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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.

1. 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 as $CD1a^+$, $CD14^+$ 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].

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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.

2. Materials and methods

2.1. 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.

2.1.1. 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 amebocyte lysate assay (Cape Cod) according to the manufacturer's instructions. Proteins used for all experiments had > 95% purity and the endotoxin content was lower than 0.3 pg/μg. Finally, proteins were dialyzed against 2 mmol/L sodium phosphate buffer pH 7.4 and stored at − 20 °C.

2.1.2. 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 × 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).

2.1.3. Characterization of Bet-MN by Fourier-transform 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). 20 μL of each sample were deposited onto the Bio-ATR cell, 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.

2.1.4. 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 μL of increasing amounts of free mannan or Bet-MN [5–300 μg/mL] in assay buffer (50 mM sodium phosphate, 0.1 M NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2 \times 4H_2O$, pH 7) were added to 500 μL ConA [150 μg/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 μL 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.