of disordered regions (1680-1650 cm^{-1}) were observed. Notably, there were no major differences between coupled and free mannan spectra in the mannan fingerprint region 1200-900 cm^{-1} (Fig. 1C). The change of the double band at 990-970 cm^{-1} can be explained by the change of the hydrogen bonding pattern around the glycosidic bond [35]. As the IR absorption of the protein and sugar bands are not overlapping (Fig. 1A), and IR absorption linearly follows the Lambert-Beer law over a highly dynamic range of concentrations [36], we used this technique for accurate quantification of sugar and protein moieties within the neoglycoconjugates. As a reference we constructed standard curves with known amounts of Bet v 1 and mannan.

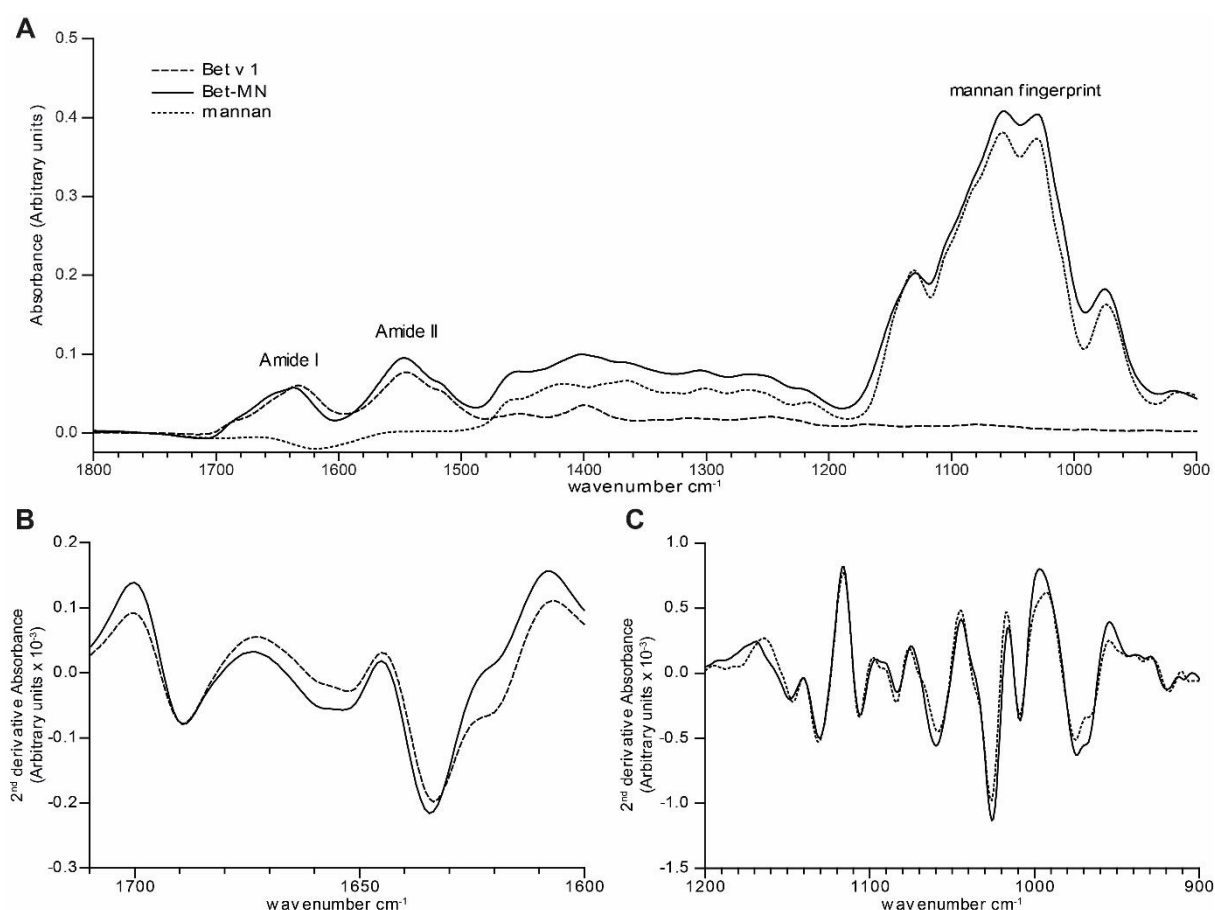


Fig. 1. Bet v 1 fold is not affected by the coupling of mannan. (A) FTIR-ATR spectra of Bet v 1. (B) Overlay of Bet v 1 and Bet-MN second derivative of the amide I band (1700-1600 cm⁻¹) shows a typical beta sheet structure (strong 1625-1635 cm⁻¹ and 1695-1685 cm⁻¹), slight increase in absorption at 1680-1650 cm⁻¹ region suggests the presence of more disordered regions compared to native Bet v 1. C) Overlay of mannan and Bet-MN second derivative of the mannan fingerprint region 1200-900 cm⁻¹.

Importantly, the mannan moiety in the Bet-MN neoglycoconjugates retained its biological activity as confirmed by the specific recognition by the mannose binding lectin ConA. The addition of 25 mM ConA agonist, methyl α -D mannopyranoside competitively inhibited this interaction (Supplementary Fig. 3). As previously shown for papain [18], Bet-MN showed 10⁶ times less capacity to induce IgE mediated basophil degranulation in relation to Bet v 1 (Supplementary Fig. 4), an important prerequisite for epicutaneous immunotherapy of allergies.

Mannan neoglycoconjugates facilitate cellular uptake and activation

Next we compared the uptake of pHrodo labeled Bet v 1 and Bet-MN neoglycoconjugates by BMDCs and investigated the subsequent activation of the cells (Fig. 2). Bet-MN neoglycoconjugates were

internalized much more efficiently than Bet v 1 (Fig. 2A) suggesting an active transport mechanism. In order to confirm this hypothesis, we assessed the uptake of Bet-MN and Bet v 1 in the presence of inhibitors of the major endocytic pathways (Fig. 2B). Uptake of soluble Bet v 1 was only inhibited by Amiloride, suggesting internalization by pinocytosis. As expected, Bet-MN was internalized by Clathrin-dependent receptor mediated endocytosis since it was blocked by the addition of Chlorpromazine, which inhibits the assembly of Clathrin-coated pits. The mechanism proved to be mannan-specific, as addition of mannan competitively inhibited the internalization of Bet-MN. Furthermore, high doses of the Caveolae inhibitor Filipin showed to have a negative effect on uptake, suggesting either the presence of an alternative Caveolae-dependent uptake mechanism or a disruption of lipid rafts [28], where CLRs are known to form microdomains [37, 38]. The concentrations of the inhibitors used were chosen as previously reported [39] [40], and their effect on BMDC viability was tested by using the Viability Dye eFluor® 780 (eBioscience) reproducing the experimental conditions previously described. Under these conditions, no decrease in cell viability was observed (data not shown).

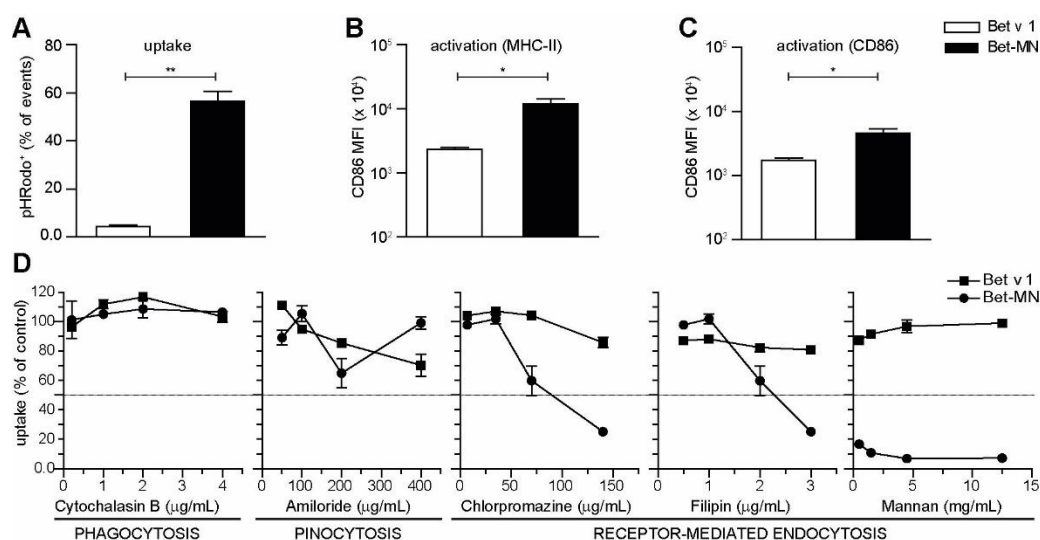


Fig. 2. Bet-MN neoglycoconjugates induce increased antigen uptake by mannan-specific receptors and promote DC activation. (A) BMDCs were incubated with 30 $\mu\text{g/mL}$ fluorescently labeled Bet v 1 or Bet-MN. (B) Expression of CD86 and (C) MHC-II on CD11c gated cells was analyzed by flow cytometry. ** P -value <0.01 , * P -value <0.05 Student's t -test. (D) Bet-MN uptake is receptor mediated and mannose-dependent. BMDCs were pre-incubated 2 h with the endocytosis inhibitors, Cytochalasin B (phagocytosis), Amiloride (pinocytosis), Chlorpromazine (Clathrin mediated endocytosis), and Filipin (Caveolae dependent endocytosis) at the indicated concentrations. Mannan was used as a competitive inhibitor of mannan-recognizing receptors and was added together with the antigens. Cellular uptake of pHrodo labeled Bet v 1 and Bet-MN was measured 4 h later by flow cytometry. Data is presented as percentage of total uptake without inhibitors. Dashed line indicates 50% of control uptake.

Application of Bet-MN to laser-microporated human skin preferentially targets CD14+ skin resident DCs and Langerhans cells

We and others have previously shown that mannan neoglycoconjugates are efficiently targeted to murine CD11b⁺CD8⁺ DCs *in vivo* [18] and that human monocyte derived DCs internalize mannan-conjugated allergoids via mannose receptors *in vitro* [17]. However, little is known about APC targeting via mannan-recognizing receptors in human skin. Therefore, we tested the targeting capacity of Bet-MN neoglycoconjugates when applied intradermally (ID) or via epicutaneous immunization (ECi) through laser microporation in an *ex vivo* human skin model (Supplementary Fig.6).

To track the antigen internalization by skin DCs, labeled Bet v 1 or Bet-MN were distributed on top of the laser-microporated area and were allowed to penetrate into the skin until dry. Then, 10 skin biopsies of 0.6 cm² were collected and transferred into 1 mL cell culture medium and cultured for 48 hours. Skin cellular crawl-outs were analyzed by flow cytometry and cell populations were determined according to the expression of surface molecules HLA-DR, CD14, CD1a, CD141 and EpCAM (Supplementary Fig. 7). According to this, six populations of HLA-DR^{high} skin-resident APCs were defined: CD14⁺, CD14⁺CD1a⁺, CD141⁺ DCs, CD1a⁺CD14⁺ (from now on called DN), CD1a⁺, and Langerhans cells were gated as CD1a^{high}EpCAM⁺.

The migratory DCs consisted mainly of CD1a⁺ dermal DCs (dDCs) comprising 50% of the total HLA-DR⁺ cells, followed by CD14⁺ cells (sum of CD14⁺ and CD14⁺CD1a⁺), DN, and CD141⁺ cells (Supplementary Fig 8). Langerhans cells were found at very low rates (1-2% of total HLA-DR⁺ population).

Regardless of the route of immunization, Bet-MN neoglycoconjugates showed increased uptake by virtually all populations of skin-resident DCs compared to Bet v 1 (Fig. 3). The uptake was more evident in DC populations expressing the CD14 surface marker (i.e. CD14⁺ DCs and CD14⁺CD1a⁺ DCs) and LCs. Furthermore, a trend towards upregulation of the activation marker CD83 was observed in CD14⁺ DCs following Bet-MN immunization (Supplementary Fig 9).

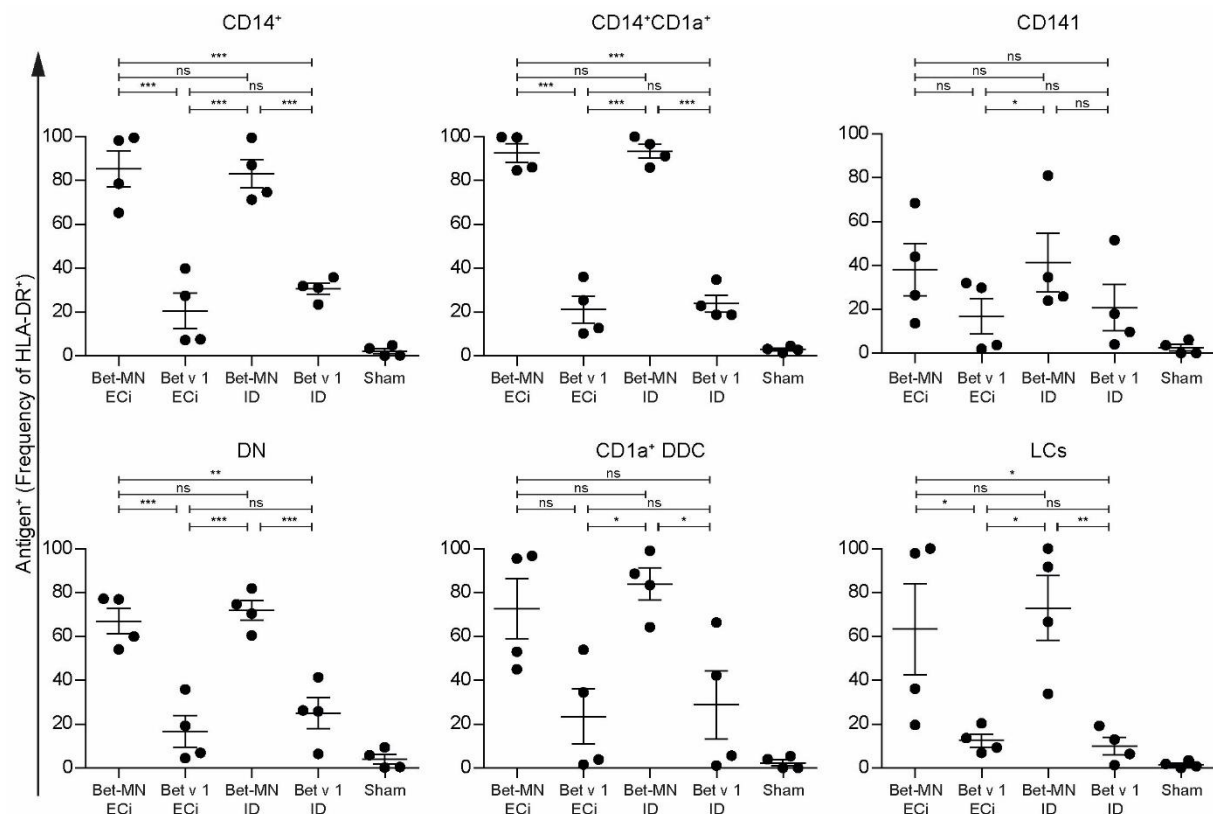


Fig. 3. Laser microporation of Bet-MN targets CD14⁺, CD14⁺CD1a⁺ and DN dendritic cell populations as well as Langerhans cells in human skin explants. Abdominal skin explants ($n = 4$) were treated with 100 μ g of fluorescently labeled Bet v 1 or Bet-MN by epicutaneous immunization (ECi) via laser microporation or intradermal injections (ID). As a negative control, laser-microporated skin was treated with PBS (sham). 10 biopsies of 0.6 cm² were collected and cultured for 48h. Cellular crawl-outs were analyzed by flow cytometry and APC populations were determined based on the expression of surface markers CD14, CD1a, CD141, and EPCAM. Differences between groups were analyzed using repeated measures one way ANOVA and Tukey's post-hoc test *** P -value < 0.001 , ** P -value < 0.01 , * P -value < 0.05 , ns not significant.

Epicutaneous immunization with Bet-MN neoglycoconjugates elicits rapid and potent antibody responses that efficiently block IgE reactivity.

In order to test the immunogenicity of Bet-MN neoglycoconjugates, we immunized mice epicutaneously via laser microporated skin or by intradermal needle injections. Fourteen days later, mice were boosted using the same antigens and routes and the antibody response was measured at day 28. In a separate experiment, another booster was applied at day 28 and antibody responses were measured at day 35. Interestingly, Bet-MN was highly immunogenic when applied via ECI while

a 10-fold weaker and delayed immunogenicity was elicited by ID. No measurable antibody response was generated against unconjugated Bet v 1, regardless the route (Fig. 4).

It has been suggested that mannan can be used as adjuvant due to its ability to trigger maturation of APCs via CLR and TLR ligation [41]. We evaluated the adjuvant effect of mannan in the ECI setting, by immunizing a group of mice with Bet v 1 mixed with free mannan. As shown in Supplementary Fig 10, no immune response was induced suggesting that a physical link between Bet v 1 and mannan is needed for Bet-MN immunogenicity.

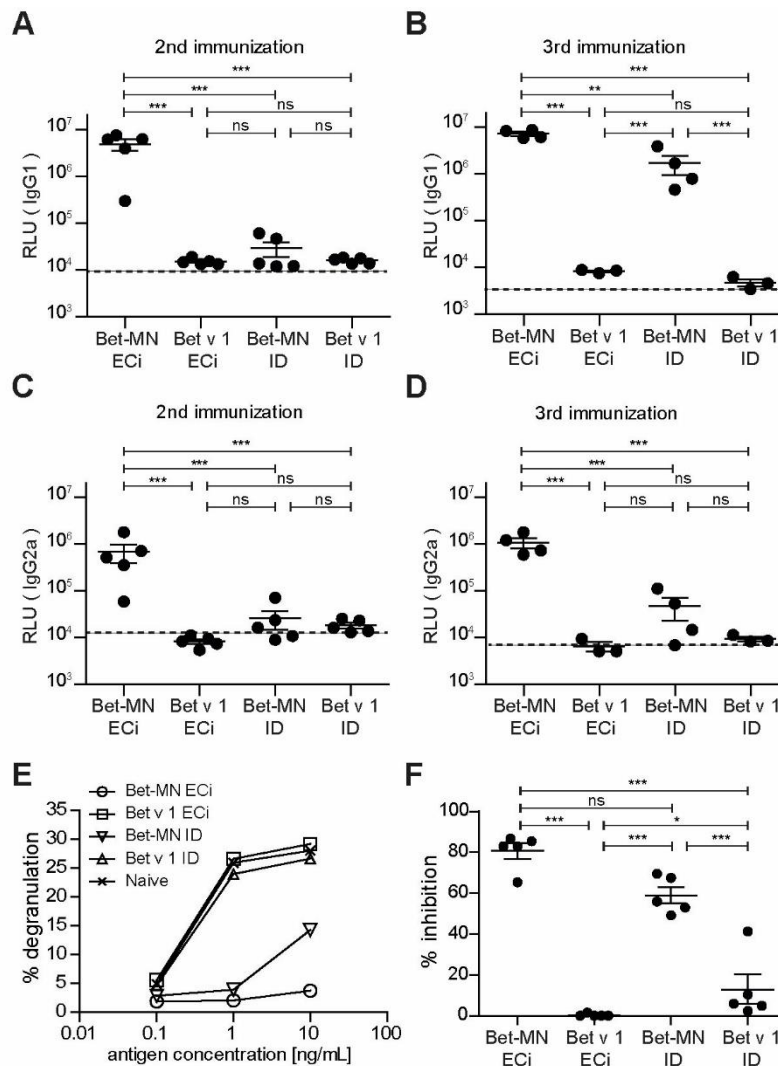


Fig. 4. Antibody responses after immunization with Bet v 1 or Bet-MN via epicutaneous immunization (ECi) using laser microporation or intradermal injection (ID). Balb/c mice ($n = 5$) were immunized at days 0 and 14. Bet v 1-specific serum IgG1 (A) and IgG2a (C) responses were measured at day 28 by a luminometric ELISA. Serum dilutions were 1:10000 for IgG1 and 1:100 for IgG2a. In a separate experiment, another booster was applied at day 28 and antibody responses were measured at day 35 (B, D). Data are shown as relative luminescence units (RLU) of a luminometric ELISA. Blocking activity was assessed as the capacity to inhibit Bet v 1 mediated degranulation of RBL cells loaded with sera from allergic patients ($n = 5$). Representative IgE blocking assay is shown in (E). IgE-blocking capacity of Bet v 1 immune sera (F) at 10 ng dose was analyzed by one way ANOVA followed by Tukey's post hoc test. * P -value < 0.001 , ** P -value < 0.01 , * P -value < 0.05 , ns not significant.**

It is now established that one of the mechanisms of allergen specific immunotherapy is the induction of IgE-blocking antibodies [42]. These are mainly high affinity IgG antibodies directed against the allergens that prevent the binding of IgE either by directly competing for the same epitope or by

increasing the steric hindrances [43]. We therefore measured the ability of the Bet-MN induced antibodies to inhibit IgE binding. Sera from mice immunized with Bet-MN efficiently inhibited the Bet v 1 induced degranulation of RBL cells, loaded with sera from Bet v 1 allergic patients (Figure 4, E and F). In agreement with the stronger immunogenicity observed, epicutaneous immunization showed a stronger inhibitory capacity than intradermal immunization. Interestingly, intradermal immunization with Bet v 1 elicited low levels of IgE blocking although no IgG1 or IgG2a could be measured (at a serum dilution of 1:100). No blocking effect was observed when Bet v 1 was immunized via the epicutaneous route.

In order to investigate whether the differences in immunogenicity observed for Bet-MN glycoconjugates upon ECI or ID reflect a general phenomenon, another antigen, the major grass pollen allergen Phl p 5, was used for the generation of neoglycoconjugates (Phl-MN) using the same procedure as described above. We immunized mice with Bet-MN, Bet v 1, Phl p 5, or Phl-MN via laser-microporated skin or intradermally twice in a 14 days interval and measured the humoral immune response at day 28. As seen in previous experiments, after two immunizations Bet v 1 only elicited a detectable humoral immune response when the antigen was coupled to mannan and immunized epicutaneously via laser micropores (Fig. 5A). As Phl p 5 is a very strong immunogen, it also elicited antibody responses when applied intradermally without conjugation. ECI boosted the antibody responses against the free protein, indicating an adjuvant effect of the laser microporation process itself. Consistent with the data observed for Bet v 1, mannan glycoconjugation to Phl p 5 had a synergistic effect on the antibody responses only when applied via laser microporation (Fig. 5B). Moreover, epicutaneous immunization with Bet-MN or Phl-MN induced high levels of IL-17 and IL-22 and, to a lesser extent, Interferon γ and IL-2. Interestingly, typical TH2 cytokines like IL-4, IL-5 and IL-13 were also present. Immunizations with native allergens only induced the TH2 cytokines IL-5 and IL-13 independent of the route of immunization. Similarly, ID immunization with Bet-MN and Phl-MN resulted in IL-5 and IL-13 (Fig. 5). Summing up, the natural TH2 immune response against allergens is shifted to TH1/TH17 with the combination of ECI and mannan coupling.

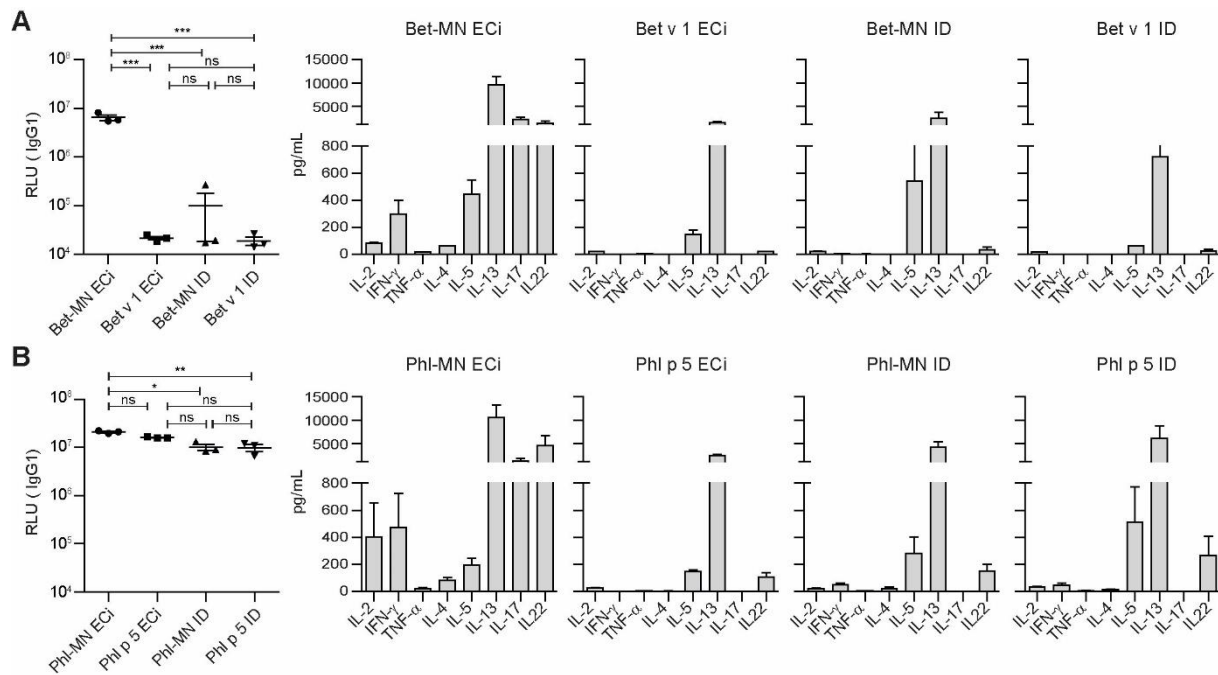


Fig. 5. Epicutaneous immunization via laser micropores with allergen-mannan neoglycoconjugates boosts the antibody responses and shifts the immune response towards TH1/TH17. Balb/c mice were immunized twice every 14 days epicutaneously via laser-microporated skin (ECi) or intradermally (ID). On day 28, animals were euthanized and specific antibody titers were measured and displayed as relative luminescence units (RLU) (A and B). Splenocytes from treated mice were re-stimulated *in vitro* with the corresponding allergen and secreted cytokines (shown as pg/mL) were quantified.

Bet-MN is efficiently retained in skin and secondary lymphoid organs while Bet v 1 is rapidly eliminated.

Since keratinocytes have been shown to express a mannose-binding receptor [44], we hypothesized that skin vaccination with Bet-MN would result in a controlled release from the vaccination site. Using a Franz static diffusion cell, we applied Cy5.5-labeled Bet v 1 or Bet-MN to full thickness (2-3mm) porcine skin either ID or via ECi and measured the transmigration of the labeled antigen into the receptor chamber. As expected, ID injected Bet v 1 was able to penetrate through the skin, while Bet-MN stayed at the injection site and was only slowly released into the lower chamber (Supplementary Fig. 11). At the same concentration, permeation of both Bet v 1 and Bet-MN was below the detection limit after ECi.

Upon immunization, the antigen is transported to the secondary lymphatic organs (SLOs) either as a soluble component of the lymph or by patrolling APCs, mainly DCs, which internalize antigen in the periphery and transport it to the LNs. Since the transport of antigen to the SLOs is a fundamental part

of immunity, we measured the distribution kinetics of Bet v 1 and Bet-MN between the skin (site of immunization), lymphoid (afferent skin draining lymph nodes and spleen) and non-lymphoid (liver + kidneys) compartments (Fig. 6). Mice were immunized with Cy5.5 labeled Bet v 1 and Bet-MN either by the epicutaneous or intradermal route. At different time points, mice were euthanized and the organs were harvested, homogenized, and the amount of antigen was quantified.

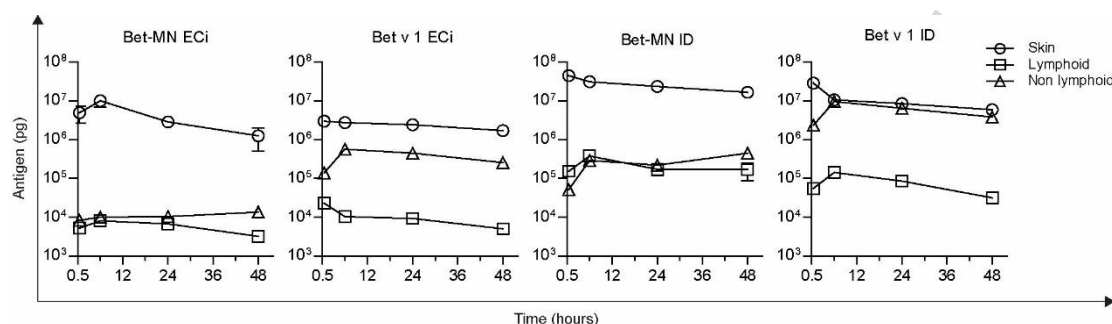


Fig. 6. Mannan conjugation reduces release of antigen into non-lymphoid compartments and favors accumulation in lymphoid tissues. Mice ($n = 3$) were immunized intradermally (ID) or epicutaneously (ECi) with either Bet v 1 or Bet-MN, and the amount of antigen in skin, lymphoid and non-lymphoid compartments was quantitated after 0,5, 6, 24, and 48 hours. Data are shown as total amount of antigen in the respective compartments in pg.

Quantification of antigen remaining at the site of immunization revealed that the amount of antigen delivered into the skin by ECI is about 50 times lower compared to ID. Interestingly, few hours after immunization unconjugated Bet v 1 was found in the non-lymphoid compartment, mainly in the kidneys. Bet-MN ID showed a more sustained release from the skin and accumulation in the lymphoid compartment compared to Bet v 1. The lower bioavailability of Bet v 1 compared to Bet-MN in lymphoid organs may explain the lack of immunogenicity observed. Surprisingly, delivery of Bet-MN via epicutaneous route was barely detected in the lymphoid compartment, which is in striking contrast to the strong immunogenicity observed previously.

CD11b+ dDCs, but not Langerin+ DCs are responsible for inducing potent humoral immunity against mannan neoglycoconjugates

In human skin, Bet-MN was efficiently internalized by CD14⁺ cells and Langerhans cells independently of the route of administration. However, in mice only the antigen applied epicutaneously via laser micropores resulted in strong antibody responses. In order to get insight into this puzzling observation, we immunized Bet-MN via laser microporation or intradermally in Balb/c-langDTR mice

in which all Langerin⁺ DCs (Langerhans and dermal Langerin⁺ DCs) were depleted prior to the immunization. Surprisingly, the ablation of all Langerin⁺ DCs in the skin resulted in a boost of immunogenicity against Bet-MN and this effect was more pronounced after ID immunization (Fig. 7). This data suggests a regulatory rather than immune potentiating function of Langerin⁺ DCs in Bet-MN immunity. Together with our previous data that identified CD11b⁺ DCs as the major antigen-carrying DC population in mice after ECI [11] we suggest that CD11b⁺ dDCs, but not Langerin⁺ DCs are responsible for inducing potent humoral immunity against mannan neoglycoconjugates after ECI.

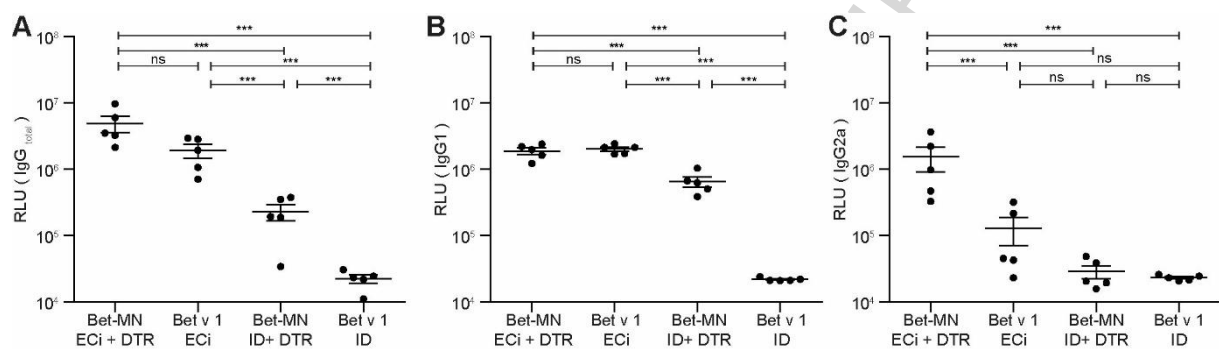


Fig. 7. Langerhans cells and Langerin⁺ dDCs are dispensable for eliciting strong antibody responses via ECI and show an immunosuppressive phenotype in the intradermal setting. All Langerin⁺ cells were conditionally ablated in Balb/c-langDTR mice (n = 5). Two immunizations of Bet v 1 or Bet-MN were applied in a 14 days interval. Bet v 1-specific IgG, IgG1, and IgG2a antibodies were measured at day 28 at serum dilutions of 1:1000, 1:400, and 1:100, respectively. Data are shown as relative luminescence units (RLU) of a luminometric ELISA. DTR was applied every 3rd day to prevent repopulation of the skin by self-renewing Langerin⁺ dermal DCs. Differences between groups were analyzed using repeated measures one way ANOVA and Tukey post-hoc test *** P-value < 0.001, ns not statistically significant.

Laser microporation induces a proinflammatory cytokine/chemokine milieu in the skin.

Recently, it has been shown that preconditioning the skin with a short laser treatment prior to influenza vaccination results in potent humoral and cellular immune responses [45]. The latter is due to local secretion of inflammatory cytokines and chemokines by skin resident cells. Taking that into account, we hypothesize that skin ablation with the fractional laser treatment would induce a local proinflammatory milieu responsible for the strong-route effect observed. The local cytokine/chemokine milieu induced solely by laser microporation (without antigen application) was

measured after 6, 24 and 48 hours post treatment. The concentration of proinflammatory cytokines (TNF- α , IL-6) and chemokines (CCL2, CCL3, CCL7, CCL-12 [not shown], CXCL-10, and TSLP) were increased in response to laser treatment, whereas IL12p70 was not altered. Other cytokines/chemokines measured (IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, IL-21, IL-22, IFN- γ , CCL-20, HSP-70, TMEM173/STING) were not significantly elevated compared to naïve controls (data not shown). From the kinetics, we could distinguish two waves of distinct cytokines/chemokines. IL-6, CCL2 and CCL7 are secreted rapidly and decline over time while TNF- α , TSLP, CXCL-10 and CCL-3 peak at 24 hours (Fig. 8). This cytokine kinetics suggests a wound healing-like process where monocytes/macrophages are recruited to the site of injury, which is resolved within three days after the laser treatment (Supplementary Fig 12) with the concomitant decrease in inflammatory mediators. The addition of mannan had no measurable additive effect in the skin cytokine/chemokine milieu induced by the laser (data not shown). However, we observed that the inflammatory CD11b⁺ infiltrate at the site of immunization lasted longer when mannan neoglycoconjugates were administered via laser micropores (Supplementary Fig. 12).

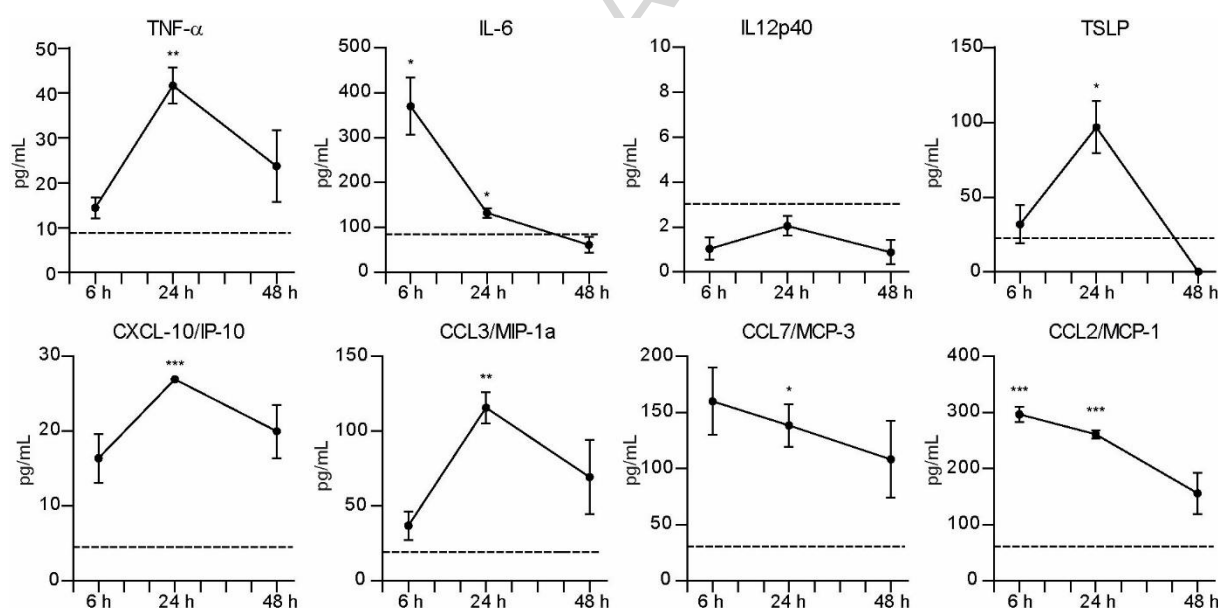


Fig. 8. Immune potentiating cytokine/chemokine milieu in mouse skin 6, 24, and 48 h following laser treatment. Balb/c mice (n=3) were laser microporated with the ablative laser system with the settings used previously for immunizations. Skin samples were excised at the indicated time points, homogenized in PBS and analyzed for cytokine/chemokine content. Samples from naïve mice served as base line controls (dashed line). ***P-value<0.001, **P-value<0.01, *P-value<0.05 Student's t-test.

ACCEPTED MANUSCRIPT

Discussion

In this work we explored the combination of epicutaneous immunization via laser microporation with dendritic cell targeting using mannan neoglycoconjugates, with the aim to increase efficacy and safety of allergy vaccines. The clinically relevant major birch pollen allergen Bet v 1 was used as a model molecule. To conjugate Bet v 1 to mannan, we employed a protocol based on mild oxidation of mannan followed by conjugation via aminative reduction. Resulting Bet-MN neoglycoconjugates had an average size of about 10 nm hydrodynamic radius (Supplementary Fig. 2). A fine control of the oxidation degree was achieved by kinetic and stoichiometric control of the oxidation reaction, thus preventing extensive destruction of mannose epitopes that may result in an impaired C-type lectin receptor recognition [18] and immunogenicity [17]. Similarly, aminative reduction was performed under mild conditions with an excess of oxidized mannan to ensure optimal coupling. This strategy resulted in a high coupling yield while preserving mannan and Bet v 1 structures as demonstrated by FTIR (Fig. 1), ConA binding (Supplementary Fig. 3) and mannan specific cellular uptake (Fig. 2).

Since the presence of IgE-mediated systemic or local side effects at the site of immunization is a main concern for allergy vaccines [6, 46], we tested the capacity of Bet-MN to trigger IgE dependent degranulation. Bet-MN was about six orders of magnitude less allergenic than the soluble Bet v 1 as revealed by RBL degranulation assay (Supplementary Fig. 4). The mannan-dependent inhibition of IgE binding to Bet v 1 is likely due to steric hindrance rather than direct modification of the binding site, since no lysine is directly involved in IgE binding [47, 48]. The low allergenicity of Bet-MN neoglycoconjugates makes them ideal “hypoallergenic” candidates for safe allergy vaccines as they cannot trigger IgE-dependent immediate responses. The conjugation conditions optimized for birch pollen allergen Bet v 1 also rendered the grass pollen allergen Phl p 5 hypoallergenic. Similarly, we have previously shown that hypoallergenic variants of the model allergen papain can be generated by mannan coupling [18] thus demonstrating the broad applicability of this strategy to generate hypoallergenic vaccines.

DC targeting and maturation capacity of pHrodo labeled Bet-MN was studied *in vitro* using BMDCs. The results showed that Bet-MN boosted both uptake and activation compared to soluble Bet v 1. The targeting capacity was conferred by the mannan moiety as free mannan could competitively inhibit the internalization of Bet-MN. Furthermore, the uptake mechanism depended on receptor mediated endocytosis pointing to the involvement of endocytic CLRs [4, 15, 49]. Moreover, Bet-MN showed a strong activation of complement (Supplementary Fig. 5) which also can facilitate internalization via complement receptors [50, 51], and antigen delivery to follicular DCs [52]. An

increased uptake, together with upregulation of MHC-II and costimulatory molecules favors the immunogenicity of Bet-MN conjugates *in vivo* as compared to Bet v 1.

In human skin explants, mannan coupling boosted antigen uptake by all analyzed skin populations. Compared to the native allergen, Bet-MN was targeted more efficiently to CD14⁺ dermal DCs and LCs (Fig. 3). CD14⁺ dermal DCs overexpress mannose receptor and have a high endocytic capacity that explains the preferential uptake of Bet-MN [53]. The involvement of LCs is in agreement with previous findings showing that (i) mannose residues are recognized by Langerin [54] (ii) mannan can block internalization of viral and fungal antigens by Langerhans cells [55, 56], and (iii) FITC-mannan painted on mouse skin is efficiently internalized by Langerhans cells LCs [57]. There were no route-specific differences in the cellular uptake or DC targeting capacity of Bet-MN in human skin explants. This result highlights the suitability of laser microporation for efficient targeting of antigens to human skin DCs. Moreover, we observed a trend for the upregulation of the activation marker CD83 in CD14⁺ and CD14⁺CD1a⁺ DC populations after immunization with Bet-MN in skin explants (Supplementary Fig 9). The relatively low increase of the DC activation marker relative to untreated skin may be explained by a pre-existing strong activation of the crawl-outs due to the extensive manipulation of the skin from the moment of the surgery. Under such conditions, also strong TLR agonists such as LPS, p(I:C), or glucopyranosyl lipid A only induce modest upregulation of costimulatory molecules [58].

In the mouse model, epicutaneous immunization of Bet-MN via laser micropores elicited strong antibody responses after only two immunizations (Fig 4 A, C). In contrast, soluble Bet v 1 was not immunogenic in any of the conditions tested. This is in agreement with previous observations that in the absence of adjuvant, even four intradermal immunizations with soluble Bet v 1 are only poorly immunogenic. One reason for the low immunogenicity of soluble Bet v 1 is its early degradation in the endolysosomal compartment of APCs leading to a reduced presentation of T cell epitopes [24]. Additionally, we were able to show that covalent linking between sugar and protein is needed to enhance the immunogenicity of Bet v 1 since the addition of free mannan has no effect in the immune response (Supplementary. Fig. 10). In contrast, mannan coupling leads to increased antigen internalization (Fig. 2) and therefore a higher probability to reach MHC class II antigen loading compartments. Moreover, mannan coupling might prevent premature endosomal degradation of Bet v 1 by limiting the access of proteases due to steric hindrances as observed for other glycosylated antigens [59].

The T helper responses elicited by ECi with allergen-mannan conjugates were TH1/TH17 skewed, according to the fungal origin of the mannan [60] (Fig. 5). Interestingly, also higher levels of IL-13 were found in the Bet-MN ECi groups. While traditionally considered a TH2 cytokine, IL-13 has been

recently shown to be frequently produced also by TH1 and TH17 cells [61]. In contrast, ECI with unconjugated Bet v 1 or intradermal immunizations with Bet v 1 or Bet-MN resulted in increased amounts of IL-5 although no or only low-level antibody responses were detected. The TH17 polarization observed with allergen-mannan neoglycoconjugates applied via laser microporated skin may turn out problematic when treating allergic diseases where a clear TH17 etiology has been established like atopic dermatitis [62, 63] and certain types of asthma [64, 65]. On the other hand, our own data show that induction of inflammatory responses (TH1/TH17) against allergens is one of the common mechanisms of protection against grass pollen allergy in healthy individuals [66]. Moreover, a high degree of instability and plasticity of TH17 cells towards regulatory functions has been recently described [67], supporting that this phenotype represents one possible mechanism of naturally acquired immunity against allergy. Mannans from other sources may also be employed to induce different immunological outcomes. One possible candidate would be mannosylated lipoarabinomannan (ManLAM) from mycobacterium tuberculosis, which has been shown to promote TH1/Treg polarization [68].

Remarkably, we showed that the antibodies induced by Bet-MN immunization are able to block the IgE-dependent degranulation of basophils loaded with sera from Bet v 1 allergic patients (Fig. 4 E, F). This is due to the presence of IgG antibodies directed against the IgE epitopes of Bet v 1, thus preventing the crosslinking of Fcε receptors on basophils, eosinophils and mast cells, which are responsible for the immediate allergic symptoms. The induction of blocking antibodies is acknowledged as one of the main therapeutic mechanisms of allergy vaccines [42], thus highlighting the suitability of Bet-MN for allergy treatment.

There was a clear route effect on the immunogenicity of mannan neoglycoconjugates. Intradermal immunization resulted in poor immune responses while ECI elicited strong antibody responses (Fig. 4 and 5). This dichotomy is puzzling since there were no detectable route-related differences in the antigen uptake or APC targeting by Bet-MN in human skin explants (Fig. 3). Hence, we further dissected the contribution of key players involved in the immunity to skin vaccination; e. g. the nature of the antigen, CLR targeting, amount of antigen reaching SLOs, local danger signals from laser-induced skin damage, and attraction of immune cells. As previously mentioned, recombinant Bet v 1 is inherently a poor antigen without adjuvantation. Therefore, we additionally used the highly immunogenic allergen Phl p 5 to vaccinate mice intradermally or via ECI. As expected, intradermal immunization of Phl p 5 elicited good antibody titers, however ECI improved the immune response against the wild-type allergen, and mannan coupling boosted the immune response even further, but only when applied via laser micropores, thus confirming the results obtained with the weak antigen Bet v 1 (Fig. 5).

In human skin explants mannan coupling enhanced antigen uptake by all skin resident APCs, particularly CD14⁺ cells and LCs, independently of the route of administration (Fig. 3). Therefore, we asked whether the improvement in antibody titers is due to additional factors derived from the laser treatment as suggested by Wang *et al* [45]. Control experiments revealed that laser microporation induced a strong proinflammatory cytokine/chemokine milieu at the site of antigen application, including TNF- α , IL-6, CCL2, CCL3, CCL7, CXCL-10, and TSLP, (Fig 8). This inflammatory environment promotes the maturation and migration of APCs resulting in potent immune responses. Thus, danger associated molecular patterns induced by the microporation process itself may synergize with mannan induced immune modulation to boost immunity.

The synergism of laser-induced damage and mannan coupling in boosting the antibody responses becomes more evident when analyzing the amount of antigen effectively reaching the SLOs. Intradermal immunization of Bet-MN resulted in two orders of magnitude higher amount of antigen in the SLOs compared to epicutaneous immunization (Fig. 6). It is well established that immune responses against foreign antigens are initiated in the SLOs and that the mode of antigen transport into skin draining lymph nodes (SDLN) impacts the subsequent immune response [69-71]. In this context, ECI using Bet-MN generates a proinflammatory environment (Fig. 8 and Suppl. Fig. 12) and a controlled release of antigen at the site of immunization (Fig. 6 and Suppl. Fig. 11) that favor active transport of the Bet-MN to the SLOs by activated inflammatory cells. On the other hand, intradermal injection lacks the inflammatory component, thus promoting a passive transport via the lymph.

Summing up, ECI using laser microporation disrupts the upper layers of the skin, and the antigen is deposited on top of the pores. Due to the laser-induced skin damage, inflammatory cells, including DCs, are activated and recruited to the site of immunization where they encounter the antigen and become activated. Upon activation, cells migrate to the SLOs and enter the T cell areas, where the immune response is initiated. Conversely, intradermal immunization delivers the antigen into deeper layers of the skin and therefore most of the antigen is passively drained to the SLOs. High molecular weight antigens such as Bet-MN reaching the lymph nodes via afferent lymphatics are diverted to the subcapsular sinus (SCS) of SDLNs. Here, antigens are captured by SCS macrophages and SCS-lining DCs, which transport them to the interfollicular regions and T cell areas. This transport is further enhanced by complement opsonization [72], which we also demonstrated for Bet-MN (Suppl. Fig. 5).

As shown for Bet-MN ID, this process is highly efficient and leads to the accumulation of antigen in the lymph nodes. However, since there is no additional adjuvant present other than mannan, DCs activation in the LNs after ID injection may be suboptimal, leading to poor T cell activation and weak immune responses [71].

Since mannan coupling efficiently targeted CD14⁺ dDCs and LCs (Fig. 3), we further investigated the role of LCs and Langerin⁺ dDCs in the immune response after ECI or ID of Bet-MN. Surprisingly, conditional ablation of all Langerin⁺ DCs resulted in enhanced immunogenicity of Bet-MN, both after ECI and ID injection (Fig. 7). These data suggest that in our experimental system, Langerin⁺ DCs not only are dispensable for mounting effective humoral immune responses against Bet-MN, but rather play a suppressive role. While both Langerhans cells and Langerin⁺ dDCs have been shown to be inducers of regulatory T cell responses under steady state conditions [73], the situation under inflammatory conditions is less clear [74]. In line with our observations, depletion of Langerhans cells (but not Langerin⁺ dermal DCs) aggravated skin inflammation in a psoriasis mouse model [75]. Similarly, Treg generation was severely impaired in a mouse model of cutaneous Leishmaniasis in absence of Langerhans cells [76]. This indicates that laser microporation of the skin obviously generates an inflammatory environment that promotes strong immune responses mainly mediated by CD11c⁺CD11b⁺ dDCs [11]. In humans, skin CD14⁺ DCs which preferentially uptake Bet-MN, may have a similar role.

Based on our observations, we propose skin vaccination via laser microporation with allergen neoglycoconjugates as an efficient way to elicit strong anti-allergic immune responses. The main advantages of the glycoconjugation are the hypoallergenic nature of the resulting neoglycoconjugates, the superior targeting to APCs via CLRs and the possibility to bias the immune response depending on the nature of the polysaccharide used. Skin barrier disruption via laser micropores allows for efficient delivery of the vaccine to skin-resident DCs while creating a proinflammatory milieu that ensures the efficient recruitment and maturation of APCs.

In conclusion, our work shows that mannan conjugation is an efficient mechanism to generate safer and highly immunogenic allergy vaccines. In addition, we showed that a potent vaccine results from the combination of targeting the antigen to specialized DC populations and optimal activation of these DCs rather than increased delivery to lymphoid organs. Finally, mannan conjugation combined with epicutaneous immunization induces potent IgE-blocking antibodies and non-TH2 responses against Bet v 1, both mechanisms accounting for the therapeutic effect of allergy vaccines. Therefore, application of neoglycoconjugates via laser microporation represents a promising platform of highly efficient, safe, and painless vaccines for allergy treatment.

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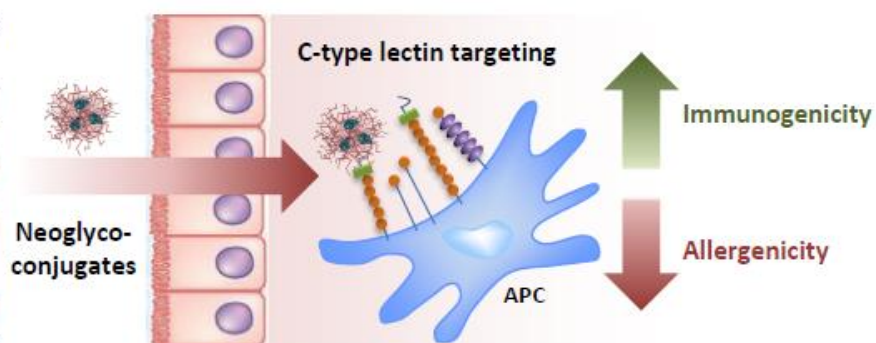
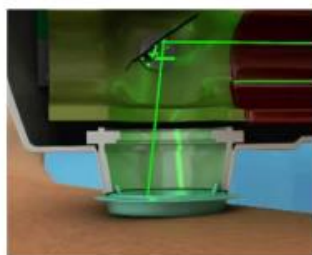
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Epicutaneous immunization
via laser microporation



Graphical abstract

Synergistic effects of dendritic cell targeting and laser-microporation on enhancing epicutaneous skin vaccination efficacy

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Supplementary Methods

Luminescence-based ELISA assay

Antigen-specific IgG1 and IgG2a were determined by luminescence-based ELISA assay as previously described [1]. Briefly, white 96-well high bind immunoplates (Greiner) were coated with 50 μ L/well recombinant antigen [1 μ g/mL] diluted in PBS for 24 h. Next, plates were washed with PBS/0.1% Tween 20 using the 96PW automatic ELISA-plate washing device (Tecan) and blocked with 200 μ L blocking buffer (PBS, 2% skim milk, 0.1% Tween 20) for 1 hour at RT. Afterwards, plates were washed again and different dilutions of the sera in blocking buffer were incubated for 1 hour at RT, washed again, and 50 μ L of a peroxidase (HRP)-conjugated detection antibody diluted 1:1000 in blocking buffer were added to the wells, recognizing either total murine IgG (Biolegend), IgG1 (BioRad), and IgG2a (antibodies online). After incubation for 1 hour at RT, the luminometric assay was developed with Luminol diluted 1:2 in H₂O, according to the manufacturer's instructions (BM chemiluminescence substrate, Roche). Briefly, 50 μ L substrate were added to each well and chemiluminescence (photon counts/second) was determined after 3 minutes of incubation using an Infinite M200 Pro plate reader (Tecan).

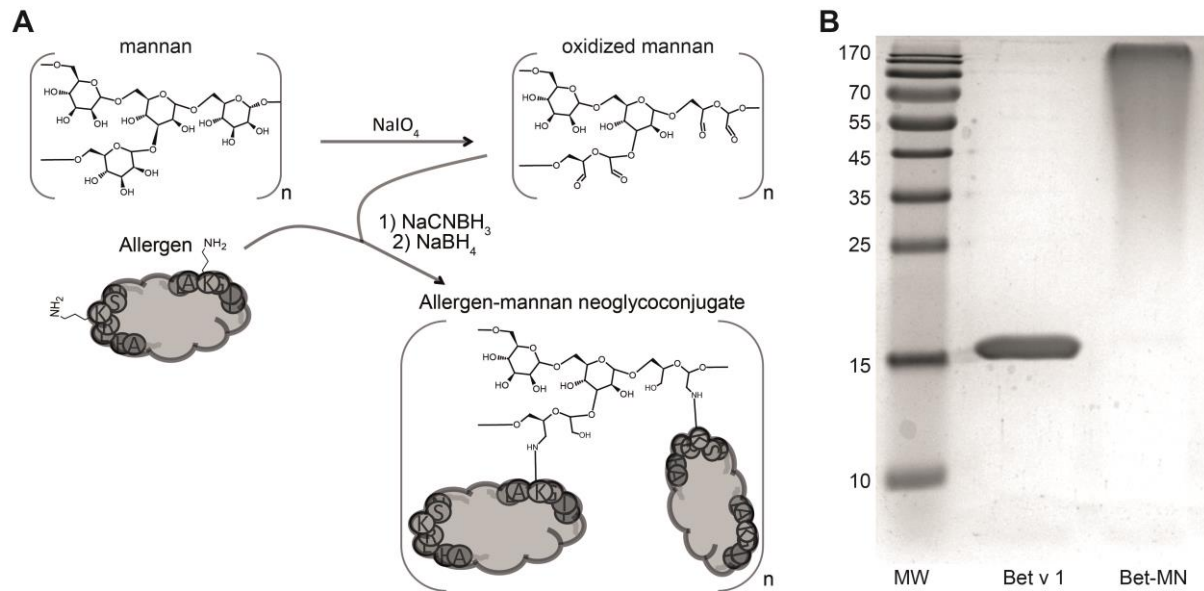
Detection of blocking antibodies

The ability of IgG generated by intradermal or epicutaneous immunization of mice with Bet v 1 or Bet-MN to inhibit Bet v1-induced degranulation of rat basophil leukemia cells (RBL-2H3) transfected with the cDNA encoding the human high affinity IgE receptor was assessed as described [2]. Briefly, RBL cells were sensitized by overnight incubation with sera from five Bet v 1 allergic patients at a final dilution of 1:50 or 1:100. Bet v 1 was pre-incubated with 10% heat-inactivated (2 h, 56°C) pools of mouse sera from the different immunization groups or naïve mice overnight at 4°C and then added at concentrations of 10ng, 1ng or 0.1ng/mL to the sensitized RBL cells to induce degranulation via cross-linking of bound Bet v 1-specific IgE.

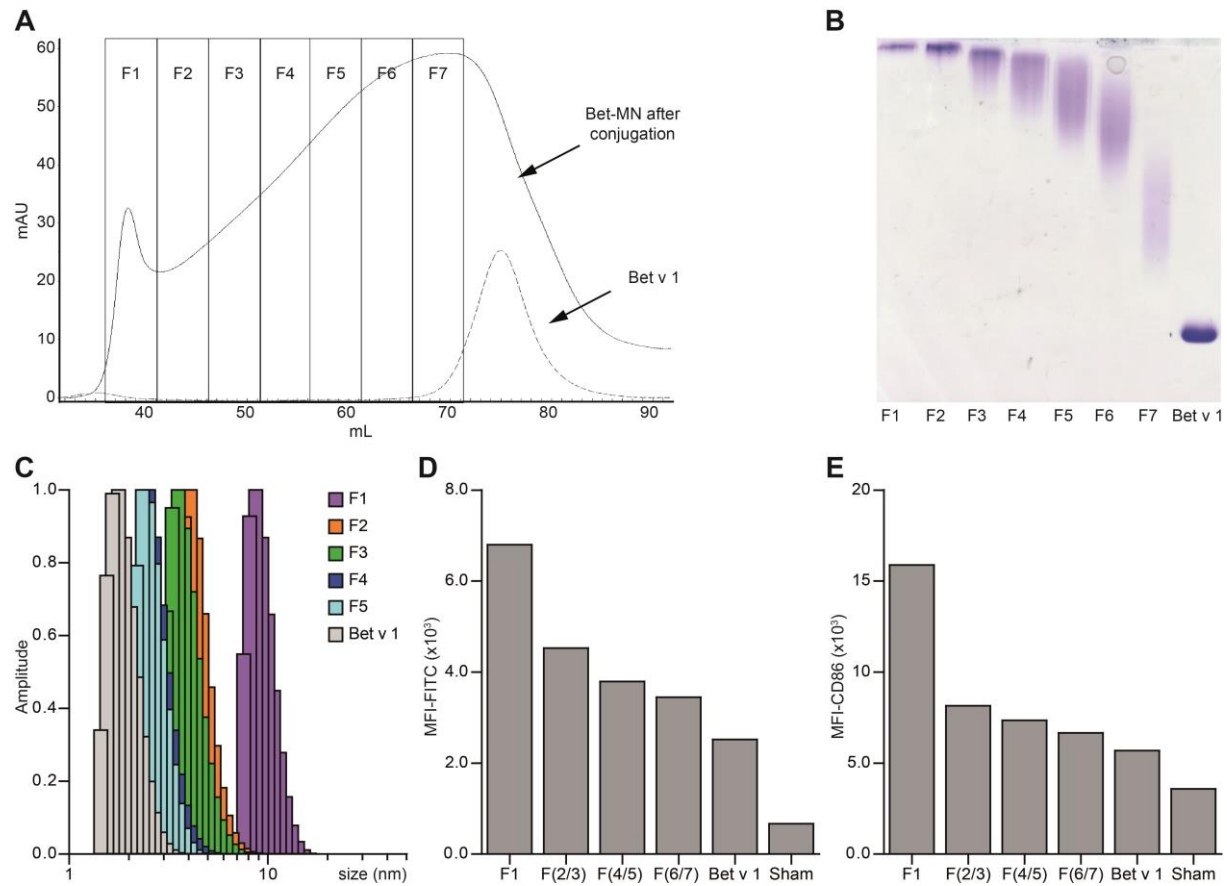
Lymphocyte cultures

Splenocytes were cultured as described previously [1]. Briefly, mice were sacrificed and spleens were minced and sedimented for 5 min in 1 mL MEM medium. For lysis of red blood cells, single cell suspensions were added to ACK lysing buffer for 7 min followed by two washing steps in 10 mL MEM. Cells were re-suspended in MEM supplemented with 1% (v/v) heat-inactivated fetal calf serum, 100U penicillin, and streptomycin/ml, 2mM L-glutamine, 1mM sodium pyruvate, 2 μ M of 2-mercaptoethanol, 20mM Hepes, and 1x non-essential amino acids. 50 μ L/well splenocyte solution was distributed into 96-well, flat-bottomed tissue culture plates (Greiner) at a density of 2×10^5 cells/well. Recombinant antigen was added in 50 μ L supplemented MEM medium to a final concentration of 20 μ g/mL and plates were incubated for 72 h at 37°C, 95% relative humidity, 7.5% CO₂. Wells stimulated with medium alone served as negative controls. After 3 days, the supernatants of wells were harvested and stored at -20°C before they were subjected to cytokine analysis via the murine Th1/Th2/Th17/Th22 13-plex FlowCytomix multiplex kit (eBioscience)

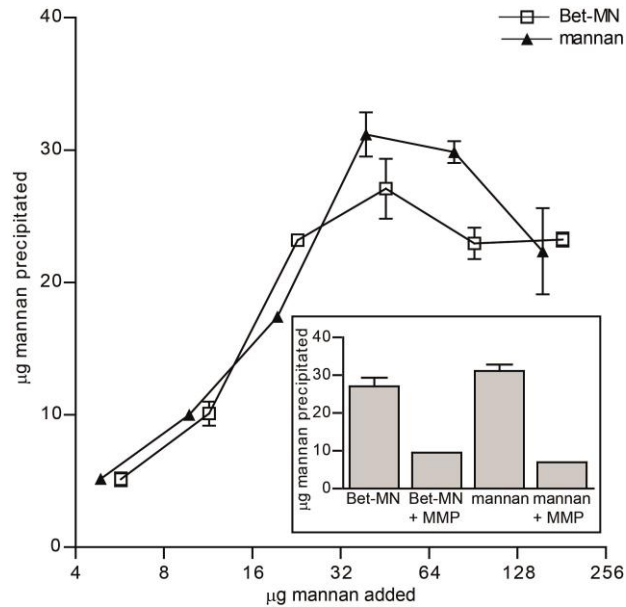
Supplementary Figures



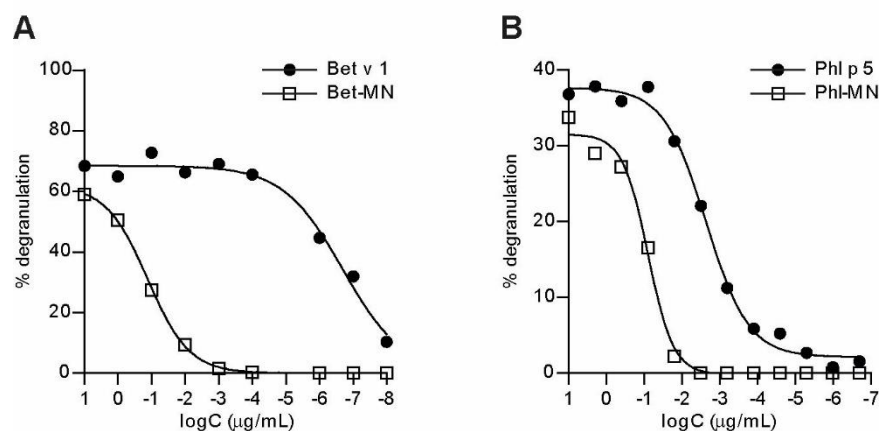
Suppl. Fig. 1 Bet v 1 was coupled to mannan via mild periodate oxidation followed by aminative reduction. A) Aldehydes were generated by the periodate oxidation of vicinal diols present in the sugar moiety and the allergen was coupled via aminative reduction in presence of sodium cyanoborohydride. Excess of aldehydes were reduced to alcohol by addition of sodium borohydride. B) Nearly 100 percent conjugation was achieved using this protocol as observed by SDS-PAGE. Molecular weight is expressed in kDa



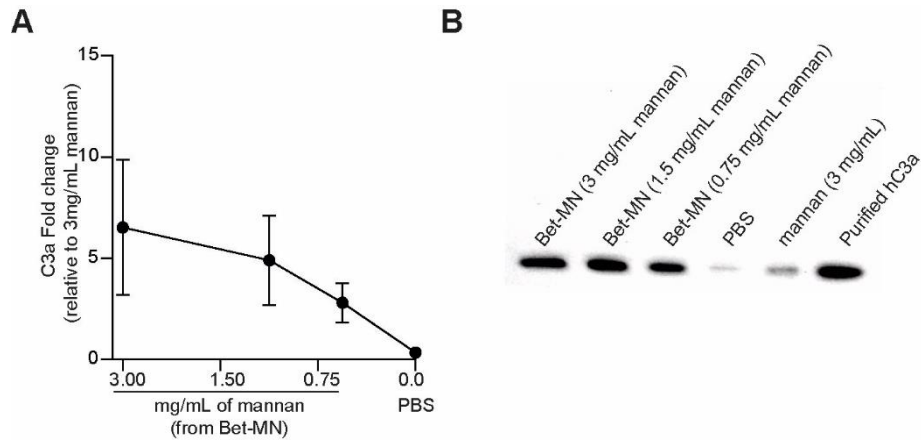
Suppl. Fig. 2 High molecular weight Bet-MN neoglycoconjugates were purified via size exclusion. (A) Overlaid chromatograms of Bet-MN glycoconjugates and Bet v 1. (B) SDS-PAGE analysis of Bet-MN neoglycoconjugates fractionated over size exclusion chromatography, (F1 to F7). (C) Hydrodynamic radius determination by dynamic light scattering (DLS). Leading peak collected in fraction 1 resulted in Bet-MN neoglycoconjugates with an average size of 10 nm as measured by DLS. Size of Bet-MN glycoconjugates positively correlates with uptake (D) and cell activation (E) by BMDCs.



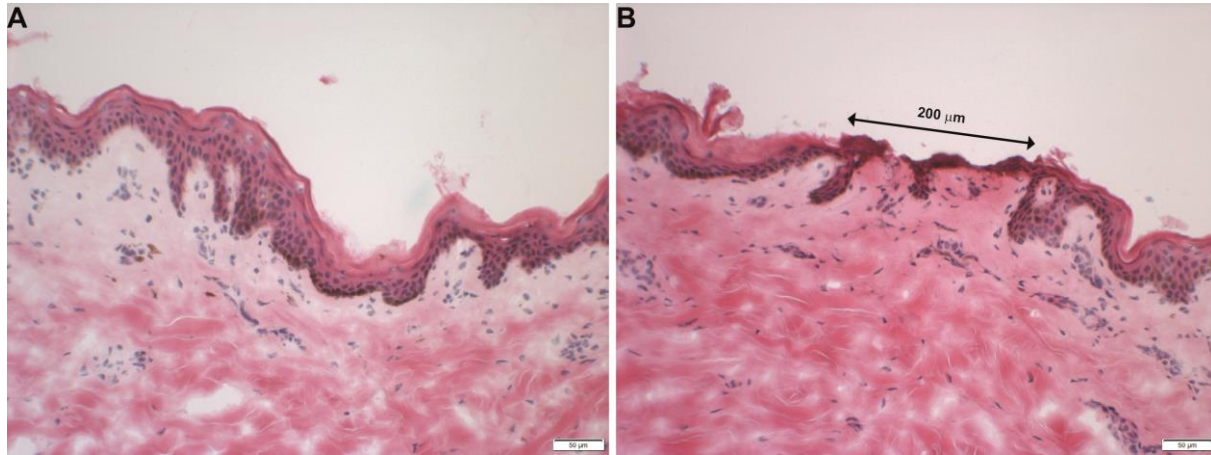
Suppl. Fig. 3. ConA binding of mannan and Bet-MN neoglycoconjugates. Increasing amounts of free mannan or Bet-MN were added to ConA, in assay buffer and the amount of mannan in the precipitate was measured by the anthrone method. In order to confirm the specificity of the ConA/Mannan interaction, 25 mM of the ConA agonist MMP was added.



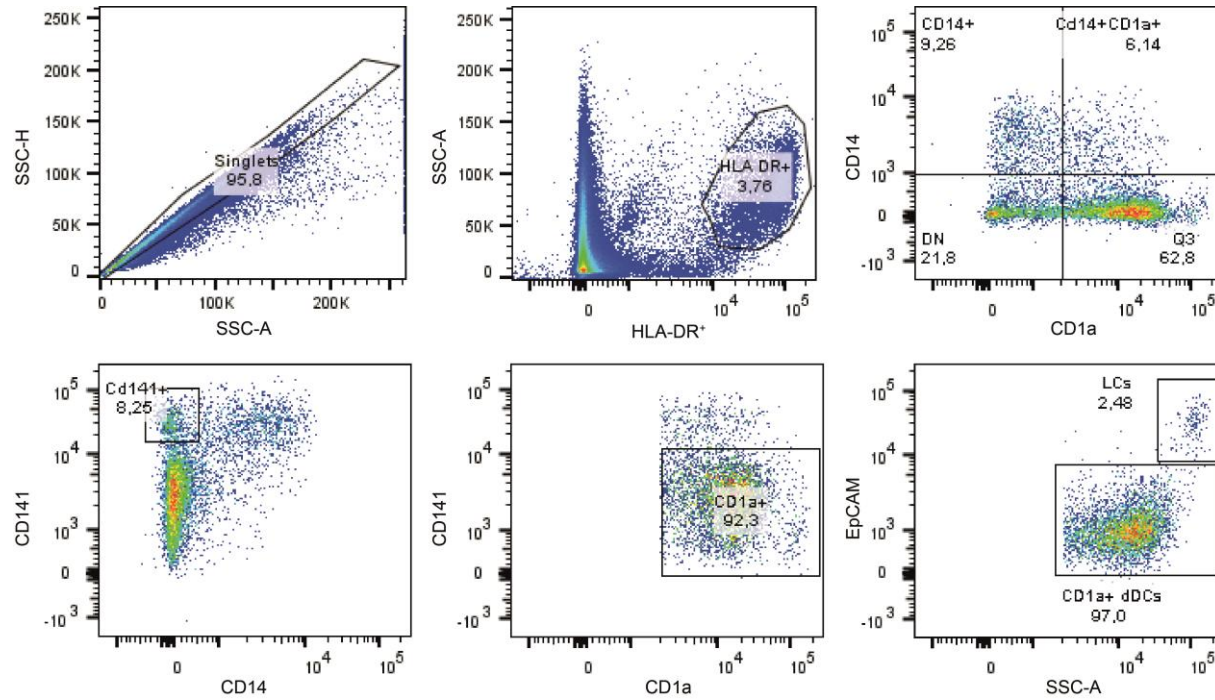
Suppl. Fig. 4. Bet-MN and Phl MN shows reduced allergenicity compared to unmodified allergens. RBL cells were loaded with either Bet v 1-specific or Phl p 5-specific IgE positive serum. Then, different concentrations of native or mannan-modified allergens were added and degranulation was determined by measuring activity of the released β -hexosaminidase in the supernatant. Data is shown as percentage of maximum degranulation obtained by Triton X-100 cell lysis.



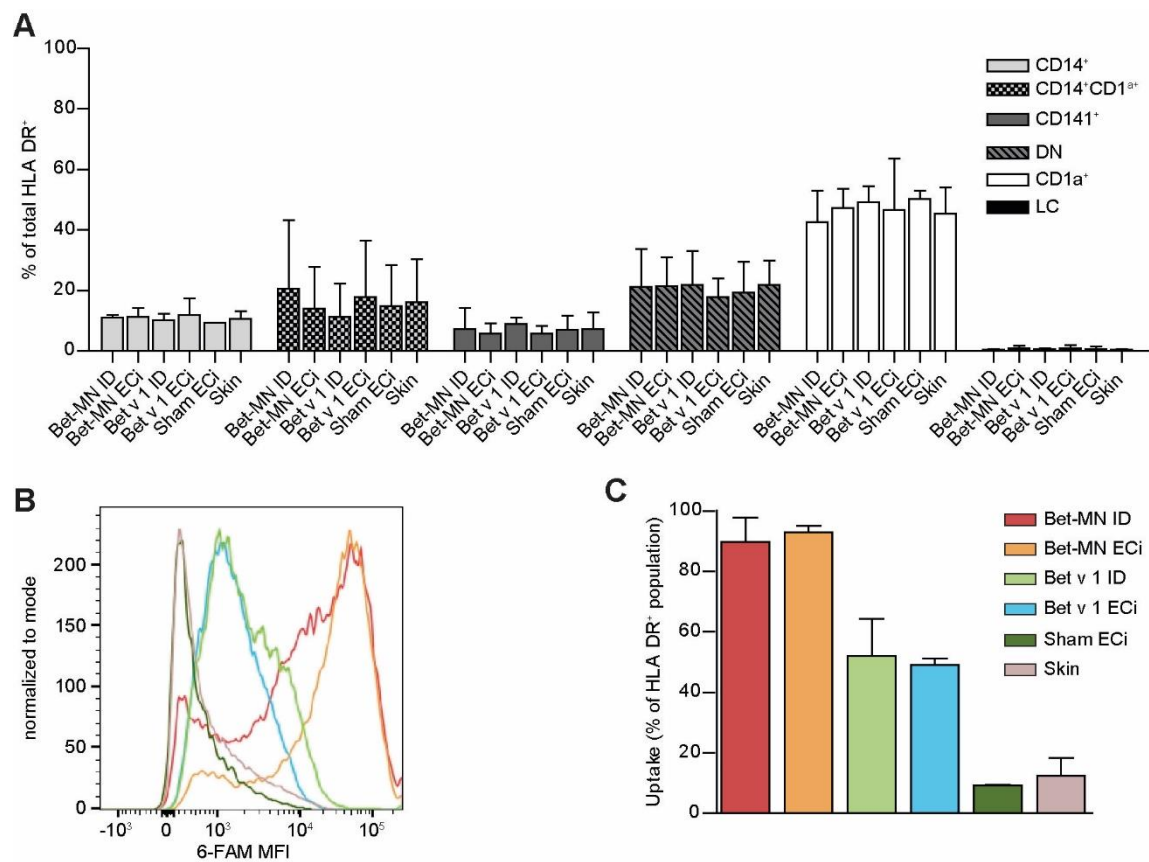
Suppl. Fig. 5. Bet-MN induces superior complement activation compared to soluble mannan. Sera from human donors (n = 4) were incubated with different concentrations of Bet-MN (2, 1, 0.5 mg/mL) containing 3, 1.5, and 0.75 mg/mL mannan, respectively or with 3 mg/mL mannan or PBS. Complement activation was shown by Western blotting of cleaved C3a. Purified human C3a served as a positive control. Data are shown as integrated band intensity relative to activation with 3 mg/mL of soluble mannan (A). Representative Western Blot of one donor (B).



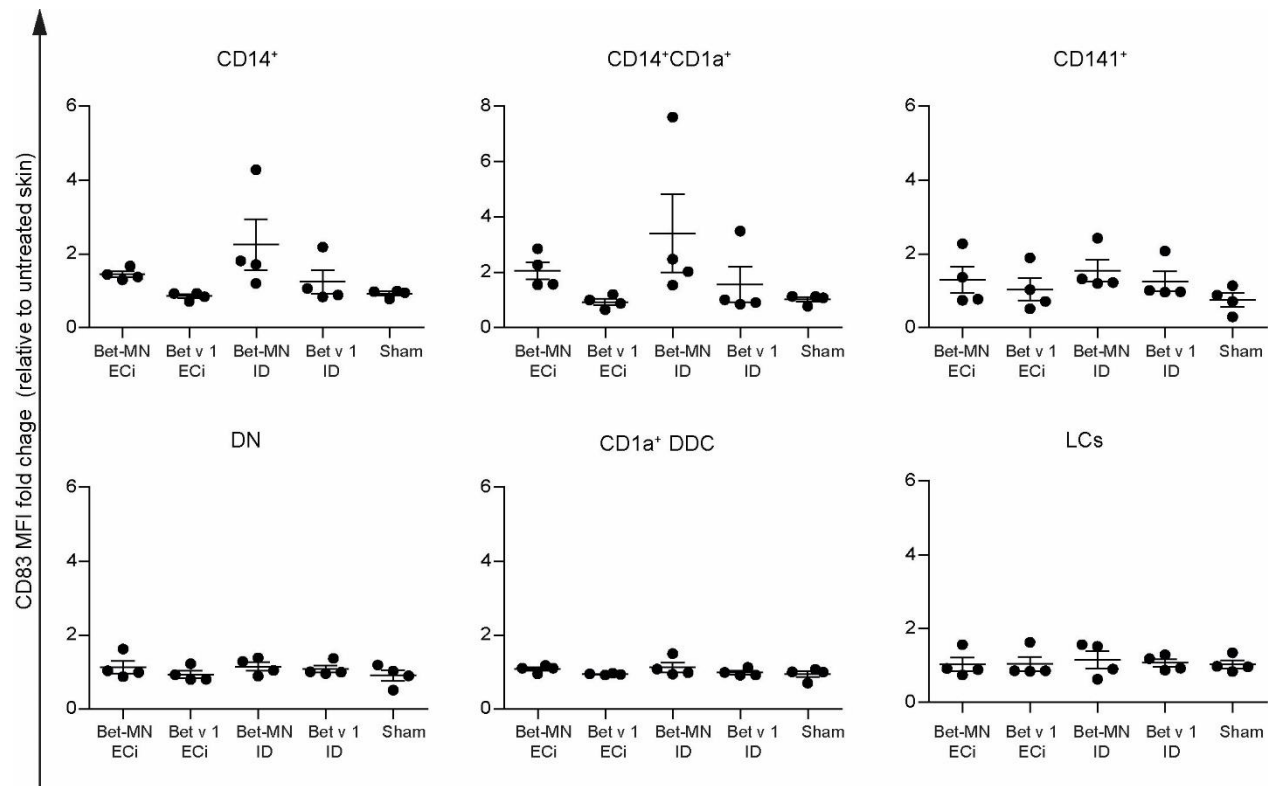
Suppl. Fig. 6. Laser generated micropores on human skin explant. Skin biopsies of intact skin (A) or laser micropores (B) were immediately taken after laser microporation of skin explants and frozen in OCT compound. 6 μm sections were stained with H&E. Pictures, 25 X magnifications were acquired. Line shows the length and position of the micropore.



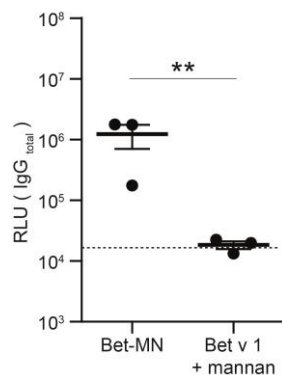
Suppl. Fig. 7. Gating strategy for analyzing DCs in crawl-outs from human skin explants. HLA-DR⁺ cells were gated from total singlet cells. Six dendritic cell populations were gated as follows, CD14⁺CD1a⁻ (CD14⁺ DCs), CD14⁺CD1a⁺ (CD14⁺CD1a⁺ DCs), CD141⁺CD14⁻ (CD141⁺ DCs), dermal CD14⁻CD141⁻CD1a⁺EPCAM⁻ (CD1a⁺ dDCs) and CD14⁻CD141⁻CD1a^{high}EPCAM⁺ (LCs) and CD14⁻CD1a⁻ (DN).



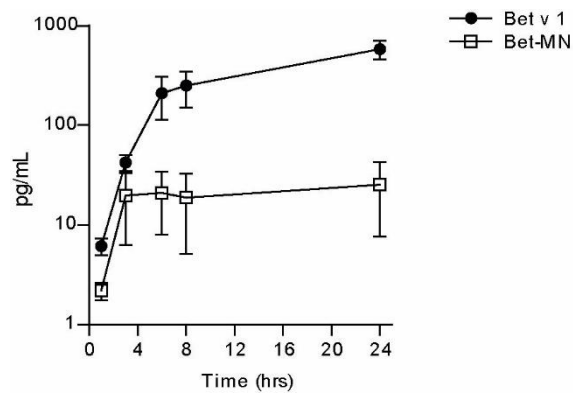
Suppl. Fig. 8 Different migratory DCs populations were found in human skin crawl-outs. (A) Percentage of DCs populations in cellular crawl-outs from human skin treated ID or ECi with Bet v 1 or Bet-MN. (B, and C) Mean fluorescence intensity (MFI) of antigen-loaded cells and percentage from the total HLA-DR⁺ population.



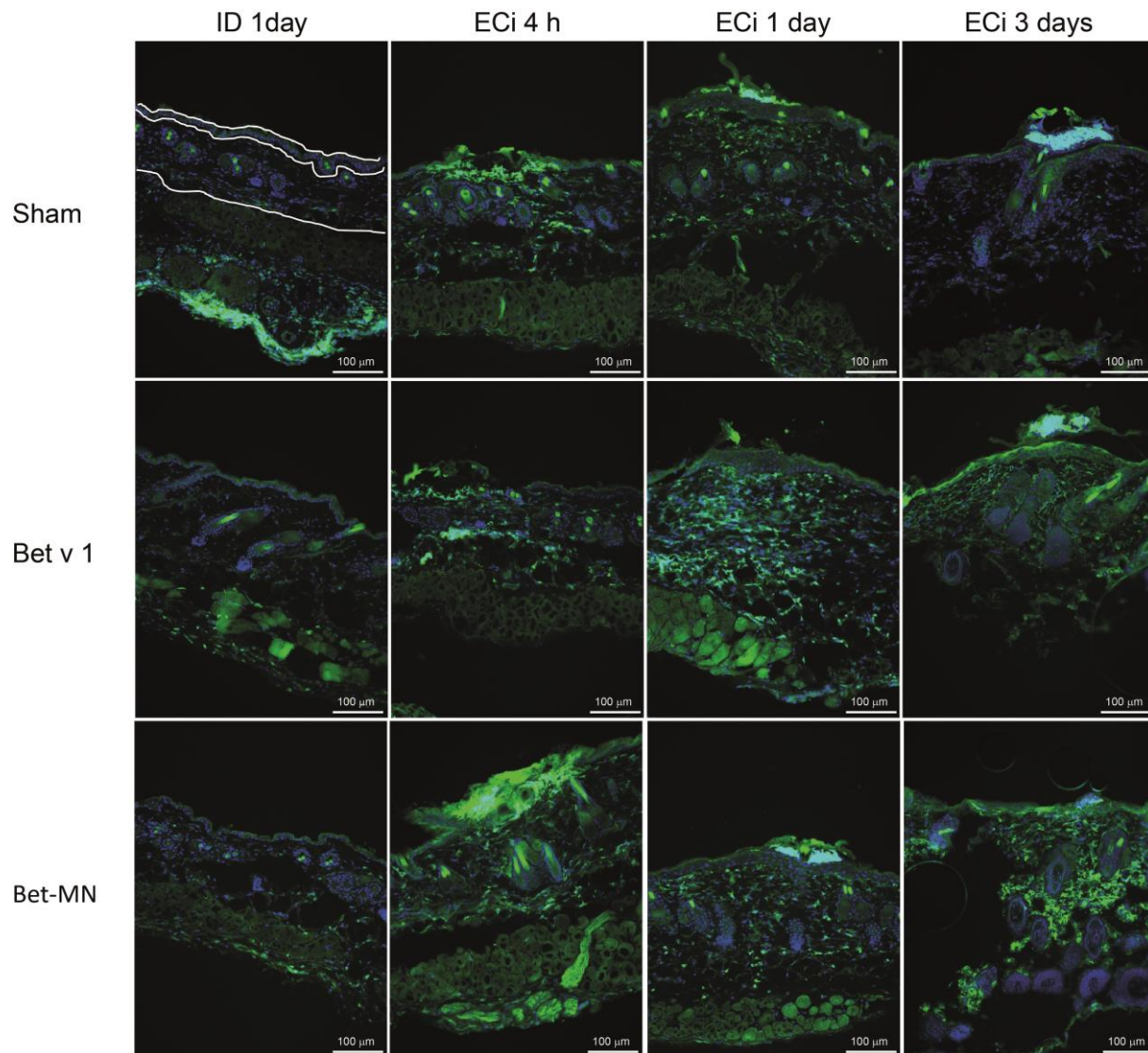
Suppl. Fig 9. Expression of CD83 DC activation marker on skin DC crawl-outs. Abdominal skin explants (n = 4) were treated with 100 µg of fluorescently labeled Bet v 1 or Bet-MN by epicutaneous immunization (ECi) via laser microporation or intradermal injections (ID). As negative controls, laser-microporated skin was treated with PBS (sham) and adjacent, untreated skin samples were also used. 10 biopsies of 0.6 cm² were collected and cultured for 48h. Cellular crawl-outs were analyzed by flow cytometry and APC populations were determined based on the expression of surface markers CD14, CD1a, CD141, and EPCAM. Data is presented as fold-change of the mean fluorescence intensity relative to untreated skin. Differences between groups were analyzed using repeated measures one way ANOVA and Tukey's post-hoc test. No statistically significant differences (p-value > 0.05) were found.



*Suppl. Fig. 10. A physical link between Bet v 1 and mannan is needed for Bet-MN immunogenicity. Balb/c mice (n = 3) were immunized epicutaneously via laser microporated skin at days 0 and 14, with Bet-MN or Bet v 1 mixed with mannan (Bet + mannan) in a mannan/protein ration 4/1 (w/w). At day 28, animals were sacrificed and Bet v 1 specific antibodies were measured and displayed as relative luminescence units (RLU) Samples from naïve mice served as base line controls (dashed line). ** $p < 0.01$, Student's *t* test.*



Suppl. Fig. 11. Bet-MN is retained at the immunization site while Bet v 1 is able to permeate skin when injected intradermally. Skin penetration was measured in a Franz static diffusion cell and pig skin was used as a surrogate of human skin. Full thickness (2-3 mm) pig skin was treated with 20 µg of Cy5.5 labeled Bet v 1 or Bet-MN either by intradermal injections or epicutaneously via laser micropores. The concentration of antigen diffusing to the lower chamber was determined at different time points by measuring the Cy5.5 fluorescence (Ex 675 nm/Em 710 nm). Passive diffusion of Bet v 1 and Bet-MN applied via laser microporation to the lower chamber was below the detection limit of our assay.



Suppl. Fig. 12. Laser microporation resembles a wound healing-like process which resolves within 72 h. Contrary to ID injections ECI induces a CD11b⁺ cell infiltrate around the micropores. CD11b⁺ cell infiltrate remains for at least 72 h when Bet-MN was applied epicutaneously while Bet v 1 has no effect compared to sham treated skin. Transversal skin sections (6 μm) were stained with anti-mouse CD11b AF488 (Green) and nuclei were counterstained with DAPI (Blue). Dashed white lines delimit the epidermal compartment. Solid line shows the boundary between dermis and subcutaneous adipose tissue.

References

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