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Context matters: Th2 polarization resulting from pollen composition and not from protein-intrinsic allergenicity

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Supplementary Materials and Methods

Recombinant protein expression, purification and characterization of Bet v 1

The expression and purification of rBet v 1 was performed as previously described[1]. In brief, rBet v 1 was expressed in *Escherichia coli* BL21 Star™ (DE3) cells (Invitrogen, Carlsbad, CA, USA) and purified by protein precipitation with 200 mM sodium chloride, followed by low-pressure chromatography using 10 ml of phenyl Sepharose and a DEAE Sepharose column (GE Healthcare Biosciences, Little Chalfont, UK). The levels of endotoxin contamination were determined by EndoZyme® recombinant Factor C (rFC) assay (Hyglos GmbH, Bernried am Starnberger See, Deutschland). The endotoxin amounts did not exceed 0.3 ng/ml. The recombinant protein was physicochemically characterized and stored lyophilized at -20°C.

Aqueous birch pollen extract

A total of 5 mg of *Betula pendula* (Allergon AB, Ängelholm, Sweden) pollen was dissolved in PBS and shaken for 24 hours at 4°C. The suspension was centrifuged three times for 5 min each at 12,000 x g at 4°C. The supernatant was collected and filtered through a 0.2-µm pore-size sterile filter (Merck Millipore, Merck KGaA, Darmstadt, Germany).

Purification of natural Bet v 1

For the purification of natural Bet v 1 (nBet v 1), an extract of 5 mg of *Betula pendula* (Allergon AB, Ängelholm, Sweden) pollen, prepared in a buffer consisting of 50 ml of endotoxin-free H_20 and 0.5 M NaCl, was shaken for 5 min at room temperature at 1,400 rpm and then centrifuged for 5 min at 12,000 x g at 4°C; the resulting supernatant was filtered (0.45-µm filter, GE Healthcare Biosciences, Little Chalfont, UK). Natural Bet v 1 was purified by a combination of hydrophobic chromatography on a 10-ml phenyl Sepharose column and size-exclusion chromatography using a Superdex 75 10/300 GL column (both from GE Healthcare Biosciences). The purified protein was physicochemically characterized and stored at -20°C. The nBet v 1 preparation represented a heterogeneous mixture of the following isoforms, as determined by mass spectrometry: Bet v 1a (MS-score 492.42, coverage 93.13), Bet v 1f (MS-score 507.42, coverage 73.75), Bet v 1g (MS-score 401.48, coverage 70.63), Bet v 1m (MS-score 454.27, coverage 67.50) and several other Bet v 1-derived fragments.

Determination of LPS level in BPE

To assess the amount of nLPS in BPEs, TLR4- and TLR2-specific NF-κB reporter gene assays were performed (as described elsewhere[2]). 400 ng of NF-κB-luciferase reporter plasmid (kindly provided by Min Li-Weber and cloned into pGL3Neo in the laboratory of J. Horejs-Hoeck) was transfected into HEK293 cells alongside a TLR4 receptor mix (with a TLR4:MD2:CD14 ratio of 3:1:1) or a TLR2 receptor mix (with a TLR2:CD14 ratio of 1:1) using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. TLR4, TLR2, CD14 and MD-2 were kind gifts from Andrei Medvedev and Douglas Golenbock. To estimate the LPS concentration, NF-κB activation in response to BPE was compared with the NF-κB activation induced by a LPS standard (starting from 10 pg/ml to 100 ng/ml). LPS from *E. coli* O111:B4 was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). For the data analysis a representative values present on both logarithmic trend lines (Fig E1c) in the parallel area (red line) were chosen for the calculation of the amount of LPS in BPE. Based on the LPS standard curve the amount of LPS in BPE was determined as 0.4 ng/ml

LPS per 1 µg/ml BPE. For the calculation of the amount of LPS the TLR4/CD14/MD2 data were used. The assay was performed and quantified in triplicates.

In vitro antigen uptake and maturation of mBMDCs

The isolation of BMDCs from C57BL/6 mouse bone marrow was performed as described previously[3]. In brief, bone marrow cells were extracted from female mouse femora and cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 1% penicillin-streptomycin, 20% granulocyte-macrophage colony-stimulating factor (GM-CSF) supernatant and 200 µM β-mercaptoethanol (mBMDC medium). After 10 days of culture, the cells were either frozen or used fresh for *in vitro* DC uptake or stimulation experiments. BMDCs were treated over a certain period of time (24, 14, 6, 3, 1 or 0 hours) with 0.5 µg of rBet v 1 or nBet v 1 per $2x10^5$ cells. For antigen uptake, rBet v 1 was labeled with pHrodo™ Red succinimidyl ester (Thermo Fisher Scientific). As a reference, nLPS without the protein was dissolved in mBMDC medium and incubated similarly. The nLPS samples contained 400 pg/ml of LPS per µg/ml rBet v 1. Cells were stained with allophycocyanin (APC)-conjugated anti-mouse CD11c antibody (clone N418; eBioscience, Inc., San Diego, CA, USA), Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD86 antibody (clone GL-1; BioLegend, San Diego, CA, USA), PerCP/Cy5.5 anti-mouse CD40 antibody (clone 3/23; BioLegend, San Diego, CA, USA) or BV421 anti-mouse CD80 antibody (16-10A1; BioLegend, San Diego, CA, USA) and analyzed via flow cytometry. Measurements were performed on a FACSCanto II instrument (BD Biosciences, San Jose, CA, USA). Dead cells were excluded upon staining with the fixable viability stain 450 (BD Biosciences), and granulocytes and monocytes with the V450 Rat anti-Mouse LY-6G and LY-6C (BD Biosciences). For compensation and data analysis, BD FACSDiva software (BD Biosciences) was used. Un-stimulated cells were only treated with BMDC medium. As a positive control, 100 ng/ml LPS was used. The aqueous birch pollen extract was used at the same concentration of total protein as rBet v 1 (0.5 µg/$2x10^5$ cells). Statistical analysis was performed using ANOVA with a Bonferroni post-test. The data represent the mean of duplicate experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ indicate significantly different values versus the unstimulated cells. The data are derived from at least two independent experiments.

Stimulation of human moDCs and analysis of cytokine profile

MoDCs were isolated from PBMCs of healthy, non-atopic donors as well as of atopic patients and cultured as previously described[4]. Immature moDCs ($1x10^6$ cells/ml) were checked for viability with Aqua® dye (Invitrogen, Carlsbad, CA, USA), expression of CD1a (eBioscience, Inc., San Diego, CA, USA) and loss of CD14 (BD Biosciences) expression by flow cytometry. Cells were stimulated for 24 hours with 1000 ng/ml of rBet v 1. As a control, moDCs were treated with medium (unstimulated control). In another experiment, the stimulation effect of 30 µg/ml of BPE on moDCs was compared with the naturally occurring amount of LPS (nLPS) in the extract. By using a Bet v 1-specific sandwich ELISA (see section: Bet v 1 depletion by immunoprecipitation), the concentration of BPE was calculated to obtain amounts of nBet v 1 equivalent to the 1000 ng/ml of rBet v 1. The assay was performed without LPS co-stimulation. The maturation markers CD40 (eBioscience), HLA-DR, CD80, CD83 and CD86 (BD Biosciences) were analyzed by FACS analysis on a Navios flow cytometer (Beckman Coulter, Brea, CA, USA). The supernatant of the stimulated cells was collected and analyzed for cytokine expression, including CCL17, IL-1β, IL-10 (BD Biosciences), IL-6, IL-12p70 and IL-23 (eBioscience). Statistical analysis was performed using one-way ANOVA with a Bonferroni post-test to compare all

groups. All statistical calculations were performed using GraphPad Prism 5 software. The study was approved by the ethics committee of the medical faculty of Technical University of Munich.

In vitro stimulation of human naïve $CD4^+$ T-cells and analysis of mRNA expression

Naïve $CD4^+$ T-cells were isolated from PBMCs of healthy and anonymous blood donors by magnetic cell separation according to manufacturer's instructions (Naïve $CD4^+$ T-cell Isolation Kit II, Miltenyi Biotec, Bergisch Gladbach, Germany). The blood samples were provided by the university hospital for blood group serology and transfusion medicine in Salzburg. T-cells were seeded in 48-well plates at a density of $2x10^6$ cells per mL in IMDM (PAA Laboratories GmbH, Austria) supplemented with 5% FCS i.a. (PAA), 2 mM L-Glutamine, 100 U/mL penicillin (Sigma-Aldrich, Inc.,St. Louis, MO, USA), 100 µg/mL streptomycin (Sigma) and stimulated with 0.1 µg/mL αCD3 (Anti-Human CD3 Functional Grade® Purified, Clone: OKT3, eBioscience), 2.5 µg/mL αCD28 (Purified NA/LE Mouse Anti-Human CD28, BD Pharmingen) and 0.01% and 0.1% of BPE. After 7 days cells were re-stimulated for another 6 hours and T-cell cytokine mRNA expression was measured by qPCR. Total RNA was isolated by using TRI Reagent (Sigma) and reverse-transcribed into cDNA with RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). mRNA expression was analyzed relatively to the large ribosomal protein P0 (RPLP0) by quantitative real-time PCR (Rotor-Gene Q, Rotor-Gene Q Series Software, Quiagen, Hilden, Germany) with iQ™ SYBR® Green Supermix (Bio-Rad, CA, USA) and the following primers: RPLP0: forward 5' - GGCACCATTGAAATCCTGAGTGATGTG - 3', reverse 5' - TTGCGGACACCCTCCAGGAAG - 3', IL-5: forward 5' - CCTTGGCACTGCTTTCTACTCATCG - 3', reverse 5' - GGTTTACTCTCCGTCTTTCTTCTCCACA - 3', IL-13: forward 5' - TGTGCCTCCCTCTACAGCCCTCAG - 3', reverse 5' - TCAGCATCCTCTGGGTCTTCTCG - 3', IL-2: forward 5' -TCCCAAACTCACCAGGATGCTCAC - 3', reverse 5' -AATGTTGTTTCAGATCCCTTTAGTTCCAGA - 3', TNF-α: forward 5'-CAAGCCTGTAGCCCATGTTG -3', reverse 5'- GAGGTTGACCTTGGTCTGGTA -3'. Statistical significance was determined by one-way ANOVA, Tukey post-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
According to the Austrian national regulations, informed consent in the case of anonymous blood cells discarded after plasmapheresis (buffy coats) is not required. Thus, there is no requirement for additional approval by the local ethics committee.

Bet v 1 depletion by immunoprecipitation

Depletion was performed by immunoprecipitation using mouse monoclonal anti-Bet v 1.0101 antibodies, which were generated by hybridoma technology and purified using Bet v 1.0101-coupled NHS Sepharose™ 4 Fast Flow (GE Healthcare Biosciences, Little Chalfont, UK) material. A total of 1 ml of Protein G Sepharose™ 4 Fast Flow (also GE Healthcare Biosciences) was pre-incubated with PBS containing 0.1% BSA to saturate the nonspecific binding sites of the Protein G Sepharose™ material. A total of 0.5 ml of BPE (1 mg/ml) was incubated with 250 µg of the anti-Bet v 1.0101 antibody and shaken overnight at 4°C. The suspension was incubated with 1 ml of the Protein G Sepharose™ slurry over 30-60 min at room temperature. The suspension was centrifuged for 15 min at 14,000 x g, and the supernatant containing the Bet v 1-depleted extract was collected. The concentration of the depleted extract was determined by Bradford assay. The depleted extract was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. For the immunoblot assay, the same monoclonal anti-Bet v 1.0101 antibody was used as a primary antibody. As a secondary antibody, an alkaline phosphatase-conjugated rabbit anti-mouse IgG + IgM antibody (Jackson ImmunoResearch Europe Ltd., Oaks Drive Newmarket, Suffolk, UK) was used at a

concentration of 1 µg/ml. The amount of Bet v 1 within the depleted extract was determined by sandwich ELISA using the mouse monoclonal anti-Bet v 1.0101 antibody in combination with an affinity-purified polyclonal rabbit anti-Bet v 1.0101 antibody (1 µg/ml). The detection antibody was an alkaline phosphatase-conjugated goat anti-rabbit antibody (1 µg/ml). The depleted extract was compared with the untreated extract and an rBet v 1 standard. For the rBet v 1-replaced extract sample, the exact amount of rBet v 1 was added to restore the level to 12.5%, which was the level of Bet v 1 in the original, untreated extract. Further, the depleted extract was analyzed using mass spectrometry with a Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with nanoelectrospray and nano-HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific).

Immunization of 4get mice

4get mice (Jackson laboratory, Bar Harbor, ME, USA) were immunized with either BPE (n=5) or rBet v 1 (n=3) in PBS, without added adjuvants. For the immunizations, 65 µg of BPE were injected, and the amount of injectable rBet v 1 was calculated according to the total Bet v 1 quantified within the BPE. Skin-draining inguinal lymph nodes of immunized mice, as well as of naïve mice were collected. Lymphocytes were stained with an APC-conjugated anti-mouse CD4 antibody and analyzed for IL-4/eGFP expression using flow cytometry (BD Biosciences, San Jose, CA, USA). In a second experiment, a different batch of BPE (n=5) was used and compared with a Bet v 1-depleted version of this extract (nBet v 1-depleted extract, n=5) as well as a reconstituted version of the depleted extract (rBet v 1-replaced extract, n=5). The results were compared with those of naïve 4get mice. Statistical analysis was performed using one-way ANOVA with a Bonferroni post-test to compare all groups (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). All *in vivo* experiments were performed according to national guidelines approved by the Austrian Federal Ministry (BMWF-66.012/0010-II/3b/2013).

References

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Figure Legends

FIG E1. TLR2- (**a**) and TLR4-specific (**b**) NF-κB activation was induced either by a birch pollen extract or LPS. Either the TLR2 or the TLR4 receptor complex was overexpressed in HEK293 cells. Cells were stimulated either with different concentrations of LPS or the birch pollen extract, starting from 10 pg/ml to 100 ng/ml. By comparing the activation signals obtained by LPS, the level of LPS in the BPE was quantified by using linear regression. For the calculation of the amount of LPS the standard curve of the TLR4/CD14/MD2 data was used (**c**). The assay was performed and quantified in triplicates.

FIG E2. Activation (**a**) and cytokine expression (**b**) of human moDCs induced by rBet v 1. Error bars indicate mean and SEM (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

FIG E3. Depletion of nBet v 1 from birch pollen extract and replacement with rBet v 1. The birch pollen extract was depleted of Bet v 1 by immunoprecipitation using allergen-specific monoclonal antibodies. With this procedure, it was possible to reduce the amount of nBet v 1 measured within the extract from 12.5% to 2%. SDS-PAGE analysis of the samples used in the 4get experiment (Fig 2b): rBet v 1, birch pollen extract, nBet v 1-depleted extract and rBet v 1-replaced extract (**a**). Immunoblot of SDS-PAGE samples using a monoclonal mouse anti-Bet v 1 antibody as the primary antibody (**b**). The reduction of nBet v 1 within the nBet v 1-depleted extract was quantified by sandwich ELISA (**c**). The rBet v 1-replaced extract was reconstituted equivalently to the 12.5% of nBet v 1 found in the original extract. The question whether or not all nBet v 1 isoforms were reduced by immunoprecipitation was addressed by mass spectrometry (**d**). For this purpose, the control proteins Bet v 2 and Bet v 7 were used to normalize the mass spectrometry data. All the isoforms found in the BPE (Bet v 1.0101, Bet v 1.0102, Bet v 1.0104, Bet v 1.0106 and Bet v 1.0201) were markedly reduced after the immunoprecipitation.

FIG E4. Activation of $CD11c^+$ mBMDCs isolated from 4get mice upon stimulation with BPE, nBet v 1-depleted BPE (dBPE) or rBet v 1 +/- nLPS for 24 hours. The expression of the surface activation markers CD80 and CD40 was analyzed.