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Genetically engineered cell factories produce glycoengineered vaccines that target antigen-presenting cells and reduce antigen-specific T-cell reactivity



To the Editor:

Allergen-specific immunotherapy is a promising approach to reduce or remove allergic symptoms by inducing tolerance.¹ As an alternative to current allergy vaccines derived from extracts of natural allergens, recombinant vaccines offer the opportunity for *de novo* antigen design,² with enhanced targeting to antigen-presenting cells (APCs) and potentially increased ability to induce tolerance.^{3,4}

Using the type 1 allergen Bet v 1 as our model antigen, we evaluated the effects of combining a nonglycosylated antigen to a

glycomodule⁵ to target the APC-expressed family of C-type lectin receptors (CLRs). CLRs are known to recognize glycan structures and promote rapid internalization of antigens on binding (see Fig E1, A, in this article's Online Repository at www.jacionline.org).^{6,7} We used our recently developed glycoengineered cellular platform⁸ to produce Bet v 1 with defined carbohydrates and evaluated APC uptake, efficacy using patient-derived T cells, and induction of tolerance in a murine model.

Currently, allergen-specific immunotherapy is primarily administered subcutaneously or sublingually. Focusing on human skin, we examined the CLR expression pattern in skin-localized APCs. We observed expression of the major CLRs, including mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), and macrophage galactose-type lectin (MGL), in the dermal compartment of skin biopsy specimens (see Fig E1, B). Next, we examined expression of MR, DC-SIGN, and MGL in *in vitro*-differentiated human monocyte-derived dendritic cells (moDCs) and macrophages (M1 and M2) by means of transcriptomics and flow cytometry (see Fig E1, C-E). Expression of CLRs peaked after 3 days of differentiation, with simultaneous and significant expression of DC-SIGN, MR, and MGL in moDCs (see Fig E1, D and E). Cytokine measurements confirmed the nature of the APCs (see Fig E1, F) and their relevance as a cellular model.

To identify the optimal carbohydrate structure or structures for specific APC targeting, we evaluated uptake of chemically synthesized carbohydrate derivatives (N-acetylgalactosamine polyacrylamide [GalNAc-PAA], N-acetylglucosamine-PAA, Lewis X-PAA, and mannose-PAA) and a GalNAc glycosylated molecule with varying numbers of GalNAc residues (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). Both mannosylated and high-density GalNAc glycosylated structures were readily taken up by the APCs, and therefore GalNAc and mannose structures were selected for further studies.

We next designed Bet v 1 allergen constructs for the expression of full-length Bet v 1 fused to glycomodules, with consensus glycosylation motifs ensuring the incorporation of either O- or N-linked glycans. The fusion constructs were expressed in glycoengineered Chinese hamster ovary (CHO) cell lines and *Pichia pastoris* (Fig 1, A, and see Fig E2, C-G, and Table E1 in this article's Online Repository at www.jacionline.org). This strategy allowed us to generate fusion proteins decorated with high-mannose N-linked glycans from MGAT1 knockout CHO cells, linear α 1-2 O-linked mannose residues from *P. pastoris* (Pichia), or O-GalNAc residues from COSMC knockout CHO cells for efficient targeting of MR, DC-SIGN, and/or MGL on APCs (Fig 1, A, and see Fig E2, C-G).

Additionally, we expressed the O-glycan fusion construct in *Escherichia coli* and inserted GalNAc residues by means of *in vitro* glycosylation. Glycosylation profiles were verified by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) of released glycans supplemented with lectin profiling (see Fig E2, C-G). As a control for glycan-mediated effects, we treated fractions of the glycoallergens with periodate, a treatment known to effectively destroy lectin recognition (see Fig E2, E).

Because moDCs were the only cell type to express high levels of DC-SIGN, as well as MR and MGL, we used moDCs to evaluate uptake of glycan-modified Bet v 1 fusion proteins. Internalization of all glycan-modified Bet v 1 fusion proteins was enhanced 1.5- to 6-fold compared with that of their

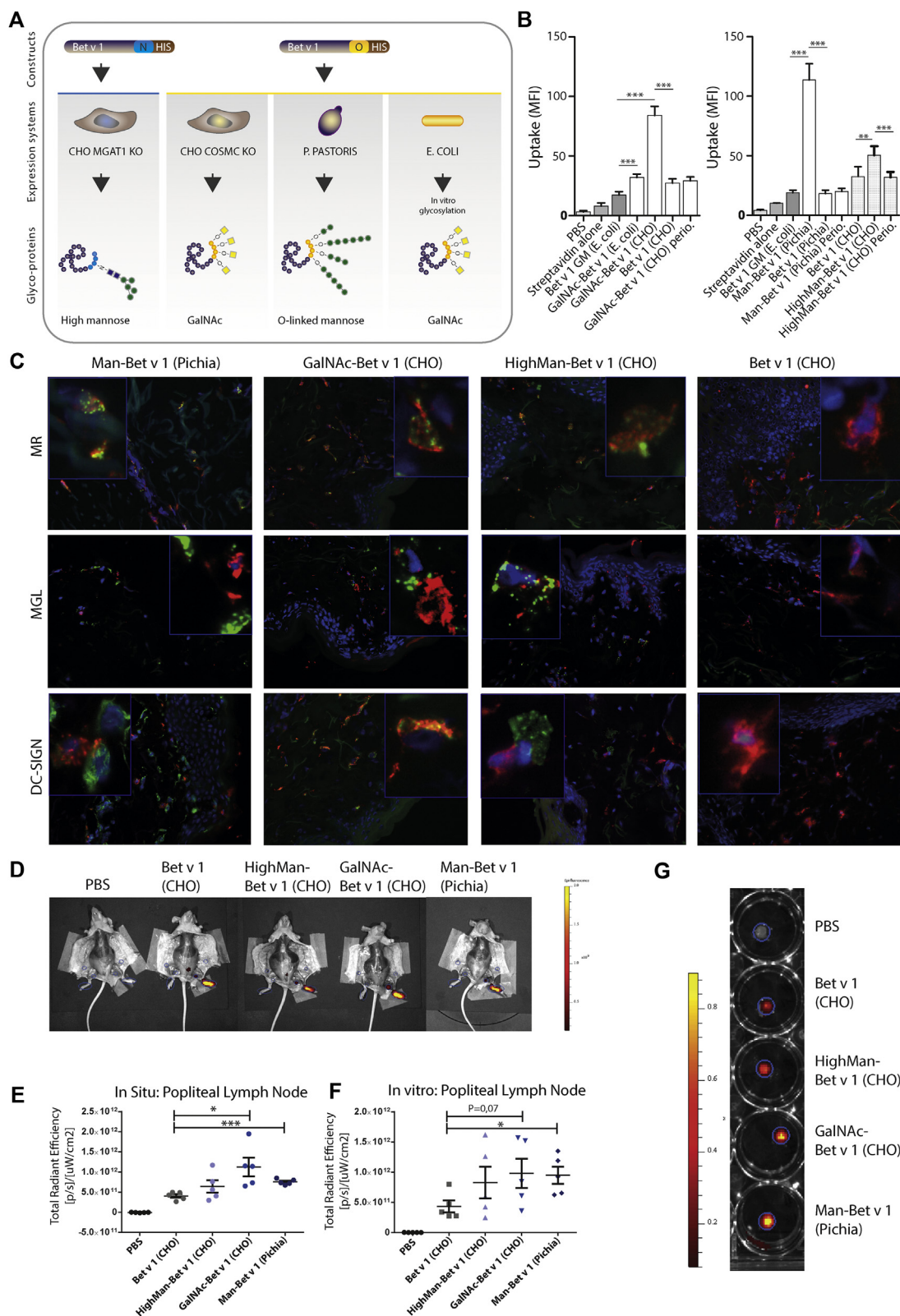


FIG 1. GalNAc and mannose glycosylation significantly increase uptake of Bet v 1 allergens in human and murine APCs. **A**, Schematic illustration of the allergen glycomodule design and production. **B**, Human moDC uptake of Bet v 1 variants, as analyzed by flow cytometry. Data are presented as mean fluorescence intensity (MFI) $\pm$ SD for all donors (n = 3-8). **C**, Immunofluorescence staining of human skin sections after intradermal microinjection of indicated allergen. Skin sections were stained with streptavidin-Alexa Fluor 488 (green) to detect the allergen and anti-CLR antibodies (red) to visualize colocalization. 4',6-Diamidino-2-Phenylindole, Dihydrochloride/DAPI (blue) was used as a counterstain. **D**, IVIS *in situ* scanning of open midsections 24 hours after footpad injection of Bet v 1 glycoforms. Circles mark the sites of draining lymph nodes for focused scanning. **E**, *In situ* quantification of fluorescent signal (Fig 1, D). The graph depicts means $\pm$ SEMs. **F** and **G**, *In vitro* quantification of the fluorescent signal after excision of the lymph nodes. * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$.

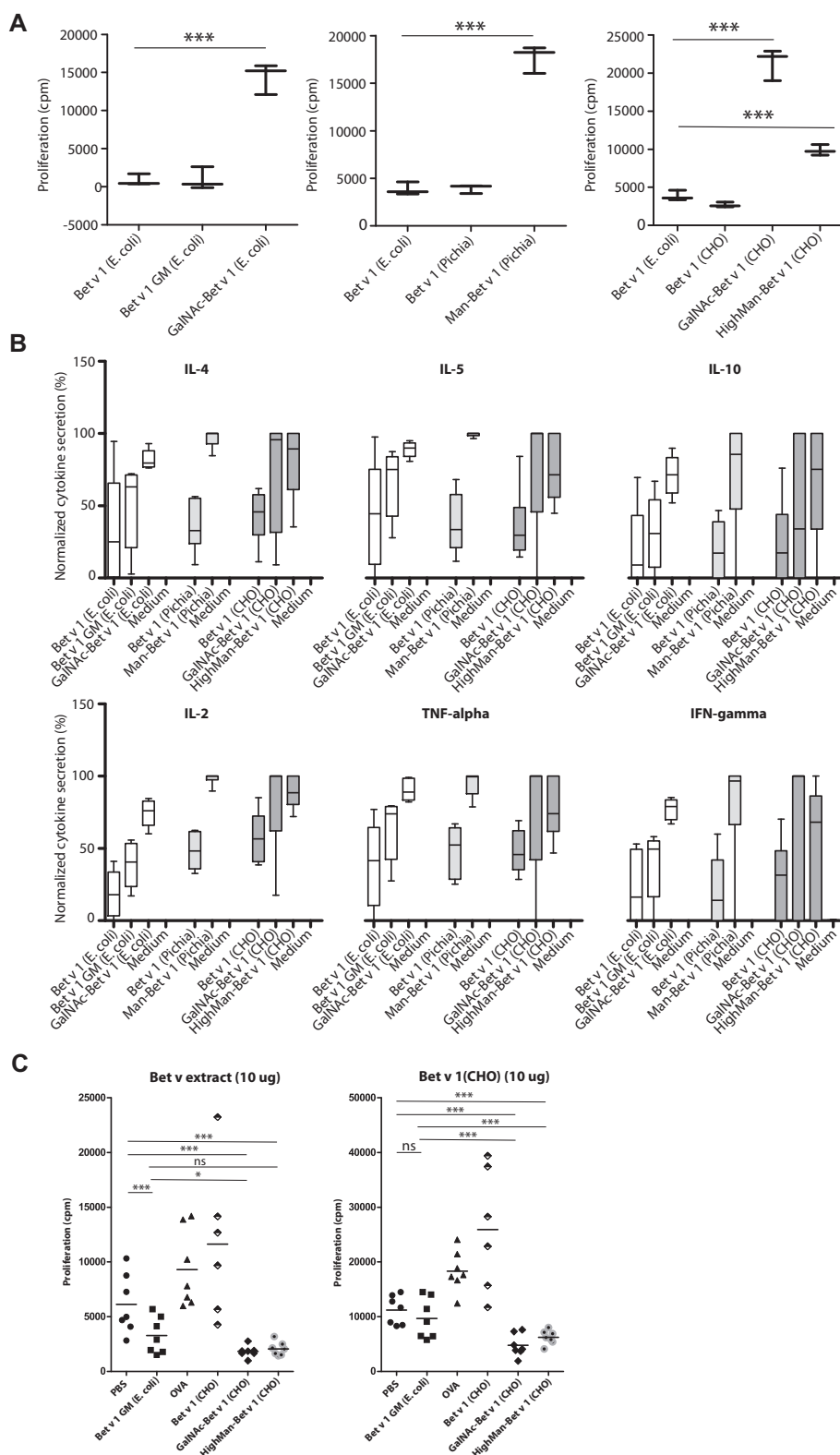


FIG 2. Bet v 1 glycosylation enhances the human T-cell response and increases effects in an *in vivo* model of type 1 allergy. **A**, T-cell proliferation of a representative allergic patient after 72 hours of *in vitro* restimulation with autologous moDCs pulsed with the indicated Bet v 1 products depicted as counts per minute (cpm). *E coli* (Donor C), CHO, and *P pastoris* (Donor 1) are shown. Donor numbers are from Fig E8, in which all individual donors are shown (n = 6-7). **B**, Select normalized cytokine release data after 48 hours (from the cocultures in Fig 2, A), as measured by an 18-plex bead assay. Additional cytokine data are depicted in Figs E9 (relative values) and E10 (in picograms per milliliter for selected donors). **C**, Murine spleen cell proliferation after *in vitro* restimulation. The x-axis indicates prophylactic treatment before challenge. Proliferation was measured by tritiated thymidine incorporation after 4 days of *in vitro* expansion with natural Bet v extract or Bet v 1 (CHO), as indicated. * $P \leq .05$ and *** $P \leq .001$. ns, Not significant.

nonglycosylated counterparts (Fig 1, B). This uptake was mediated through clathrin-coated pits, EEA-1- and Rab-5-positive endosomes. Additionally, live imaging revealed a rapid uptake of glycosylated allergen (see Video E1 and Fig E3 in this article's Online Repository at www.jacionline.org). Importantly, periodate treatment of the glycomodified allergens eliminated the increased uptake and supported the glycan-specific effect (Fig 1, B). This was confirmed by *ex vivo* uptake of *in vitro*-glycosylated GalNAc-Bet v 1 (*E coli*) in primary plasmacytoid dendritic cells (pDCs) isolated directly from human blood (see Fig E4 in this article's Online Repository at www.jacionline.org), exemplifying *in vivo* relevance. Additionally, the increased uptake was confirmed in human tissue by means of injection of glycosylated Bet v 1 intradermally *ex vivo* (Fig 1, C). Glycosylated products accumulated and localized to APCs, expressing MR and MGL, whereas the nonglycosylated allergens were virtually undetectable (Fig 1, C). In contrast, DC-SIGN⁺ cells only seemed to internalize GalNAc-Bet v 1 (CHO).

The APC nature of the cells internalizing the glycosylated allergen was verified by means of extraction and quantification of Bet v 1 uptake in cells colabeled with CD11c (see Fig E3, C). The high-density linear mannose structures produced by *P pastoris* mediated the most significant increase in uptake, closely followed by GalNAc glycosylated Bet v 1. The single high-mannose N-glycan also promoted uptake, although less efficiently (Fig 1). These findings are consistent with previous reports suggesting that clustered glycans are more potent compared with their linear and nonclustered counterparts.⁹

To facilitate comparison of the physiologic effects of the glycan-mediated increased uptake between human and murine models, we conducted CLR expression profiling of murine APCs (see Fig E5, A, in this article's Online Repository at www.jacionline.org). In murine bone marrow-derived dendritic cells (BM-DCs), we again found increased uptake of Lewis X-PAA and GalNAc-PAA but not mannose-PAA. This uptake pattern correlated with the very low MR expression seen in murine BM-DCs (see Fig E5).

We next investigated the biodistribution of fluorescence-labeled glycosylated Bet v 1 *in vivo* and found a significant increase in allergen uptake with targeting to the popliteal lymph nodes for GalNAc and linear mannose glycoforms compared with the nonglycosylated Bet v 1. Interestingly, there was a tendency for greater uptake of GalNAc-modified antigen in murine cells in contrast to human cells, where linear clustered mannose seems to dominate (Fig 1, D-G, and see Fig E5). This uptake pattern correlates with the expression level of MGL and MR in APC populations (see Figs E1 and E5, A). The potential differences in uptake between species might be relevant when testing glycosylated human vaccine candidates in murine models and could make it difficult to conclude which glycan structure would be most relevant in the human setting.

We next wanted to clarify the global effect of glycans on dendritic cell stimulation. We performed RNA sequencing analysis of moDCs stimulated with the Bet v 1 allergens by using LPS as a reference (see Fig E6 in this article's Online Repository at www.jacionline.org). Although LPS stimulation resulted in a well-defined transcriptional change, a comparison between moDCs stimulated with glycosylated Bet v 1 and periodate-treated Bet v 1 yielded no significant transcriptional change and thereby no indications of any major immune

pathways differentially activated (see Fig E6). This suggests that the major effect of glycans on allergens is related to increased uptake. This is supported by cytokine measurements on protein level of *ex vivo*-stimulated human pDCs, showing that cytokines are not produced at detectable levels in pDC cultures stimulated with Bet v 1 for 24 hours (see Fig E7 in this article's Online Repository at www.jacionline.org). However, future studies could include several time points to fully clarify the immunomodulatory effect and compare early and late cytokine responses.

To investigate whether this increased uptake could alter immune reactivity, we performed *in vitro* experiments using T cells from allergic patients and *in vivo* experiments in an animal model. First, we evaluated the proliferation and cytokine release of primary T-cell lines from donors with birch pollen allergy. Cocultures of T cells with autologous moDCs pulsed with glycosylated Bet v 1 induced significantly increased proliferation compared with nonglycosylated Bet v 1, regardless of the glycophenotype (Fig 2, A, and see Fig E8 in this article's Online Repository at www.jacionline.org). Regarding cytokines, we observed a mixed picture with increased levels of both proinflammatory cytokines and anti-inflammatory cytokines, such as IL-10, after glyco-Bet v 1 priming (Fig 2, B, and see Figs E9 and E10 in this article's Online Repository at www.jacionline.org).

To assess function, we evaluated the *in vivo* effect of glycosylating Bet v 1 in a prophylactic mouse model of type 1 allergy and compared the efficacy to nonglycosylated *E coli*-produced Bet v 1 (Bet v 1 noGM [*E coli*]), as well as nonglycosylated CHO-produced Bet v 1. Because GalNAc products had the overall highest uptake in murine cells (Fig 1 and see Fig E5), we chose to prophylactically treat the mice with GalNAc-Bet v 1 (CHO). We included the HighMan-Bet v 1 (CHO) variant also to test a mannose-containing product and ensure that all proteins for the *in vivo* analysis were produced in the same production cell. We showed significantly reduced *ex vivo* spleen cell proliferation, with a uniform low proliferative response across all experiments when compared to the nonglycosylated Bet v 1 (Fig 2, C, and see Fig E11, A, in this article's Online Repository at www.jacionline.org). Reduced T-cell proliferation was also seen for GalNAc-Bet v 1 (CHO) after restimulation with the natural Bet v extract in different concentrations (Fig 2, C, and see Fig E11, A).

In addition, prophylactic treatment with GalNAc-Bet v 1 (CHO) significantly reduced the IgE response compared with control treatment (PBS; see Fig E11, B). The reduction in IgE levels is at the same level as Bet v 1 (*E coli*), suggesting that the glycan module does not disrupt the positive effect of prophylactic Bet v 1 treatment on IgE level while significantly reducing the T-cell proliferative response. We cannot fully exclude that introduction of the glycan module could have an effect on the overall 3-dimensional structure of Bet v 1 and thereby conformational epitopes. However, because pretreatment with Bet v 1 containing the glycan module can protect mice sensitized with Bet v 1 without the module, we believe the effect is minimal.

In summary, the use of a genetically engineered production platform combined with a glycomodule design provides a flexible solution with freedom to choose different posttranslational moieties and antigens. For allergen-specific immunotherapy, our results suggest that fusion proteins containing high-density

GalNAc modification is the prime candidate for a clinically relevant Bet v 1 vaccine. However, the specific design will most likely be dependent on the biophysical and physiologic properties of the individual antigen. The described glycoengineered production platform will be useful for the design of next-generation therapeutic vaccines.

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Prophylactic and therapeutic inhibition of allergic airway inflammation by probiotic *Escherichia coli* O83



To the Editor:

Oral supplementation with probiotic bacteria is a promising alternative for allergy prevention. However, a recent meta-analysis has demonstrated that orally delivered bacteria only prevented the development of certain allergic manifestations, such as atopic eczema, but had limited efficacy on respiratory allergies.¹ Similarly, oral delivery of the gram-negative probiotic bacterial strain *Escherichia coli* A0 34/86 (*E coli* O83; serotype O83:K24:H31; a commercially available oral live vaccine, Colinfant Newborn) to high-risk infants reduced development of atopic diseases in later life but had no beneficial effects on respiratory allergies.²

Here, we address whether the delivery of *E coli* O83 directly through the airways might be more effective in preventing the development of allergic disease in the lung. To test this, mice were intranasally treated with *E coli* O83 or an attenuated α -hemolysin-deficient mutant *E coli* O83 before systemic sensitization and local airway challenge with ovalbumin (OVA). Detailed methods are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Prophylactic intranasal treatment with *E coli* O83 during sensitization and challenge with OVA ([Fig 1, A](#)) completely inhibited airway hyperreactivity (AHR) to methacholine, as measured by using whole-body plethysmography ([Fig 1, B](#)) and invasive assessment of pulmonary resistance and compliance (see [Fig E1](#) in this article's Online Repository at www.jacionline.org). Treatment with *E coli* O83 reduced airway eosinophilia ([Fig 1, C](#)), T_H2 cytokine production (see [Fig E2, A and B](#), in this article's Online Repository at www.jacionline.org), and mucus secretion ([Fig 1, D](#), and see [Fig E2, E](#)) but did not induce pulmonary IFN- γ and IL-10 levels (see [Fig E2, C and D](#)) when compared with values in sham-treated/OVA-sensitized control mice. Of note, although OVA-specific antibody production was not influenced (see [Fig E2, F-I](#)), *E coli* O83 still inhibited the effector phase of the allergic response.

LPS is a major constituent of the outer membrane of gram-negative bacteria, such as *E coli* O83, and potently stimulates the immune system through Toll-like receptor (TLR) 4. Studies on the effect of LPS in experimental models of asthma have produced conflicting results; some report a beneficial effect,³ and others show an exacerbation.⁴

To determine the role of TLR4 in *E coli* O83-mediated protection, we treated wild-type (WT) and TLR4-deficient (TLR4KO) mice with *E coli* O83 as in [Fig 1, A](#). Unlike in WT mice *E coli* O83-treated TLR4KO mice had strong eosinophilic airway

1 **Supplementary Materials and Methods section for Mathiesen *et al.***

2 ***Ethics statement***

3 Buffy coats were obtained from anonymous healthy blood donors. Written informed consent was obtained
4 from the blood donors at the Department of Clinical Immunology in Copenhagen and used without the
5 possibility of identifying case-specific information. Research use for these buffy coats was approved by the
6 ethical committee, Copenhagen County. Allergic patient samples were obtained at ALK-Abelló, Hørsholm,
7 after written consent and ethical approval of the local ethical committee. All information about the donors
8 were handled in accordance with the Danish Act on Processing of Personal Data. All animal experiments
9 were approved by the local animal facility and the Danish Veterinary and Food Administration (approval
10 numbers: 2014-15-0201-00048 and 2011/561-65). The animals were monitored daily after injection to
11 detect any unwanted side effects and sacrificed humanely at the end of the experiment. Human skin
12 samples were obtained from reductive abdominal surgery at BodyLift Center, Virum. Written informed
13 consent was obtained, and the study was performed in accordance with the Declaration of Helsinki.
14 Biopsies of human skin was obtained from healthy donors, with informed consent approved by local
15 ethical committee (H-16020701).

16 **Design, expression and purification of Bet v 1 products and Bet v extract**

17 *Design, expression, and purification of CHO Bet v 1*

18 Expression constructs containing the entire coding sequence of Bet v 1 were cloned into pcDNA3.1 vector
19 synthesized by Genewiz, USA.

20 3 constructs were made, Bet v 1 without glyco-module, with a chimeric glyco-module for generation of
21 allergen carrying O-linked glycans, and Bet v 1 with a glyco-module for generation of allergen carrying N-
22 linked glycans ¹ were designed and transfected in relevant CHO cells after codon optimization for CHO cell
23 expression.

24 *INF- BET V 1 +His*

25 MKYTSYILAFQLCIVLGS LGCDLPGEFGVFNYETETTSVIPAA RLKAFILDGDNLF PKVAPQA ISSVENIEGNGGPGTIKKISFP
26 EGLPFKYVKDRVDEVDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATPDGGSILKISNKYHTKGDHEVKAEQVKASKEMGETL
27 LRAVESYLLAHSDAYNHHHHHHH

28 *INF- BET V 1 + MAA-E1+His (O-glyco-module underlined)*

29 MKYTSYILAFQLCIVLGS LGCDLPGEFGVFNYETETTSVIPAA RLKAFILDGDNLF PKVAPQA ISSVENIEGNGGPGTIKKISFP
30 EGLPFKYVKDRVDEVDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATPDGGSILKISNKYHTKGDHEVKAEQVKASKEMGETL
31 LRAVESYLLAHSDAYNLQAPTRAPSTTPAPSTTPAPAPTRAPSTTPAPSTTPAPAPTRAPSTTPAPSTTPAPAPTRAPSTTPAP
32 STTPAPHHHHHHH-

33 *INF- BET V 1 +N-glycan-module-E1+His (N-glycan module underlined)*

34 MKYTSYILAFQLCIVLGS LGCDLPGEFGVFNYETETTSVIPAA RLKAFILDGDNLF PKVAPQA ISSVENIEGNGGPGTIKKISFP
35 EGLPFKYVKDRVDEVDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATPDGGSILKISNKYHTKGDHEVKAEQVKASKEMGETL
36 LRAVESYLLAHSDAYNLQLEAAAAAANATVAAASGDVWDIHHHHHHH

37 Glyco-engineered CHO-GS cells were maintained as suspension cultures in serum-free media supplemented
38 with 4 mM L-glutamine in 500-ml Corning culture bottle at 130 rpm (Infors, USA) at 36.5°C in a 5% CO₂
39 atmosphere. CHO-GS cells producing only truncated O-glycans (*Cosmc*) ² were transfected with the O-

glycan glyco-module construct to generate GalNAc carrying Bet v 1, as well as one without the glyco-module. Glyco-engineered CHO cells producing only high mannose N-glycans (*Mgat1*)³ were transfected with the N-glycan glyco-module construct to generate high mannose carrying Bet v 1. Cells were co-transfected with GFP plasmid, followed by FACS to enrich clones expressing GFP. Stable transfectants were selected in 0.4 mg/ml Zeocin (Invitrogen). High-expressing clones (>1000ng/ml of secreted allergen in media, measured by Bet v 1 ELISA) were selected for recombinant allergen production followed by nickel affinity purification (Invitrogen, US), and characterized by Bet v 1-specific western blot, Bet v 1-specific ELISA, lectin blots and/or mass spectrometry of released glycans.

Design, expression, purification, and in vitro glycosylation of *E. coli* Bet v 1

Expression constructs containing the entire coding sequence of Bet v 1 were cloned into the expression vector T7/Cascade pMAB36 (Biomedal) and expressed in *E. coli* cells (strain BL21(DE3)4S2, Biomedal) as recombinant protein with a C terminal glyco-module fused to an Intein-LYTAG domain. Following larger scale culture, the produced Bet v 1 was His-purified and analyzed by SDS-PAGE. Part of the Bet v 1 product was in vitro glycosylated using recombinant human GalNAc-T2 as described previously⁴

The construct was designed as depicted below:

MBP-Fx- BET V 1 + MAA-E1+His (O-glycan module underlined)

KIEEGKLVIWINGDKGYNGLAIEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIIFWAHDRFGGYAQSGLLAEITPD
KAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADG
GYAFKYENGKYDIKDVGVNDAGAKAGLTFLVDLIKHKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGV
TVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELVKDPRIAATMENAQKG
EIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSVPIEGRVFNJETETTSVIPAARLFKAFILDGDNLFKVA
PQAISVENIEGNGGPGTIKKISFPEGLPFKYVKDRVDEVDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATPDGGSILKISNKY
HTKGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYNAPTRAPSTTPAPSTTPAPAPTRAPSTTPAPSTTPAPAPTRAPS
TTPAPSTTPAPAPTRAPSTTPAPSTTPAPHHHHHHH

E. coli expressing Bet v 1 was inoculated in 10 ml of growth medium, followed by expansion into 1 L cultures and IPTG induction of Bet v 1 expression. Cells were harvested and disrupted by sonication, followed by purification on an amylose column. *E. coli* media (300 ml) containing Bet v 1 was dialyzed against 5 L 50 mM Tris (pH 8) containing 150 mM NaCl, followed by ni-NTA affinity purification and analysis by SDS-PAGE.

E. coli produced Bet v 1 with the glyco-module were subjected to buffer exchange (from PBS to 25 mM Tris-HLC pH 8, 100 mM NaCl) using PD-10 columns (GE Healthcare). Bet v 1 was glycosylated in vitro using recombinant human glycosyltransferase GalNAc-T2 in reaction mixtures containing 25 mM sodium cacodylate buffer (pH 7.4), 10 mM MnCl₂, 0.25% Triton X-100, and 2 mM UDP-GalNAc at 37°C overnight. Samples from enzyme reactions were analyzed by MALDI-TOF MS on a Voyager-DE™ Pro Biospectrometry Workstation (Applied Biosystems, Stafford, TX) as described below. *E. coli* produced Bet v 1 with the glyco-module was subsequently purified by nickel affinity purification.

Ni-NTA affinity column

Columns were prepared by adding 0.6 ml of 50% Ni-NTA agarose slurry to an Econo-Column 10 mm in length with an inner diameter of 1 cm and column volume (CV) of 2.5 ml. The column was washed with 10 CVs of Milli-Q water before equilibration with 10 CVs of 1X equilibration buffer, 25 mM Tris pH 8.0, 300 mM NaCl.

The media collected from Bet v 1-producing cell lines was centrifuged at 4000 rpm for 2 min and then passed over Whatman filter paper (or 0.45 μ m filter membrane), followed by 1:4 dilution using 4X equilibration buffer (100 mM Tris pH 8.0, 1.2 M NaCl). The sample was loaded on the column using gravity flow ON, followed by 25 CVs of 25 mM Tris (pH 7.5) containing 300 mM NaCl and 10 mM imidazole (wash) and eluted with 3 ml of 25 mM Tris (pH 7.5) containing 300 mM NaCl and 250 mM imidazole. The eluted sample was collected in 1 ml fractions. Imidazole was removed by buffer exchange to PBS on a PD10 column (GE Healthcare) and the final protein concentration measured by BCA assay (ThermoFisher Scientific). Purification of the product was validated by SDS-PAGE and Western blot. Samples were stored at 0-4°C until use.

Design, expression, purification of Bet v 1 in *P. pastoris*

Translated coding amino acid sequences for Bet v 1 with and without the O-glycan glyco-module were identical to their counter parts expressed in CHO cells, with the glyco-module in *P.pastoris* serving to attract O-mannosylation. The pUC57 Bet v 1 construct with O-glycan glyco-module was codon optimized for *P.pastoris* and purchased from Genscript. Bet v 1 without glyco-module was generated from pUC57-Bet v 1 with glyco-module by initial removal of the glyco-module using NotI-HF and PstI-HF (NEB).The primer set GCACCACCACCACCACCACCTAGGC and GGCCGCTAGTGGTGGTGGTGGTGGTGGTGCTGCA was annealed and ligated in to restore the His7 tag and stop codon. Bet v 1 construct with and without the glyco-module were cloned in fusion to the alpha mating factor of pPicZαA (Invitrogen) using EcoRI and NotI restriction sites for secretion of the mature (glyco)proteins via the secretory pathway. Linearized pPicZalfaA-Bet v 1 with and without glyco-module were transformed into the *P. pastoris* KM71H strain by electroporation and plated on yeast extract peptone dextrose sorbitol (YPDS) plates containing Zeocin (100 µg/ml for pPicZalfaA-Bet v 1+glyco or 1000 µg/ml for pPicZalfaA-Bet v 1) to score multi copy high-expressers. The highest expressing transformants were scored by Western blot analysis using rabbit anti-Bet v 1 (0909 Mix of 4 Abs [1:50000]) and swine anti-rabbit polyclonal (Dako P 0217) as described previously⁵, except expression was scored after 2 days of incubation at 28°C and methanol was added to a final concentration of 1% (v/v) after 24 h.

For production of Bet v 1 in *P. pastoris* with and without glyco-module, 250 ml of buffered complex glycerol medium (BMGY) supplemented with 100 µg/ml Zeocin was inoculated with the highest expresser and grown at 28°C (280–300 rpm, ON) until an OD₆₀₀ of 2-6. Cells were pelleted by centrifugation and re-suspended in 1000 ml complex buffered methanol medium (BMMY) to induce expression and incubated for 48 h at 26°C (280–300 rpm) with the addition of methanol to a final concentration of 1% (v/v) after 24 h. Cells were pelleted by centrifugation and the supernatant (medium fraction) collected for purification.

Allergen-containing media fractions (1 L) were diluted in 4 L of binding buffer (50 mM NaHPO₄, 300 mM NaCl) and the pH adjusted to 8. A 5-ml HisTrap™ Excel column (GE Healthcare) was equilibrated with 5 CVs of binding buffer (50 mM NaHPO₄, 300 mM NaCl, pH 8). The diluted media was applied to the column overnight (flow rate 5 ml/min) using an Äkta FPLC (Amersham Pharmacia Biotech) system, washed with 10 CVs of wash buffer (50 mM NaHPO₄, 10 mM imidazole, 300 mM NaCl, pH 8), and bound protein eluted in four fractions of 5 ml elution buffer (50 mM NaHPO₄, 500 mM imidazole, 300 mM NaCl, pH 8) with a flow rate of 1 ml/min. Eluted fractions were analyzed for protein by UV absorbance (Äkta FPLC Monitor UV900) and SDS-PAGE.

Bet v 1+glyco-module containing elution fractions were subjected to a second His-tag purification. Protein-containing fractions were pooled and dialyzed against binding buffer (50 ml fractions in 5 L of binding buffer) for 24 h twice. Roche Complete HisPur resin (1 ml) was equilibrated in binding buffer and added to

1 the pooled dialyzed fractions, incubated at 4°C for 4 h under mild agitation, and the beads collected and
2 applied to a homemade column. The column was washed with 50 CVs wash buffer and protein eluted with
3 elution buffer in 1 ml fractions. For high-expressing Bet v 1 without glyco-module, 1 ml of Roche Complete
4 HisPur resin was applied to a homemade column and equilibrated with 5 CVs of binding buffer. Bet v 1-
5 containing fractions were applied and allowed to run through by gravity flow. The column was washed with
6 50 CVs of wash buffer and protein eluted with elution buffer in 1 ml fractions. Additionally, resulting
7 glycosylated products were subjected to profiling by Western blot, Bet v 1 ELISA, MALDI-TOF, and lectin
8 blots.

9 Bet v extract

10 The extracts are prepared from 5 g defatted pollen, extracted in a ratio 1:5, 2 hours, +5°C in 125mM
11 NH₄HCO₃, followed by centrifugation and dialysis at MWCo 3.5kD followed by 0.2µm filtration and freeze
12 drying in bulk.

13 **Characterization and modification of Bet v 1 products**

14 N-glycan and O-glycan profiling of purified Bet v 1

15 Approximately 40 µg of purified Bet v 1 was digested with trypsin in 50 mM ammonium bicarbonate
16 overnight at 37°C, heated at 95°C for 15 min, and cooled to room temperature before release of N-glycans
17 by 2 U PNGase F (Roche Diagnostics, Mannheim, Germany) in 50 mM ammonium bicarbonate at 37°C
18 overnight. Released N-glycans were separated from tryptic digest using in-house packed Stagetips (Empore
19 disk-C18, 3M) and incubated in 100 mM ammonium acetate (pH 5.0) for 60 min at 22°C. O-glycans were
20 chemically released by reductive β-elimination. Released glycans were permethylated using the sodium
21 hydroxide protocol and purified on a Sep-Pak C18 cartridge (Waters). Glycans were permethylated
22 according to standard procedures, extracted with chloroform, and desalted by washing the organic phase
23 repeatedly with deionized water. The organic phase was evaporated over a stream of nitrogen gas and the
24 permethylated glycans dissolved in 20 µl of 50% (v/v) methanol. Finally, 1 µl of sample was co-crystallized
25 with 1 µl of matrix composed of 2,5-dihydroxybenzoic acid (10 mg/ml) in 70% (v/v) acetonitrile, 0.1% (v/v)
26 trifluoroacetic acid, and 2.5 mM sodium acetate. Permethylated glycans were analyzed by positive mode
27 MALDI-TOF (Autoflex Speed, Bruker Daltonics, Bremen) operated in the reflector mode with data
28 acquisition (2,000 shots/spot) in the m/z 900-4500 Da mass range and O-glycans from 700-3500 Da mass
29 range. Spectra were analyzed using Flex analysis software (version 3.4, Bruker Daltonics) and peaks
30 annotated with suggested glycan structures using GlycoWorkBench 2.0

31 Bet v 1 ELISA

32 Bet v 1 ELISA was performed as described previously ⁶.

33 Lectin ELISA

34 Bet v 1-specific polyclonal rabbit IgG (ALK, Hørsholm, Denmark) was diluted 1:10,000 and coated onto the
35 ELISA microplate (Immuno MaxiSorp F96 plates [Nunc]) for 16 h at 4°C. Total proteins (Bet v 1 or
36 Asialofetuin, 10 µg) were added to the plates, washed with PBS containing 0.05% Tween 20, and treated
37 with PVP blocking solution (50 mM Tris HCl pH 8.0, 1% polyvinylpyrroliden, 150 mM NaCl) for 2 h at 4°C.
38 Each well was washed three times, followed by the addition of 10 µg/ml biotinylated VVA or Con A (Vector
39 Labs) for 16 h at 4°C. Streptavidin-HRP (Dako, Denmark) was used for detection (4 h at room temperature)
40 followed by addition of the TMB+ one-step substrate system (Dako, Denmark). Reactions were stopped with

1 0.5 M H₂SO₄ and read at 450 nm. Data were analyzed and expressed as fold increase compared to
2 background noise (asialofetuin).

3 SDS-PAGE and Western blots

4 Proteins were separated by SDS-PAGE under reducing conditions in precast 4–20% gradient gels (Bio-Rad)
5 and electrotransferred to PVDF membranes (Millipore). For SDS-PAGE gels were comassie stained following
6 standard procedure. For the Western-blots membranes were treated with PVP blocking solution (50 mM
7 Tris HCl pH 8.0, 1% polyvinylpyrroliden, 150 mM NaCl) for 30 minutes at room temperature before being
8 subjected to 10 µg/ml biotinylated VVA or Con A (Vector Labs) or anti Bet v 1 Ab (ALK-Abelló, Hørsholm,
9 Denmark) in blocking buffer supplemented with 0.05% Tween 20, 1 mM MgCl₂/CaCl₂/MnCl₂/ZnCl₂ for 16 h
10 at 4°C. Detection was achieved by adding Streptavidin-HRP (Dako, Denmark) or anti-IgG-HRP (Dako,
11 Denmark) for 30 minutes at room temperature followed by ECL chemiluminescence reagent (Amersham).

12 Quantification and modification of Bet v 1

13 All protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific)
14 according to the manufacturer's instructions.
15 When indicated, Bet v 1 was biotinylated using the EZ-link sulfo-NHS-SS- Biotinylation kit (Pierce, Rockford,
16 IL, USA) according to the manufacturer's instructions.
17 When indicated, periodate treatment was used to disrupt the glycan: 10mM sodium metaperiodate was
18 added to 500 µg of Bet v 1 in oxidation buffer (0.1M sodium acetate, pH 5.5) for 30 minutes at room
19 temperature. The sodium metaperiodate was removed using PD-10 columns. Glycan oxidation was verified
20 using lectin blots and lectin ELISA. For in vivo tracking 500 µg of each allergen construct was conjugated
21 using the VivoTag 680XL Protein Labeling Kit (PerkinElmer) according to the manufacturer's instructions.
22 For live imaging in vitro Bet v 1 was labeled with NHS-fluorescein (Thermo Scientific). NHS-fluorescein
23 dissolved in DMSO was added in a 10-fold molar excess to a 2 mg/ml Bet v 1 solution in coupling buffer (20
24 mM HEPES). The solution was kept in the dark and incubated at room temperature with continuous mixing
25 for 1 h. Labeled Bet v 1 were separated from unreacted dye by buffer exchange to PBS on a PD10 column
26 (GE Healthcare). The degree of labeling was calculated according to the manufacturer's instructions.

27

28 **Immunohistochemistry**

29 Human skin biopsies were fixed in 4% Formalin, and cut in 5mm sections. Paraffin-embedded, formalin
30 fixed sections off human skin were used for CLRs immunostaining. Antigen retrieval was performed by
31 microwaving slides submerged in citrate buffer (pH 6), followed by permeabilization using 0.3% Triton X-
32 100 (Sigma-Aldrich) before staining sections for DC-SIGN (BD Pharmingen Clone: DCN46), MR (BD
33 Pharmingen, clone 19.2) and MGL (R&D Systems, clone NS0). Slides were incubated overnight in moist
34 chambers and labeled with Alexa Fluor 548-conjugated anti-mouse or anti-goat IgG, alternatively the
35 paraffin embedded, formalin fixed sections were deparaffinized and stained with hematoxyline/eosine. For
36 *in situ* Bet v 1 injections 500 ng/ml of the indicated Bet v 1 product was injected intra dermally. After 24 h,
37 skin samples were fixed in 10% formalin, paraffin embedded, and sectioned for immunostaining.

38

39 **Transcriptome**

40 Primary derived moDCs day 3 were pulsed with 1µg/ml Bet v 1 fusion proteins or LPS for 24 h. After
41 stimulation, cells were thoroughly washed, followed by total RNA isolation using the RNeasy Plus Universal
42 Kit (Qiagen) according to the manufacturer's instructions. RNA sample integrity was measured by Nanodrop

1 spectrophotometry and an Agilent Bioanalyzer. Target RNA was fragmented and converted to (reverse
2 transcription) double-stranded cDNA (dscDNA) by N6 random primers, followed by end repair. Two specific
3 primers were used to amplify the ligation product. Finally, the PCR product was denatured and the single-
4 stranded DNA cyclized by splint oligo and DNA ligase. RNA sequencing was performed on a desktop
5 Sequencer BGISEQ-500 (BGI). Prior to analysis, the quality of the sample was assessed using a bioanalyzer.
6 Primary sequencing data were subjected to quality control to determine if a resequencing step is needed.
7 After quality control, raw reads were filtered into clean reads (remove reads with adaptors, reads in which
8 unknown bases are more than 10%, and low quality reads), which were aligned to the reference sequences.
9 Gene expression was quantified using the RSEM software package. Differentially expressed genes were
10 identified using a strict algorithm developed by BGI. Corrections were performed for false positive (type I
11 errors) and false negative (type II) errors using the FDR method. FPKM ≥ 10 was selected as the threshold.
12 Visualisation and statistical analysis was performed using Qlucore Omics Explorer.

13 ***Preparation and culture of APCs and T cells***

14 *Human moDC, M1, and M2 preparation and culture*

15 Monocytes were generated from human peripheral blood mononuclear cells (PBMCs) isolated from blood
16 donor buffy coats. For all studies, the monocytes were purified within 24h of the blood being drawn. The
17 buffy coat was separated on Lymphoprep (Fresenius Kabi, Norway) and the PBMC layer salvaged and
18 washed in PBS before being plated at 1×10^7 cells/well in 6 well plates (Falcon) in RPMI 1640 containing
19 Glutamax and 15% inactivated human AB serum (Valley Biomedical). After 1.4h of adhesion at 37°C in a
20 humidified incubator, the cells were shaken to release unbound (non-monocyte) cells and the wells washed
21 3-4 times in RPMI 1640. Finally, RPMI 1640 with Glutamax and 5% inactivated human AB serum was added.
22 Cytokines were added for individual APC-differentiation (moDC: 50ng/ml human recombinant GM-CSF and
23 25 ng/ml human recombinant IL-4, M1: 100 ng/ml human recombinant GM-CSF, M2: 100 ng/ml M-CSF,
24 [Peprotech]). Cells were cultured between 0 and 7 days as indicated, and the media refreshed on day 3 if
25 relevant.

26 *Human pDC-isolation and stimulation experiments*

27 Human plasmacytoid DCs (pDCs) were purified from PBMCs by Plasmacytoid Dendritic Cell Isolation Kit II
28 from Miltenyi Biotech (Cat. num. 130-097-415) following the manufacturer recommendations. The cells
29 were immediately after isolation setup in RPMI 1640 medium with 5% FBS and stimulated with 1 μ M of
30 indicated biotinylated allergens and incubated at 37°C for 45 min. Cells were then analyzed by intracellular
31 flow-cytometry as for the moDCs. Additionally, pDCs were incubated with allergens for 24H at which point
32 the supernatants were salvaged and an MSD analysis of cytokine section was performed.

33 *Patient-derived moDC preparation and culture*

34 PBMCs were isolated from heparinized blood by density centrifugation on Lymphoprep solution (AXIS-
35 SHIELD PoC AS). CD14⁺ cells were isolated using anti-CD14 MACS beads (Miltenyi Biotech) according to the
36 manufacturer's protocol. The isolated cells were cultured with GM-CSF (50ng/ml) and IL-4 (25ng/ml)
37 (Peprotech) for 3 or 6 days to induce the generation of moDCs.

38 *BM-DC preparation and culture*

39 Bone marrow was flushed from the femurs and tibias of female Balb/c mice (6-8 weeks old). Cells were
40 passed through a 70- μ m filter, washed, set-up in 6 well plates (2×10^6 cells/ml) and supplemented with 20
41 ng/ml recombinant murine (rm)-GM-CSF (Peprotech). When not used in experiments medium and rmGM-

1 CSF were refreshed on day 3. At day 6 non- and loosely-adherent cells were collected, washed and cultured
2 again in 6 well plates (1×10^6 cells/ml) with 20 ng/ml mrGM-CSF.

3 *T cell line generation and T-cell/DC co-culture measurements*

4 Bet v 1-specific T cell lines from birch-allergic donors were generated by repeated (2x) stimulation of
5 PBMCs with Bet v 1 as previously described⁷. After a third round of non-specific stimulation in the presence
6 of PHA, IL-2, and irradiated allogeneic PBMCs, the T cells were co-cultured with day 3 autologous moDCs
7 (Ratio: 10:1) pulsed for 2 h with different doses of the respective proteins/allergens (50, 5, and 0.5nM).
8 After 48 h, supernatants were collected and 3H-thymidine added to the cells to measure proliferation 20 h
9 later.

10 Cytokines (IFN- γ , TNF α , IL-10, IL-12p70, GM-CSF, IL-13, IL-17A, IL-18, IL-1 β , IL-2, IL-21, IL-22, IL-23, IL-27, IL-
11 4, IL-5, IL-6, and IL-9) were measured in the T cell line supernatants by using a ProcartaPlex h
12 Th1/Th2/Th9/Th17/Th22/Treg 18-plex bead assay kit (eBioscience, Austria) according to the manufacturer's
13 instructions. Normalized cytokine secretion is calculated by setting medium background to 0%, highest
14 cytokine secretion level to 100% for each donor, additional data points normalized according to these
15 values. This was done to accommodate for donor variations.

16 **Flow cytometry**

17 Intracellular flow cytometry was performed using the Cytofix/Cytoperm kit (BD Bioscience 554715)
18 following the manufacturer's recommendations. Briefly, the cells were detached by scraping, washed twice
19 in PBS, fixed in the Cytofix/Cytoperm solution, and washed in the permeabilisation wash buffer. The uptake
20 of allergen constructs was monitored by Streptavidin-AF488 (Invitrogen). Human APCs were characterized
21 in a standard surface staining assay for CLRs and CD marker expression using anti-MGL-AF647 (DENDRITICS,
22 ref DDX0010A647-100), anti-DC-SIGN/CD209 (BD Pharmingen, ref 551249), anti-MR (BD Pharmingen ref
23 555953)), anti CD11c-PE (BD pharmingen ref 555392), anti CD14 FITC/PE (Dako clone T $\ddot{U}$ K4)) anti-HLA-
24 DR/DP/DQ PE (Dako clone CR3/43)), anti-CD163-APC (Biolegend ref 333609), and secondary Abs/isotype
25 controls (goat anti-mouse IgG AF488 (ref A11029), AF594 (A11032), and AF546 (A-21045) [all Invitrogen],
26 mouse IgG-FITC, PE, APC, AF488 isotype controls [BD]). Secondary background staining or isotype controls
27 were determined for all individual samples. Samples were run on a BD FACS Calibur and analyzed by Cell
28 Quest Pro software. BM-DCs was assessed by flow cytometry using the following antibodies: CD11c-FITC
29 (eBioscience), CD206-PE (BioRad), CD209-AF647 (BioRad), CD301b-PerCP/Cy5.5 (BioLegend), F4/80-APC
30 (eBioscience), MHCII-e650 (eBioscience), and CD14-PE (BD). The cells were washed in PBS 1% BSA, stained
31 with the antibodies for 20 min on ice, washed twice in PBS 1% BSA and analyzed using BD FACSFortessa.

32 **Cytokine qPCR**

33 Total cellular RNA was collected from differentiated DCs, M1-, and M2-macrophages using a RNeasy Kit
34 (#74106, Qiagen) according to the manufacturer's recommendations. RNA was treated with DNase I
35 (#EN0525, ThermoFisher Scientific) in DNase I buffer (#B43, ThermoFisher Scientific) to remove genomic
36 DNA. RNA purity and concentration was determined using a NanoDrop ND-1000. To obtain cDNA, 1 μ g total
37 RNA was reverse transcribed using the High Capacity cDNA Reverse Transcriptase Kit (#4374966,
38 ThermoFisher Scientific) according to the manufacturer's recommendations and run on The Veriti™ Dx
39 Thermal Cycler, ThermoFisher Scientific. qPCR was performed on the StepOnePlus real time PCR system,
40 ThermoFisher Scientific using the HOT FIREPol® Probe qPCR Mix Plus (ROX). Pre-designed fluorescent
41 primer/probe mixtures were used to quantify; IL-1 β (Hs00174097_m1), TNF- α (Hs01113624_g1), IL-6

(Hs00985639_m1), IL-12p40 (Hs01011518_m1), IL-10 (Hs00961622_m1), IFN- γ (Hs00989291_m1), TGF- β 1 (Hs00998133_m1) and GAPDH (Hs02758991_g1) (all ThermoFisher). RNA expression levels were normalized to the GAPDH reference gene.

MSD analyses

V-PLEX Proinflammatory Panel 1 Human Kit (sub-selected IL-10, IL-6, IL-1 β , IL-12p70 and TNF- α) was used to analyse for cytokine production from pDC cultures, following the manufacturer recommendations. The plate was read on a MESO QuickPlex SQ 120 and analyzed by MSD discovery Workbench 4.0

Uptake and intracellular localization of allergens

For uptake experiments, human moDCs on day 3 of development were detached by scraping, washed in PBS and co-cultured for 5 min-24 h as indicated with 1 μ g/ml of biotinylated full-length allergen construct protein (with or without glycan) in PBS with Mg⁺⁺/Ca⁺⁺ and 0.5% BSA. The cells were washed twice in PBS and stained with streptavidin-AF488 as described above for flow cytometry. For intracellular localization experiments, moDCs were additionally stained for intracellular markers clathrin, caveolin, Lamp-2 (Abcam, ref ab13524), EEA-1, Rab 5 and Rab 7 (All except Lamp-2 from Endosomal Sample Kit #12666, Cell Signaling)). Relevant secondary abs used for detection: goat anti-mouse IgG AF488, AF594 and AF546 (as for flow cytometry) goat anti-rabbit IgG AF546 (Invitrogen, A10040). After staining, the cells were mounted with pro long gold antifade media with DAPI (Dako) and analyzed using a Zeiss LSM 780 confocal microscope. Live imaging was performed using the Zeiss LSM 780 confocal microscope using directly NHS-fluorescein-conjugated allergens and Lyso-Tracker (LysoTracker[®] Red DND-99, ThermoFisher Scientific). Day 6 moDCs were used, and 24 h prior to imaging the cells were transferred to 40 mm glass cover slips to allow them to attach and adjust before the experiment was performed. Human skin APCs were obtained by slicing the dermis and epidermis into 0.5-1 mm split-skin slices using a dermatome knife. The skin slices were washed in PBS and cultured in RPMI 1640 with 15% inactivated human serum (Sigma), 1% Pen.-Strep, fungizone, and gentamicin as well as 40 ng/ml human recombinant GM-CSF (Peprotech) for 48 h, allowing them to migrate out of the skin. The skin slices were removed and the resulting cells washed and stained for intracellular flow cytometry as described above. Day 3 and day 6 BM-DCs were incubated with different glycosylated (Tn, mannose, GlcNAc and LeX) and biotinylated PAA constructs (10 μ g/ml, GlycoTech Corporation) for 45 min at 37°C or on ice. Afterwards, the cells were stained with CD11c-APC (eBioscience) and streptavidin-AF488 (ThermoFisher) and the uptake analyzed by flow cytometry.

In vivo targeting to draining lymph nodes

NMRI nude mice (Taconic) were housed in individually ventilated cages at University of Copenhagen and fed a diet of alfalfa free pellets (D10001i – Brogaarden, AIN-76A: widely used, balanced, purified rodent diet) to reduce auto fluorescence in the far-red spectrum from the digestive tract during fluorescent imaging. A dilution series (20 μ g-0.2 μ g) of VivoTag680-labelled Bet v 1 fusion proteins was imaged to assess labelling and correct for the difference in signal intensity between the individual proteins. VivoTag680 was detected by IVIS Spectrum CT (Perkin Elmer) fluorescence imaging with spectral unmixing, filters 605/660, 605/680, 605/700, 605/720, 675/720, layout (FOV C), photograph binning “small”, acquisition time “auto”. Animals were anaesthetized with 2.5% isoflurane gas and injected with 20 μ g of either non-modified or glyco-modified Bet v 1 labelled with VivoTag 680 in the left foot pad. Control animals were injected with PBS. 24 h post-injection, the animals were euthanized by intracardial injection of pentobarbital and opened along the midline, dissected by peeling the skin away, revealing draining popliteal lymph nodes for imaging as described above. After *in situ* imaging, lymph nodes were resected and placed in a 24-well plate for *in*

1 *vitro* imaging of the draining lymph nodes. Total radiant efficiency ([p/s] / [μ W/cm²]) was measured by
2 gating on foot pad, popliteal and inguinal lymph nodes. Signals were corrected for non-specific background
3 from surrounding tissue by subtracting the signal from the opposite side and normalized in accordance with
4 in vitro titration of VivoTag680 labelled Bet v 1.

5 ***In vivo model of type-1 allergy***

6 *Animals*

7 Female BALB/c mice (8-10 weeks old, Taconic or own breeding) were housed under pathogen-free
8 conditions. Water and diet were supplied ad libitum.

9 *Sensitization and treatment of mice with Bet v 1*

10 Animals (n=7 per treatment group) were sensitized via two intraperitoneal injections of 10 μ g Bet v 1 (CHO)
11 emulsified in Al(OH)₃ in a total volume of 250 μ l on days 0 and 7 of the experimental protocol. In order to
12 treat the mice with the different glyco-allergens, 10 μ g was administered intra dermally on days -14, -11
13 and -8 of the protocol. The sensitization status of the mice was assessed 10 days after the last
14 intraperitoneal injection.

15 *Isolation and in vitro stimulation of splenic T cells*

16 A single cell suspension of spleen cells was prepared by meshing the spleens through a 70 μ m filter. The
17 splenocytes (3x10⁶/ml) were cultured in the presence of Bet v extract or Bet v 1 (CHO) (30 or 10 μ g/ml). On
18 day 4 3H-thymidine was added. Proliferation was measured 18 h later by scintillation counting.

19 *Analysis of Bet v 1-specific antibodies*

20 Bet v 1-specific antibodies were measured in serum by using the MagPix platform and the mouse isotyping
21 7plex kit from eBioscience. In order to measure antigen-specific antibodies the kit's detection antibody was
22 exchanged for DyLight550-labelled Bet v 1 (E.coli) (5 μ g/mL).

23 ***Statistical analysis***

24 When nothing else is noted in figure legends all bar plots depict mean +/- standard deviation, all box and
25 whisker plots have mix/min whiskers. In all scatter dot plots the line depicts the mean. Since all samples are
26 of biological origin and all repeated experiments are performed on different donors on different days the
27 observations are considered normally distributed and independent. Therefore a one-way ANOVA followed
28 by a Turkey multiple comparison test (95% confidence interval) has been applied to the in vitro uptake data
29 (Figure 1B). Variance was compared by applying an F-test to the T cell data followed by a parametric two-
30 tailed unpaired t-test (Figure 2A). For the statistical analysis of the in vivo data (Figure 2C) a one-way
31 ANOVA was performed followed by a Turkey multiple comparison test on all relevant Bet v 1 containing re-
32 stimulations as well as negative PBS control. For the bio-distribution data we used a one-way ANOVA
33 followed by individual two-tailed unpaired t-test (95% confidence interval) to assess differences between
34 the in situ and in vitro lymph node fluorescence signal. For all figures confidence level $P \leq 0.05$ (*),
35 $P \leq 0.01$ (**), $P \leq 0.001$ (***) $P \leq 0.0001$ (****) applies.

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Supplementary figure legends Mathiesen et al.

Figure E1. Characterization of human M1s, M2s, and moDCs. (A) Schematic overview of important CLRs on APCs and their ligands. (B) H&E stain of human skin in reference to human skin sections labeled with CLR antibodies (red) and counterstained with DAPI (blue). The epidermal/dermal interface is depicted with dotted lines. (C) Schematic overview of the generation of in vitro APCs. (D) Transcriptional analysis of the expression levels of relevant lineage markers and CLRs in M1, M2, and moDC cells on day 6 of differentiation. (E) Expression of lineage CD molecules and CLRs in M1, M2, and moDC cells on day 3 (left) and day 6 (right) of development. A representative histogram is shown for each marker (black), including negative control (light gray). Bar charts (left of histograms) show the mean fluorescence intensity (MFI) $\pm$ SD for all individual donors (n=3-4). M1 (black), M2 (dark gray), and moDCs (light gray). (F) The mean cytokine expression in resting APCs, as measured by qPCR (mean Ct value $\pm$ SEM). Ct values expressed relative to GAPDH.

Figure E2. Screening of human APC uptake of glycan products and validation of Bet v 1 production. (A) Uptake of PAA-sugars in human M1 (black), M2 (dark gray), and moDC (light gray) cells as measure by flow cytometry. The uptake is depicted as the mean fluorescence intensity (MFI) $\pm$ SD for all donors (n=2-4). (B) Flow cytometric histogram overlays of the uptake of in vitro glycosylated *E. coli*-produced Bet v 1 (GalNAc-Bet v 1 (*E. coli*)). Streptavidin alone (filled gray), Bet v 1 1-3 GalNAc (thin black line), Bet v 1 2-5 GalNAc (gray line and overlay in white on top of gray filled plot), Bet v 1 18-20 GalNAc (thick black line). Approximately 5000 events analysed for all samples. (C) Schematic illustration of the production of the allergen-glyco-module fusion products. (D) MALDI-TOF spectra of glycans released from the indicated Bet v 1 fusion proteins. (E) Bet v 1 capture ELISA of the indicated Bet v 1 products, followed by GalNAc detection using VVA lectin. Data were analyzed and expressed as fold increase compared to background noise (asialofetuin). (F) SDS-PAGE of indicated Bet v 1 products. Molecular weight estimates are indicated to the left. (G) VVA and ConA lectin blots of the indicated Bet v 1 products. Molecular weight estimates are indicated to the left.

Figure E3. Ex vivo uptake and intracellular routing of glycosylated allergens. (A) Confocal imaging of fixed moDCs after 7.5 min (caveolin, clathrin), 25 min (EEA-1 and Rab 5) and 2H (Rab 7 and Lamp-2) uptake of the indicated Bet v 1 fusion proteins (green) and relevant compartment label (purple) to detect uptake and intracellular routing. Co-localization appears in white, as indicated by arrows. Caveolin, Rab-7 and Lamp-2 staining omitted, as there were no co-localization. Green and red lines depict the cross section points where the whole depth of the Z-stack is shown. The large clumps of clustered green light are due to an artifact of the streptavidin staining. Representative images from one donor (n=3). (B) Selected time points from the live imaging video (Video E1) of moDCs after pulsing with NHS-fluorescein-Man-Bet v 1 (*Pichia*) (green), LysoTracker (red), and transmitted light by confocal microscopy. Arrows indicate areas with high allergen uptake. (C) Flow cytometric dot plots (logarithmic scales) depicting allergen uptake in isolated CD11c-positive primary skin cells.

Figure E4 Uptake in ex vivo human pDCs. %pDCs taking up indicated Bet v 1 allergen quantified by flow cytometry. Strep: streptavidin background control. n=2 donors, mean $\pm$ SD depicted.

Figure E5. Characterization of and PAA-sugar uptake in murine BM-DCs. (A) Characterization of CD and CLR panel expression in murine BM-DCs on day 3 and 7 by flow cytometry. Isotype control (gray filled), CD11c positive (black), relevant CD/CLR molecule expression in the CD11c-positive population (dotted black line). (B) Uptake of PAA constructs in BM-DCs on day 3 and 7. Histograms are representative of the pooled

data in the lower panel (n=4). Gray histograms represent background control uptake at 4°C, whereas black lines are specific uptake of PAA constructs at 37°C as depicted by streptavidin stain. The pooled uptake depicts MFI ±SD.

Figure E6. Targeting CLR with GalNAc and mannose Bet v 1 does not significantly change the expression profile in DCs. Transcriptomic analysis of human moDCs pulsed with the indicated allergen or LPS for 24 h. **(A)** Heatmap of genes with an FPKM ≥ 10. The gradient color indicates the minimum value in blue and the maximum in red. **(B)** Principle component analysis result for indicated components.

Figure E7. Cytokine secretion by pDCs. Cytokine production in pg/ml by pDC cultures stimulated with the indicated allergens for 24 h as analyzed by MSD assay. White bars depict data from donor 1 (approx. 98% pDC purity) and gray bars depict data from donor 2 (approx. 85% pDC purity, the last 15% primarily in lymphocyte gate).

Figure E8. Proliferative T cell response from individual allergic patients. T cell proliferation response of all allergic patient donors after 48 h of in vitro re-stimulation with autologous moDCs (day 3) pulsed with CHO **(A)**, *E. coli* **(B)**, or *Pichia* **(C)** produced Bet v 1 fusion proteins, depicted as counts per minute (cpm) after 3 h ³H-thymidine in cooperation as in Figure 2. **(D)** T cell proliferation in all donors (n=6-7) depicted as the stimulation index (cpm value after stimulation divided by cpm value of medium control) to take donor-donor variation into account. The donor panel for GalNAc-Bet v 1 (*E. coli*) is different from the others; therefore, a direct comparison between this dataset and the other glyco-forms of Bet v 1 is not possible.

Figure E9. Cytokine production by T cells from allergic donors. Cytokines from T-cell-DC co-cultures measured by an eBioscience 18-plex bead assay as in Figure 2. IL-17A, IL-1β and IL-23 were equal to or below detection limit and has therefore been omitted from the figure. n.d. (not determined).

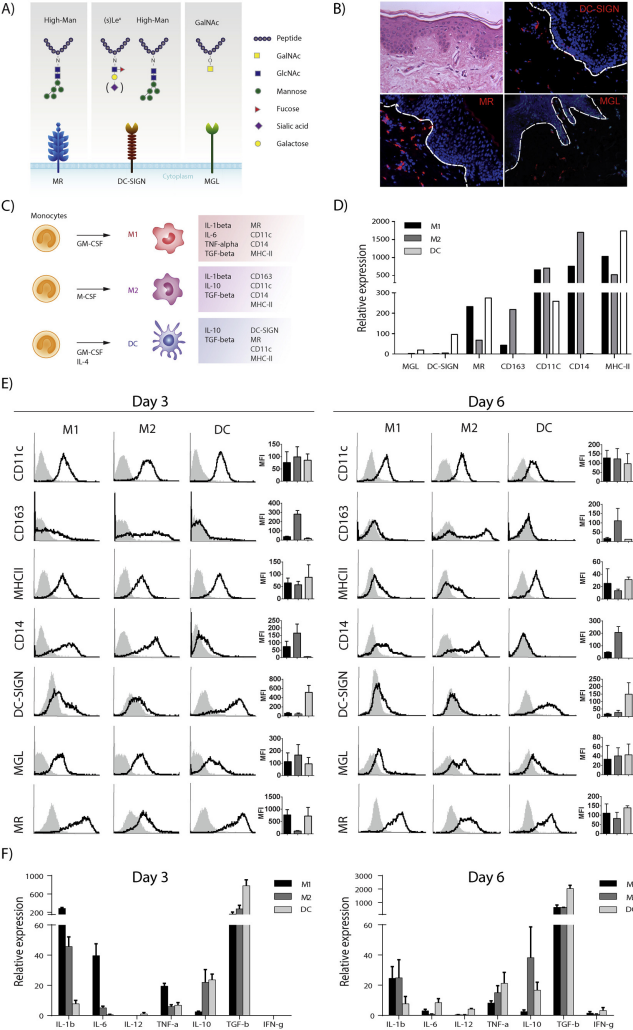
Figure E10. Cytokine concentrations from representative donor. Selected representative donor for each analyzed cytokine from Figure 2 and Figure E9 to appreciate actual pg/ml concentrations of cytokine produced in the DC-T cell co-cultures. n.d. (Not determined).

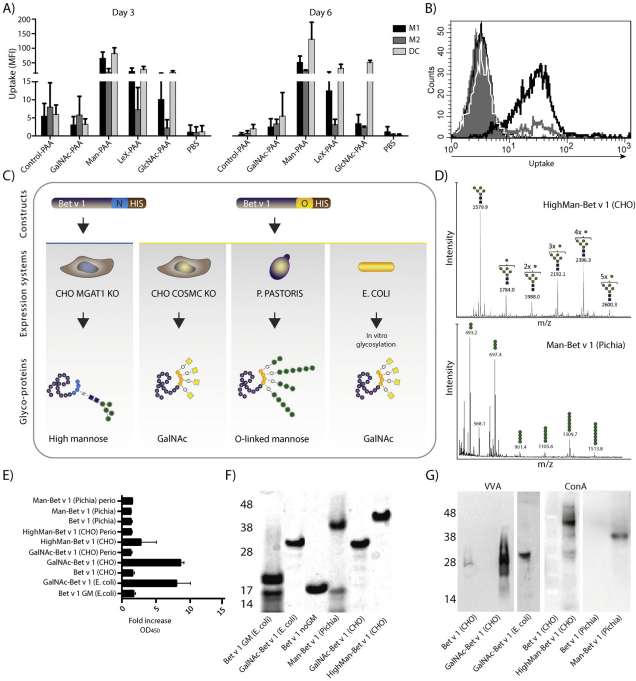
Figure E11. T cell proliferation (30 μg/ml re-stimulation). **(A)** Spleen cell proliferation (as in Figure 2C) after in vitro re-stimulation of prophylactic-treated mice in the animal model of type 1 allergy (treatment-product indicated on X-axis). Proliferation is measured by 18 h ³H-thymidine incorporation after 4 days of in vitro expansion with Bet v extract or Bet v 1 (CHO) (30 μg/ml). **(B)** Bet v 1-specific antibody production in serum from the same mice as in A and Figure 2C, depicted as fluorescence intensity (FI) as measured by the MagPix platform 7-plex kit. Mean including 95% CI interval is indicated for clarity. IgE level is altered by prophylactic treatment compared to control IgG2a, that remains unaffected, demonstrating specific effect.

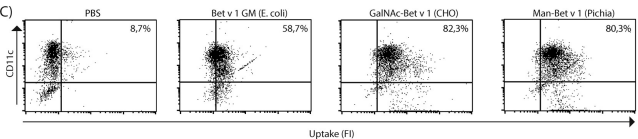
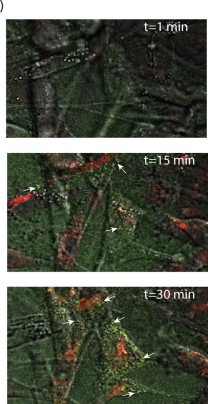
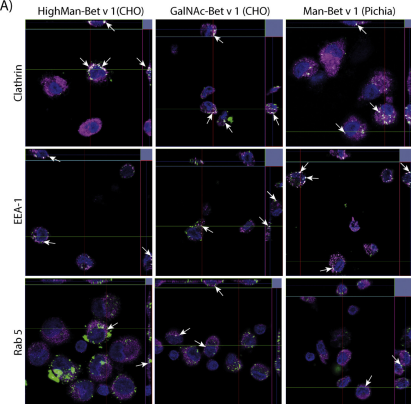
Video E1. Live uptake of Man-Bet v 1 (Pichia). Time-lapse video of confocal scans on 3 channels: red/orange Lyso-Tracker, green: Man-Bet v 1 (*Pichia*) and transmitted light to see individual cells. Site scanned with a 20 second interval (approx. 150 frames in 45 min). Depicted as 3 frames/sec. Adjustment of focus done after approximately 15 min and 30 min to compensate for focus drift during long term confocal scanning. After approximately 5-6 min uptake is visible as green dots accumulating within the cells, and after approximately 30-35 min co-localization (in yellow) with the Lyso-Tracker is visible.

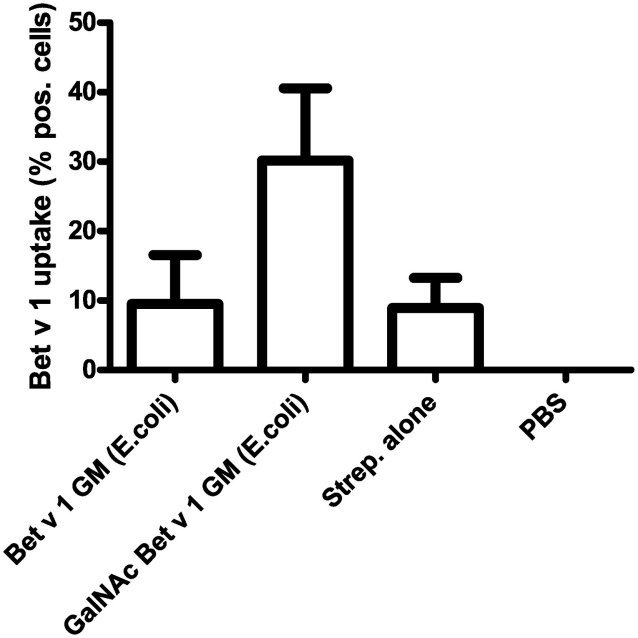
Table E1: Bet v 1 products

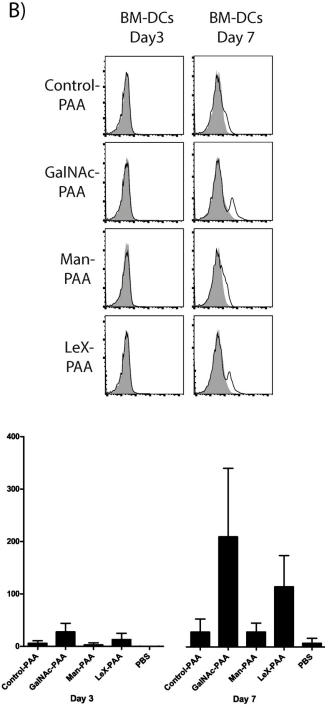
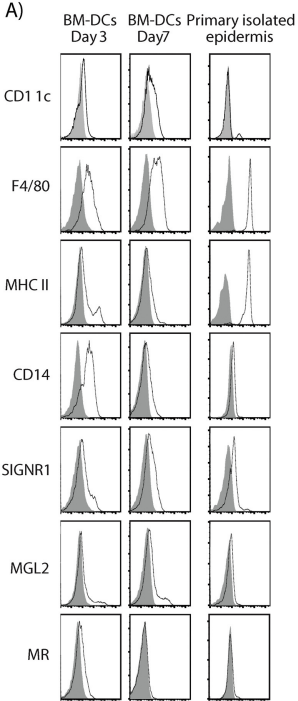
Construct name	Glycomodule	Expression system	Type of glycan
Bet v 1 (E.coli)	none	<i>E.coli</i>	none
Bet v 1 GM (E.coli)	O-glycan module	<i>E.coli</i>	none
GalNAc-Bet v 1 (E.coli)	O-glycan module	<i>E.coli</i>	>15 GalNAc residues
Bet v 1 (CHO)	none	CHO	none
GalNAc-Bet v 1 (CHO)	O-glycan module	CHO (COSMC-KO)	>15 GalNAc residues
HighMan-Bet v 1 (CHO)	N-glycan module	CHO (MGAT-1 KO)	Single N-glycan with high mannose
Bet v 1 (Pichia)	none	<i>P. pastoris</i>	none
Man-Bet v 1 (Pichia)	O-glycan module	<i>P. pastoris</i>	> 15 (Man α 1-2Man) ₂₋₇ linear Man chains



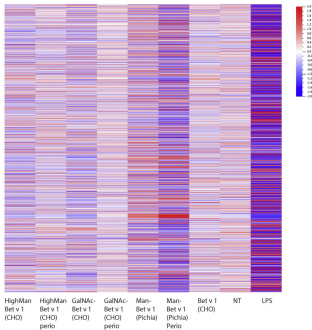




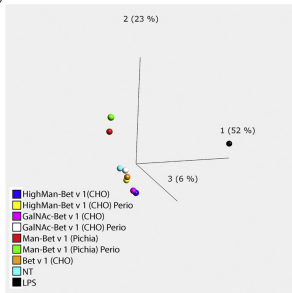


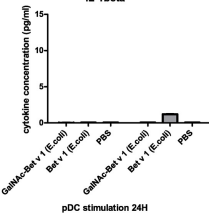


A)

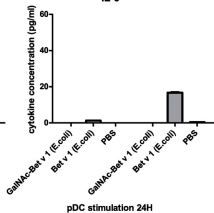


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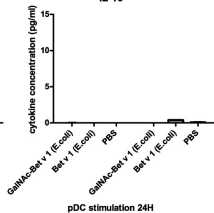


IL-1 β 

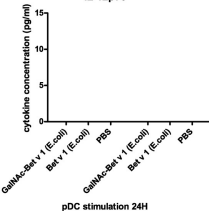
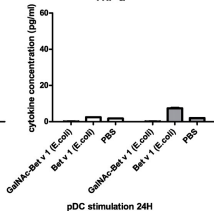
IL-6



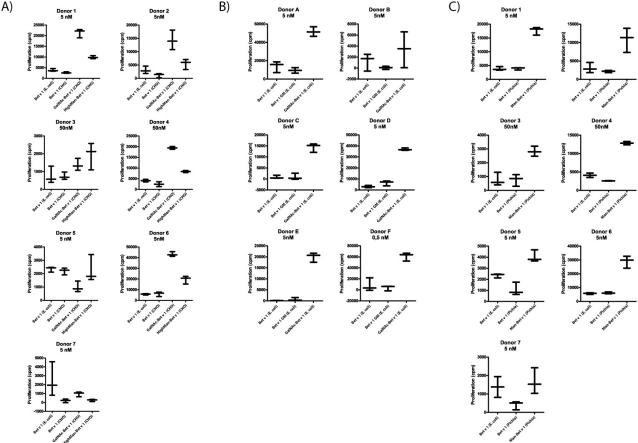
IL-10



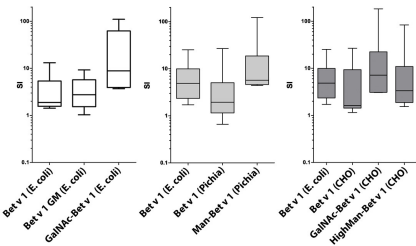
IL-12p70

TNF- α 

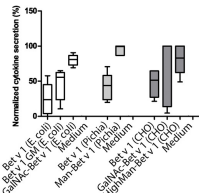
□ pDC-Donor 1
■ pDC-Donor 2



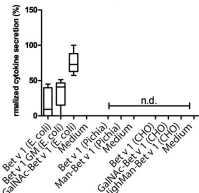
D)



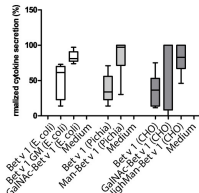
GM-CSF



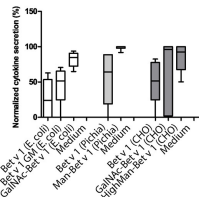
IL-12p70



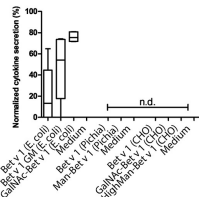
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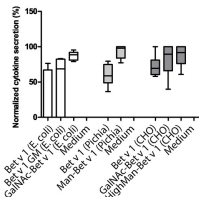
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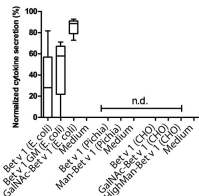
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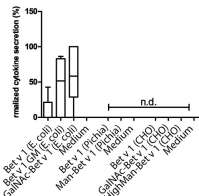
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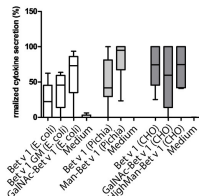
IL-21

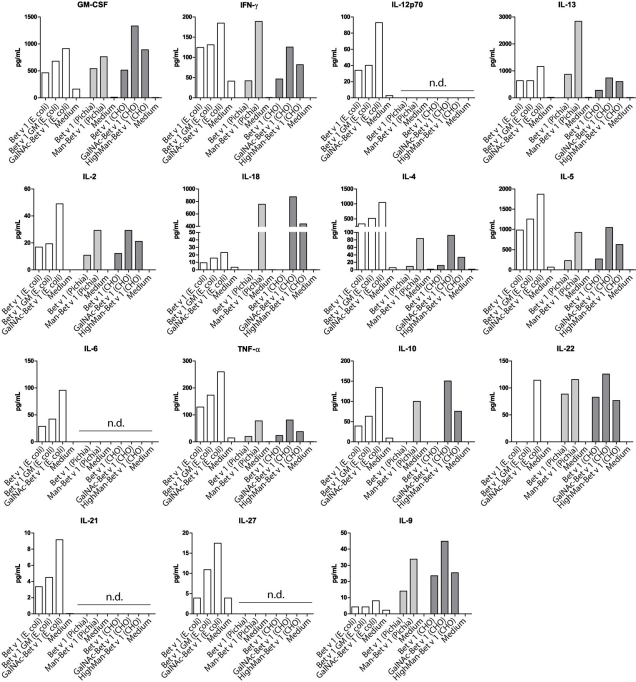


IL-27



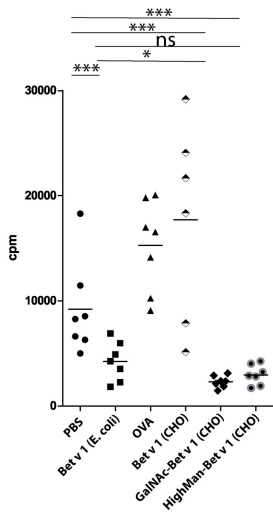
IL-9



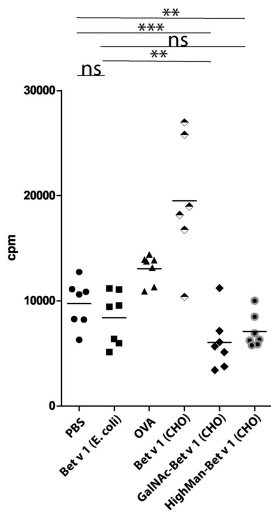


A)

Proliferation (Bet v 1 extract 30 ug)

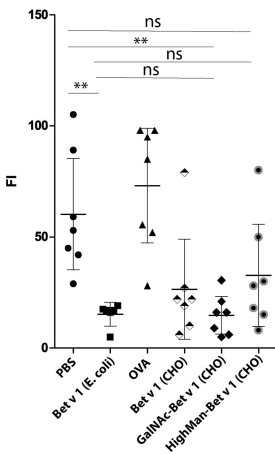


Proliferation (Bet v 1 (CHO) 30 ug)



B)

IgE



IgG2a

