Context matters: TH2 polarization resulting from pollen composition and not from protein-intrinsic allergenicity

Lorenz Aglas, Stefanie Gilles, Renate Bauer, Sara Huber, Galber R. Araujo, Geoffrey Mueller, Sandra Scheiblhofer, Marie Amisi, Hieu-Hoa Dang, Peter Briza, Barbara Bohle, Jutta Horejs-Hoeck, Claudia Traidl-Hoffmann, Fatima Ferreira

Angaben zur Veröffentlichung / Publication details:

Aglas, Lorenz, Stefanie Gilles, Renate Bauer, Sara Huber, Galber R. Araujo, Geoffrey Mueller, Sandra Scheiblhofer, et al. 2018. "Context matters: TH2 polarization resulting from pollen composition and not from protein-intrinsic allergenicity." *Journal of Allergy and Clinical Immunology* 142 (3): 984–987.e6. https://doi.org/10.1016/j.jaci.2018.05.004.

modify CHS recovery. These included *Tgfb2*, *Kcnk5*, *Lrrk2*, *Ptk2*, *Cyp11b1*, *Shoc2*, and *Dusp5* (for further discussion, see Results and Discussion sections in the Online Repository). Together with the previously identified candidates *Gilz* and *Bach2*, many of these genes were identified as members of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway (Fig 2, *E*).

Finally, we performed further genome-wide scans for linkage at each day after immune challenge up to D4, also comparing the linkage peaks detected here with those associated with nonrecovery at D7. This allowed the confirmation of previously identified loci that failed to reach statistical significance across multiple time points and identification of additional candidate genes (for further discussion, see Fig E2 and Results and Discussion sections in this article's Online Repository at www.jacionline.org).

In this study, we have demonstrated that genetic variability underlies substantial differences in CHS responses and we have identified genetic loci strongly associated with the response to CHS immune challenge in the mouse. The most important associations were with the level of inflammation (acute response to immune challenge), and level of recovery (postchallenge recovery), which both revealed strong gene candidates explaining the observed phenotype. These were identified as *Bach2* and *Gilz*, respectively, and are of significant interest because both are known modifiers of CHS and immune responses in general.[7,8] Beyond these 2 genes, we have also identified a large number of additional loci and candidate genes possibly associated with the CHS response in multiple independent analyses over time. Altogether, these findings point to the possibility that the MAPK/ERK signaling pathway is a key regulator of the development and recovery of CHS.

In conclusion, our data overwhelmingly support a strong genetic basis for the development of ACD. The intensity of the dermatitis as well as the recovery from the dermatitis were regulated by key loci where candidate genes regulate the MAPK/ERK pathway. These findings have far-reaching implications in the management of ACD and point toward new therapeutic targets.

Julien M. D. Legrand, PhD[a]
Edwige Roy, PharmD, PhD[a]
Batoul Baz, PhD[a]
Pamela Mukhopadhyay, PhD[b]
Ho Yi Wong, BSc[a]
Ramesh Ram, PhD[c]
Grant Morahan, PhD[c]
Graeme Walker, PhD[b]*
Kiarash Khosrotehrani, MD, PhD, FACD[a]*

From [a]the UQ Diamantina Institute, Translational Research Institute, University of Queensland, Brisbane, Australia; [b]the QIMR Berghofer Medical Research Institute, Brisbane, Australia; and [c]the Centre for Diabetes Research, Harry Perkins Institute of Medical Research, Perth, Australia. E-mail: k.khosrotehrani@uq.edu.au.

*These authors contributed equally as senior authors to this work.

Present address: Julien M. D. Legrand is currently at the Australian Regenerative Medicine Institute, Monash University, Clayton, Victoria, Australia.

This work was funded by the National Health and Medical Research Council (NHMRC) of Australia (grant no. 1082438). K.K. is supported by an NHMRC fellowship (grant no. 1125290).

Disclosure of potential conflict of interest: G. Morahan holds stock in Geniad Pty Ltd. K. Khosrotehrani and G. Walker are the holders of a National Health and Medical Research Council (NHMRC) project grant that supported this work and K. Khosrotehrani is the holder of an NHMRC fellowship, the details of which are listed earlier. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online June 12, 2018.
https://doi.org/10.1016/j.jaci.2018.04.028

Context matters: T_H2 polarization resulting from pollen composition and not from protein-intrinsic allergenicity

To the Editor:

Birch (*Betula verrucosa)* pollen is the main cause of spring pollinosis in the temperate climate zone of the Northern Hemisphere. Approximately 95% of birch pollen–allergic patients are sensitized to Bet v 1, the major birch pollen allergen.[1] However, it is not known whether sensitization toward Bet v 1 results from its own intrinsic properties or from immunomodulatory pollen compounds codelivered with the allergen. Therefore, we investigated whether Bet v 1 or the pollen matrix is responsible for the activation of antigen-presenting cells and the subsequent T_H2 polarization, relevant in the process of allergic sensitization.

Because dendritic cells (DCs) are the most potent activators of adaptive immune responses, we sought to investigate their role in the process of allergic sensitization toward Bet v 1. For this purpose, the activation of murine bone marrow–derived dendritic cells (mBMDCs) as well as the maturation of human monocyte–derived dendritic cells was monitored *in vitro*. Concerning the latter, PBMCs were isolated either from healthy, nonatopic donors or from atopic patients. Cells were stimulated either with recombinant Bet v 1.0101 (rBet v 1) produced in *E coli* or with an aqueous birch pollen extract (BPE). We analyzed whether DC activation is triggered by the costimulation of Toll-like receptor (TLR) 4 by LPS contaminations found in BPE, termed nLPS. Furthermore, we investigated the capacity of rBet v 1 and BPE to induce T_H2 polarization using an *in vivo* sensitization model. The methodology can be found in this article's Online Repository at www.jacionline.org.

The upregulation of maturation markers (CD40 and CD86) expressed on $CD11c^+$ mBMDCs was monitored over 24 hours (Fig 1, *A*). The nLPS concentration was determined by using a

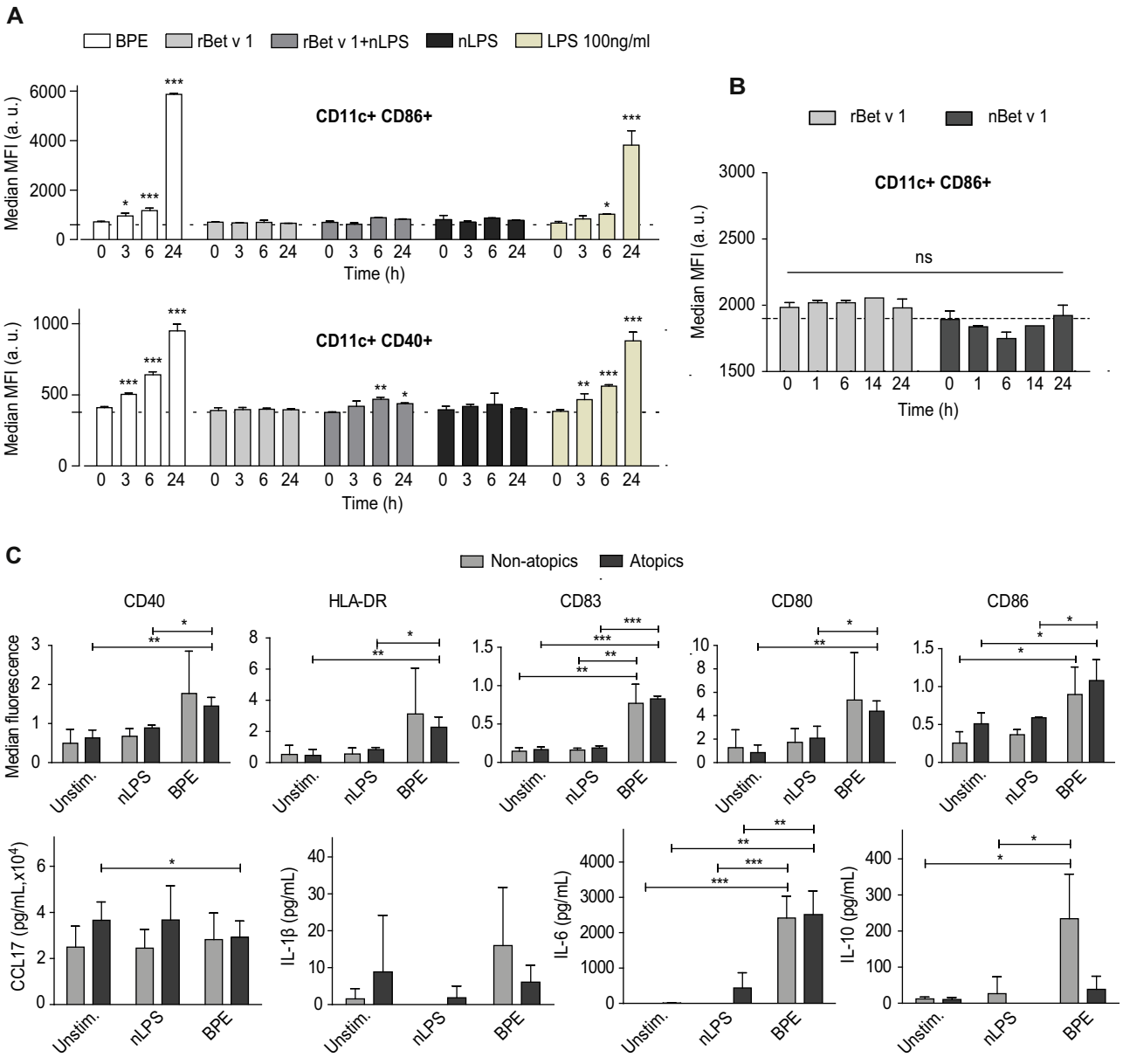

FIG 1. A, Time-dependent activation of murine BMDCs stimulated with BPE or rBet v 1 ± nLPS. **B,** Comparison of activation signal induced by rBet v 1 or nBet v 1. Expression of maturation markers **(C)** and cytokine profile of human moDCs stimulated with BPE. *a.u.*, Arbitrary units of fluorescence; *MFI*, mean fluorescence intensity; *Unstim*, unstimulated. Error bars indicate mean and SEM (**P* ≤ .05; ***P* ≤ .01; ****P* ≤ .001).

nuclear factor kappa B reporter gene assay (see Fig E1 in this article's Online Repository at www.jacionline.org). Here, 0.4 ng/mL of LPS had an equivalent capacity of 1 μg/mL of total soluble BPE protein to activate TLR4 and TLR2. As positive control, cells were incubated with LPS at a concentration of 100 ng/mL, 250-fold higher than nLPS. Compared with the basal activation of uninduced cells (Fig 1, *A*, dashed line), BPE induced a time-dependent upregulation of CD86 and CD40. Stimulation with rBet v 1, even in the presence of nLPS, was not sufficient to mimic this effect. Because rBet v 1 represents just one of many isoforms found in BPEs, we investigated whether the activation of mBMDCs differs on stimulation with a natural Bet v 1 isoform mixture (nBet v 1) purified from BPE (Fig 1, *B*). No differences were observed between both natural and recombinant Bet v 1.

Furthermore, we analyzed the maturation (CD40, HLA-DR, CD80, CD83, and CD86) and secretion of the cytokines CCL17, IL-1β, IL-10, IL-6, IL-12p70, and IL-23 of human moDCs on stimulation with the major allergen. In parallel, the maturation-inducing effects of BPE were determined and compared with nLPS alone. The concentration of nBet v 1 in BPE was determined by ELISA and rBet v 1 was used in equivalent amounts. No maturation was induced by rBet v 1 (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org). In contrast, BPE induced an upregulation of maturation markers and IL-6 secretion in comparison to the unstimulated and/or nLPS-stimulated control (Fig 1, *C*). Surprisingly, in moDCs derived from nonatopic donors, the maturation effect was less pronounced, and a significant upregulation of IL-10

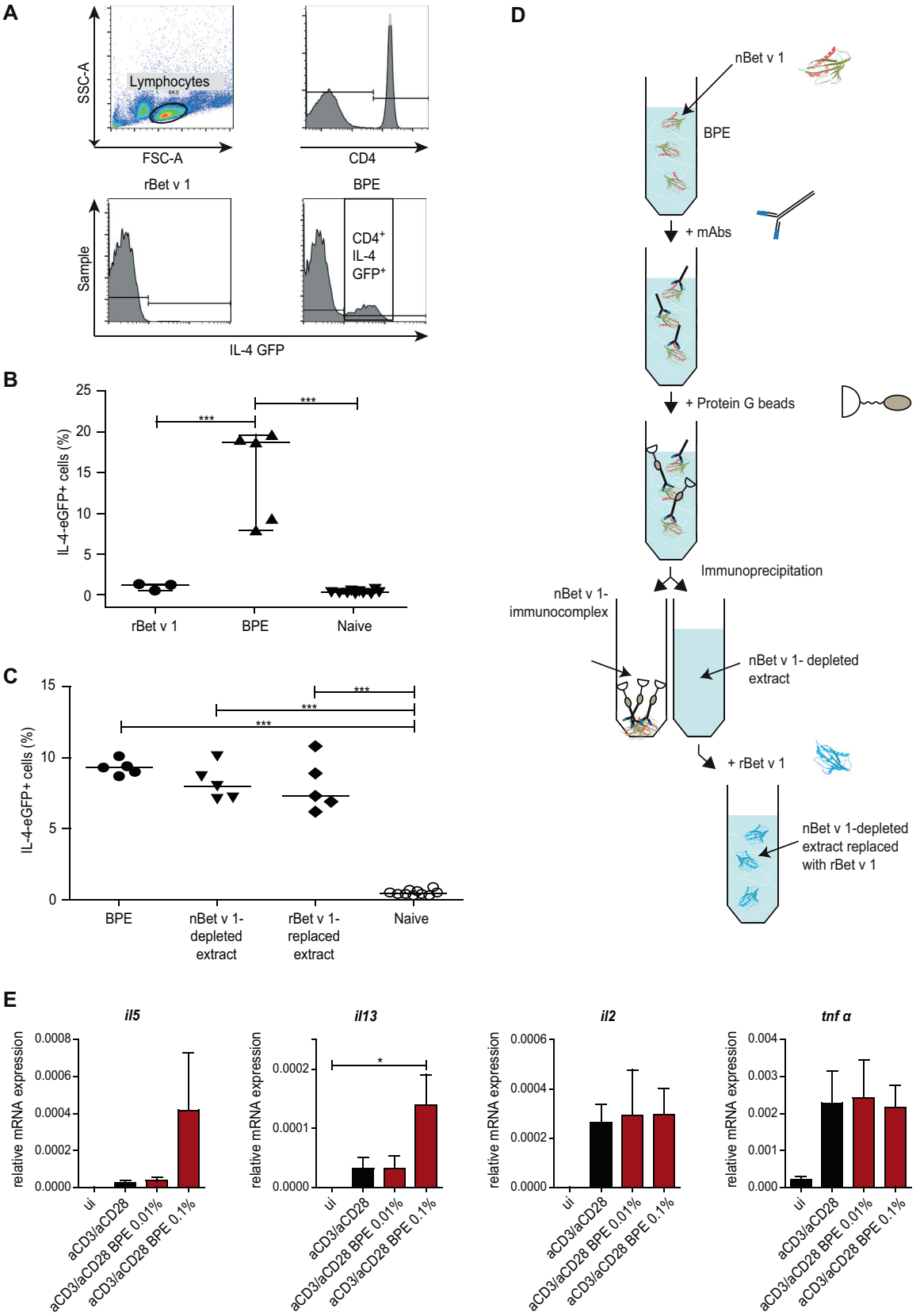

FIG 2. A, Flow cytometry gating strategy used to quantify $CD4^+$ IL-4 GFP^+ T cells. **B,** Percentage of IL-4–expressing $CD4^+$ T cells of mice immunized with rBet v 1 or BPE. **C,** Comparison of nBet v 1–depleted and rBet v 1–replaced extracts with untreated BPE; $*P \leq .05$; $**P \leq .01$; $***P \leq .001$. **D,** Schematic overview of nBet v 1 depletion of BPE using immunoprecipitation. **E,** mRNA expression of *in vitro*–stimulated human naive $CD4^+$ T cells with BPE. *FSC-A*, Forward scatter-area; *SSC-A*, side scatter-area.

was observable. Without LPS costimulation secretion of neither IL-12p70 nor IL-23 by BPE-stimulated moDCs was observable (data not shown).

The finding that BPE but not rBet v 1 is able to effectively activate DCs raised the question whether pollen-derived compounds possess adjuvant properties and thus contribute to the allergenicity of Bet v 1 *in vivo*. Therefore, we examined the capacity of BPEs and rBet v 1 to promote T_H2 polarization. We used IL-4/GFP-enhanced transcript (4get) mice for monitoring the expression of the T_H2 cytokine IL-4 in skin-draining inguinal lymphocytes (Fig 2, *A*). Strikingly, rBet v 1 did not cause T_H2 polarization; however, BPE strongly induced IL-4 activation (Fig 2, *B*). Because the involvement of the allergen itself as part of BPE in T_H2 polarization was unclear, we addressed this question by immunizing 4get mice with a Bet v 1–depleted BPE (nBet v 1–depleted extract; Fig 2, *D*; see Fig E3, *A-D*, in this article's Online Repository at www.jacionline.org) and a rBet v 1–reconstituted version (rBet v 1–replaced extract). Indeed, the T_H2-promoting effect of the BPE remained even after the depletion (Fig 2, *C*), indicating that T_H2 polarization is induced via an allergen-independent costimulus. In addition, the *in vitro* activation of mBMDCs from 4get mice was analyzed (see Fig E4 in this article's Online Repository at www.jacionline.org).

To analyze potential direct effects of BPE on naive $CD4^+$ T cells, we activated T cells by treatment with αCD3/αCD28 and BPE. Although the expression of αCD3/αCD28-stimulated T_H2-specific cytokines IL-5 and IL-13 was enhanced on additional stimulation with BPE (0.1%), BPE treatment did not affect the expression of the T_H1-related cytokines IL-2 and TNF-α.

Summarizing, we found that BPE efficiently activates murine and human DCs *in vitro* and is able to induce T_H2 polarization *in vivo* and *in vitro*, whereas Bet v 1 lacks this property. These observations strongly support a role for the pollen context. Noteworthy, T_H2 polarization upon immunization with BPE occurred independently of Bet v 1, indicating that the protein itself is not the sensitization-driving force.

Equivalent amounts of LPS (nLPS) in BPE are not capable of inducing DC maturation compared with the whole BPE, suggesting that the observed activation cannot be attributed to LPS contaminations. However, LPS may not be the effective TLR-activating factor present in BPE because other contaminants are also present in pollen extracts. Previous studies demonstrating that pollen extracts promote maturation of TLR4-deficient DCs and that recognition of allergens is largely independent of TLR correlates with our observation.[2-4] Hence, allergenicity may be explained by recognition of functional features of antigens.[5-7] BPE-stimulated moDCs derived from nonatopic donors showed a significant reduction in maturation that may arise because of an increase in the anti-inflammatory cytokine, IL-10, which has been previously associated with downregulation of maturation activity.[8]

Remarkably, Bet v 1–depleted BPE was still able to induce T_H2 polarization. Therefore, we propose that Bet v 1 sensitization occurs as a result of additional immune interactions elicited by pollen-derived components that are not necessarily associated with the major allergen itself. Whether allergic sensitization toward Bet v 1 arises as a secondary effect of a preprimed T_H2 environment[3] and/or due to the high levels of Bet v 1 in BPEs remains to be determined in future studies.[9] In this respect, the identification of T_H2-polarizing compounds in pollen will be of utmost importance to understand the mechanisms of host-allergen source interactions and for the development of allergy prophylaxis.

Lorenz Aglas, PhD[a]
Stefanie Gilles, PhD[b,c]
Renate Bauer, BSc[a]
Sara Huber, MSc[a]
Galber R. Araujo, PhD[a]
Geoffrey Mueller, PhD[d]
Sandra Scheiblhofer, PhD[a]
Marie Amisi, MSc[b,c]
Hieu-Hoa Dang, BSc[a]
Peter Briza, PhD[a]
Barbara Bohle, PhD[e]
Jutta Horejs-Hoeck, PhD[a]
Claudia Traidl-Hoffmann, PhD[b,c]
Fatima Ferreira, PhD[a]

From [a]the Department of Molecular Biology, University of Salzburg, Salzburg, Austria; [b]the Institute of Environmental Medicine, UNIKA-T, Technical University of Munich and Helmholtz Zentrum München, Research Center for Environmental Health, Augsburg, Germany; [c]CK CARE Christine Kühne Center for Allergy Research and Education, Davos, Switzerland; [d]Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, NC; and the [e]Department of Pathophysiology and Allergy Research and Christian Doppler Laboratory for Immunomodulation, Medical University of Vienna, Vienna, Austria. E-mail: Fatima.ferreira@sbg.ac.at.

This work was supported by the Austrian Science Funds (FWF Projects P23417, P27589, and SFB F4610), by the University of Salzburg priority program "Allergy-Cancer-BioNano Research Centre," by the Doctorate School Plus Program "Biomolecules" of the University of Salzburg, and by the Intramural Research Program of the National Institute of Environmental Health Sciences (grant no. Z01-ES102906-01, REL).

Disclosure of potential conflict of interest: L. Aglas has been supported by the European Union's Seventh Framework Programme FP7 (grant nos. 601763 and BM4SIT). F. Ferreira is a member of scientific advisory boards (HAL Allergy, NL; SIAF, Davos, CH; AllergenOnline, USA) and has been supported by the Austrian Science Funds (FWF). B. Bohle has been supported by the Austrian Science Funds and by the Christian Doppler Laboratory for Immunomodulation, is a board member of the Allergen Online Database, and is a member of the scientific advisory board of the Paul Ehrlich Institute (nonfinancial support). G. R. Araujo is a recipient of a European Academy of Allergy and Clinical Immunology Research Fellowship 2017 and 2018. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online May 18, 2018.
https://doi.org/10.1016/j.jaci.2018.05.004

METHODS

Recombinant protein expression, purification, and characterization of Bet v 1

The expression and purification of rBet v 1 was performed as previously described.[E1] In brief, rBet v 1 was expressed in *Escherichia coli* BL21 Star (DE3) cells (Invitrogen, Carlsbad, Calif) 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 assay (Hyglos GmbH, Bernried am Starnberger See, Germany). The endotoxin amounts did not exceed 0.3 ng/mL. The recombinant protein was physicochemically characterized and stored lyophilized at −20°C.

Aqueous BPE

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 3 times for 5 minutes each at 12,000*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 mol NaCl, was shaken for 5 minutes at room temperature at 1400 rpm and then centrifuged for 5 minutes at 12,000*g* at 4°C; the resulting supernatant was filtered (0.45-μm filter, GE Healthcare Biosciences). 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 nuclear factor kappa B (NF-kB) reporter gene assays were performed (as described elsewhere[E2]). A total of 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, Mass) 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 an 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). For the data analysis, a representative value present on both logarithmic trend lines (Fig E1, *C*) in the parallel area (red line) was 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 maturation of mBMDCs

The isolation of BMDCs from C57BL/6 mouse bone marrow was performed as described previously.[E3] In brief, bone marrow cells were extracted from female mouse femora and cultured in RPMI 1640 medium supplemented with 5% FCS, 2 mM L-glutamine, 1% penicillin-streptomycin, 20% 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 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 2×10^5 cells. 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 1 μg/mL rBet v 1. Cells were stained with allophycocyanin (APC)-conjugated antimouse CD11c antibody (clone N418; eBioscience, Inc, San Diego, Calif), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD86 antibody (clone GL-1; BioLegend, San Diego, Calif), PerCP/Cy5.5 anti-mouse CD40 antibody (clone 3/23; BioLegend), or BV421 anti-mouse CD80 antibody (16-10A1; BioLegend) and analyzed via flow cytometry. Measurements were performed on a FACSCanto II instrument (BD Biosciences, San Jose, Calif). Dead cells were excluded on 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. Unstimulated cells were treated only with BMDC medium. As a positive control, 100 ng/mL LPS was used. The aqueous BPE was used at the same concentration of total protein as rBet v 1 (0.5 μg/2×10^5 cells). Statistical analysis was performed using ANOVA with a Bonferroni posttest. The data represent the mean of duplicate experiments. $*P < .05$, $**P < .01$, and $***P < .001$ indicate significantly different values versus the unstimulated cells. The data are derived from at least 2 independent experiments.

Stimulation of human moDCs and analysis of cytokine profile

MoDCs were isolated from PBMCs of healthy, nonatopic donors as well as of atopic patients and cultured as previously described.[E4] Immature moDCs (1×10^6 cells/mL) were checked for viability with Aqua dye (Invitrogen, Carlsbad, Calif), expression of CD1a (eBioscience, Inc), 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 1000 ng/mL of rBet v 1. The assay was performed without LPS costimulation. 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, Calif). 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 1-way ANOVA with a Bonferroni posttest 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 naive $CD4^+$ T cells and analysis of mRNA expression

Naive $CD4^+$ T cells were isolated from PBMCs of healthy and anonymous blood donors by magnetic cell separation according to manufacturer's instructions (Naive $CD4^+$ 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 2×10^6 cells per milliliter in Iscove's Modified Dulbecco's Medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 5% FCS i.a. (PAA), 2 mM L-glutamine, 100 U/mL penicillin (Sigma-Aldrich, Inc, St Louis, Mo), and 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 Biosciences, San Jose, Calif), and 0.01% and 0.1% of BPE. After 7 days, cells were restimulated for another 6 hours and T-cell cytokine mRNA expression was measured by quantitative PCR. 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, Hercules, Calif) and the following primers: RPLP0: forward 5′-GGCACCATTGAAATCCTGAG TGATGTG-3′, reverse 5′-TTGCGGACACCCTCCAGGAAG-3′, IL-5: forward 5′-CCTTGGCACTGCTTTCTACTCATCG-3′, reverse 5′-GGT TTACTCTCCGTCTTTCTTCTCCACA-3′, IL-13: forward 5′-TGTG CCTCCCTCTACAGCCCTCAG-3′, reverse 5′-TCAGCATCCTCTGGGT CTTCTCG-3′, IL-2: forward 5′-TCCCAAACTCACCAGGATGCTCAC-3′, reverse 5′-AATGTTGTTTCAGATCCCTTTAGTTCCAGA-3′, TNF-α: forward 5′-CAAGCCTGTAGCCCATGTTG-3′, reverse 5′-GAGGTTGA CCTTGGTCTGGTA-3′. Statistical significance was determined by 1-way ANOVA, Tukey posttest (*$P < .05$, **$P < .01$, ***$P < .001$).

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) material. A total of 1 mL of Protein G Sepharose 4 Fast Flow (also GE Healthcare Biosciences) was preincubated 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 to 60 minutes at room temperature. The suspension was centrifuged for 15 minutes at 14,000*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. Furthermore, the depleted extract was analyzed using mass spectrometry with a Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, Mass) with nanoelectrospray and nano-HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific).

Immunization of 4get mice

4get mice (Jackson laboratory, Bar Harbor, Me) 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 was 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 naive 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). 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 naive 4get mice. Statistical analysis was performed using 1-way ANOVA with a Bonferroni posttest to compare all groups (*$P \leq .05$; **$P \leq .01$; ***$P \leq .001$). All *in vivo* experiments were performed according to national guidelines approved by the Austrian Federal Ministry (BMWF-66.012/0010-II/3b/2013).

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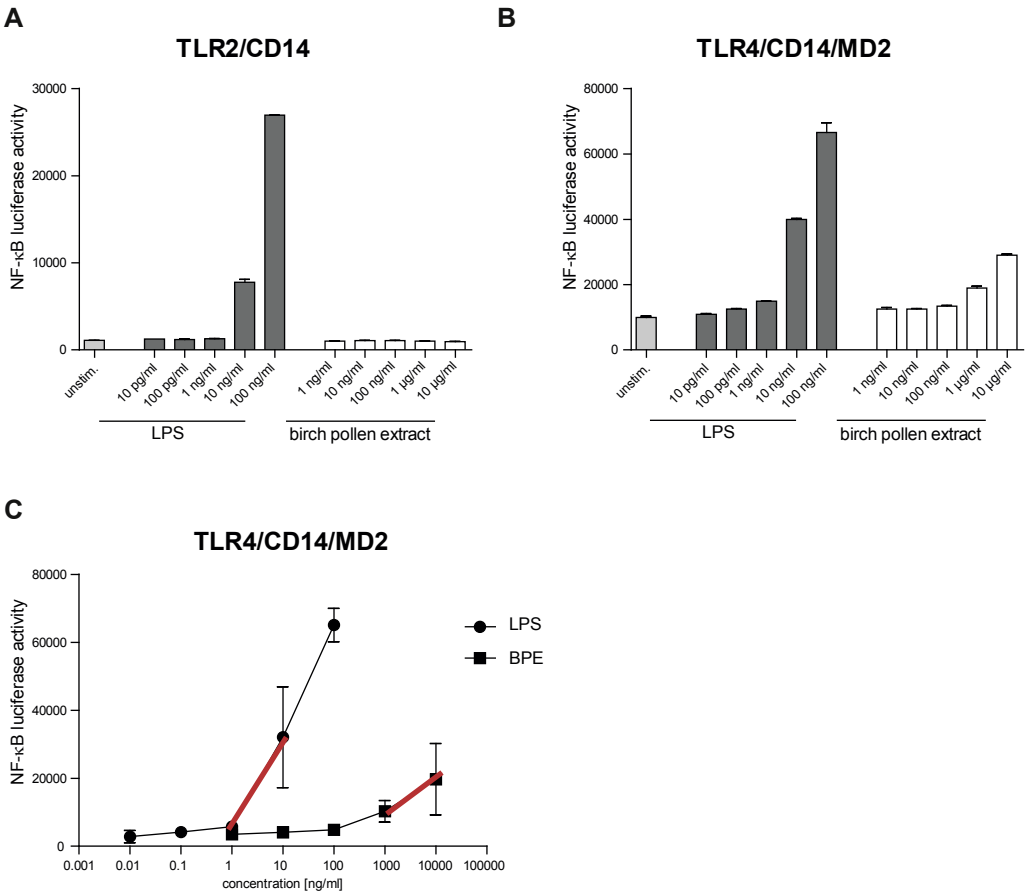

FIG E1. TLR2- **(A)** and TLR4-specific **(B)** NF-κB activation was induced either by a BPE or by LPS. Either the TLR2 or the TLR4 receptor complex was overexpressed in HEK293 cells. Cells were stimulated either with different concentrations of LPS or the BPE, starting from 10 pg/mL to 100 ng/mL. By 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. *NF-kB*, Nuclear factor kappa B; *unstim*, unstimulated.

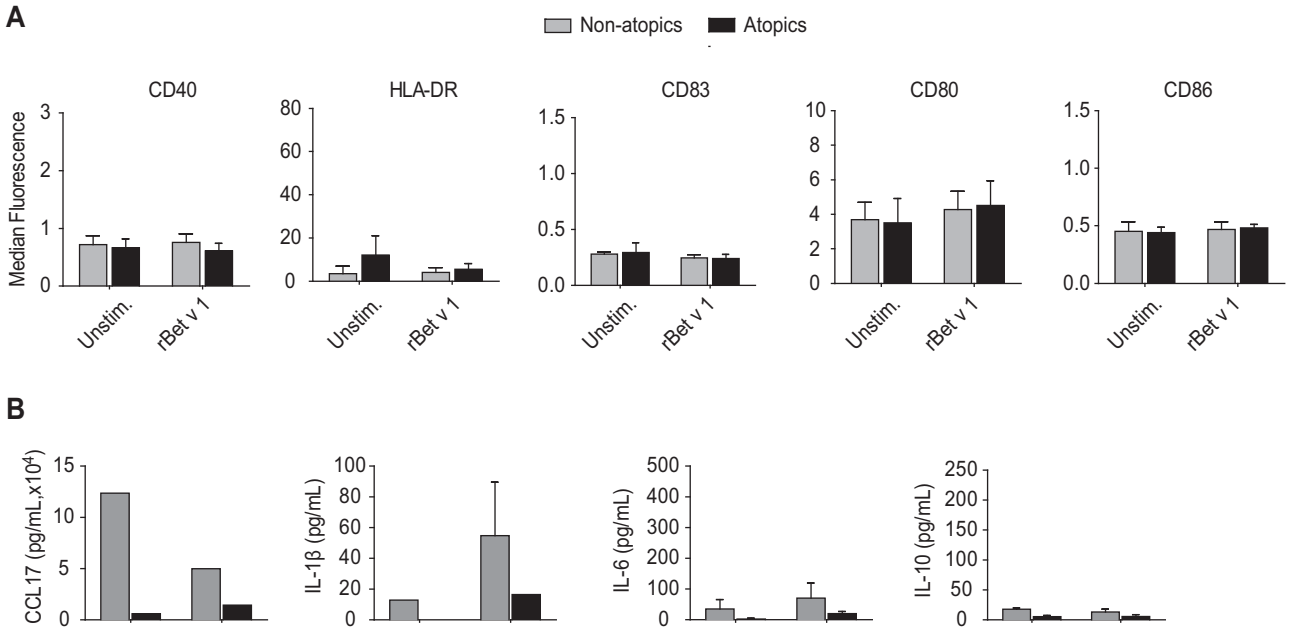

FIG E2. Activation **(A)** and cytokine expression **(B)** of human moDCs induced by rBet v 1. *Unstim.*, Unstimulated. Error bars indicate mean and SEM (*$P \leq .05$; **$P \leq .01$; ***$P \leq .001$).

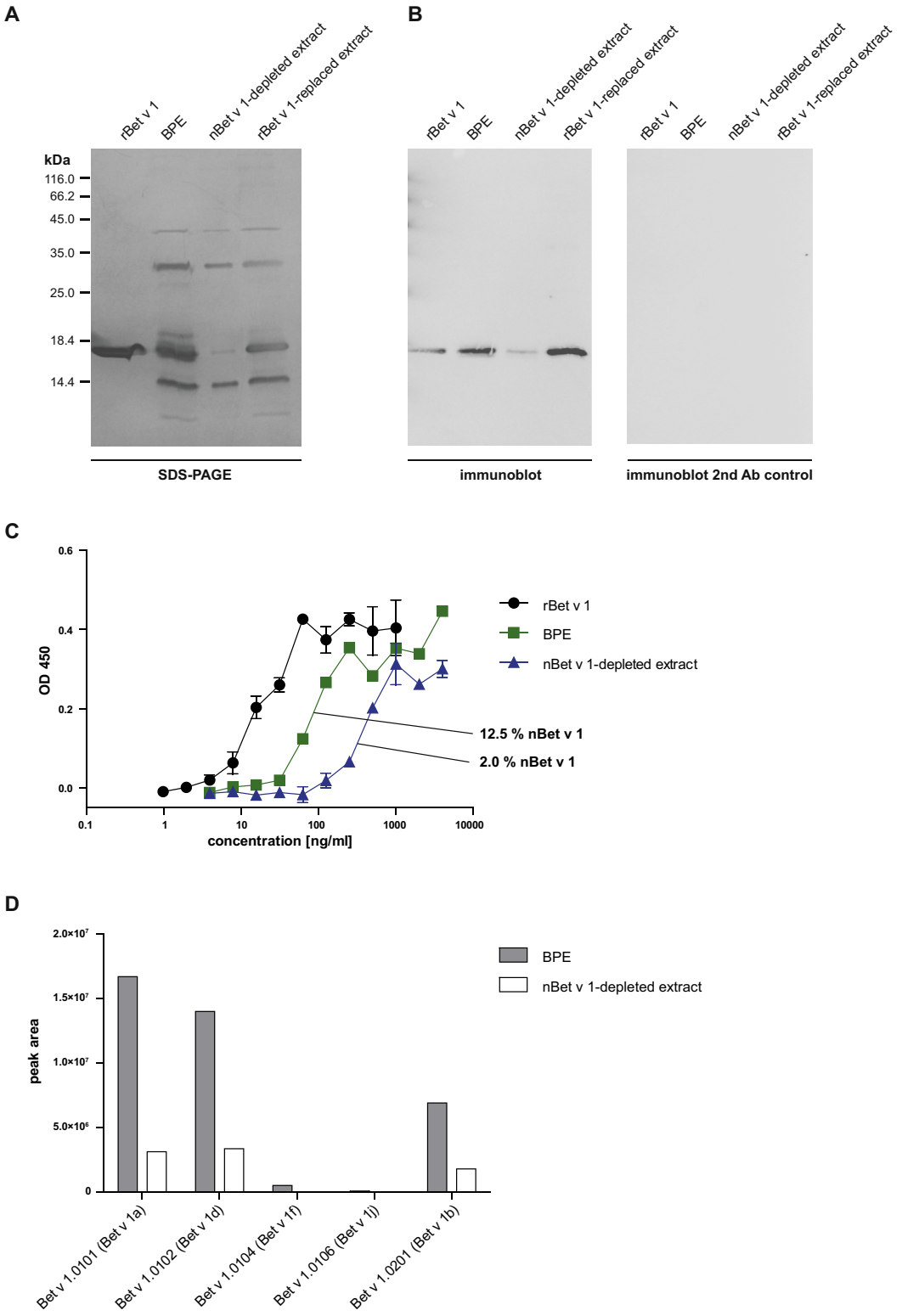

FIG E3. Depletion of nBet v 1 from BPE and replacement with rBet v 1. The BPE was depleted of Bet v 1 by immunoprecipitation using allergen-specific mAbs. With this procedure, it was possible to reduce the amount of nBet v 1 measured within the extract from 12.5% to 2%. SDS-PAGE analysis of the samples used in the 4get experiment (Fig 2, *B*): 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.

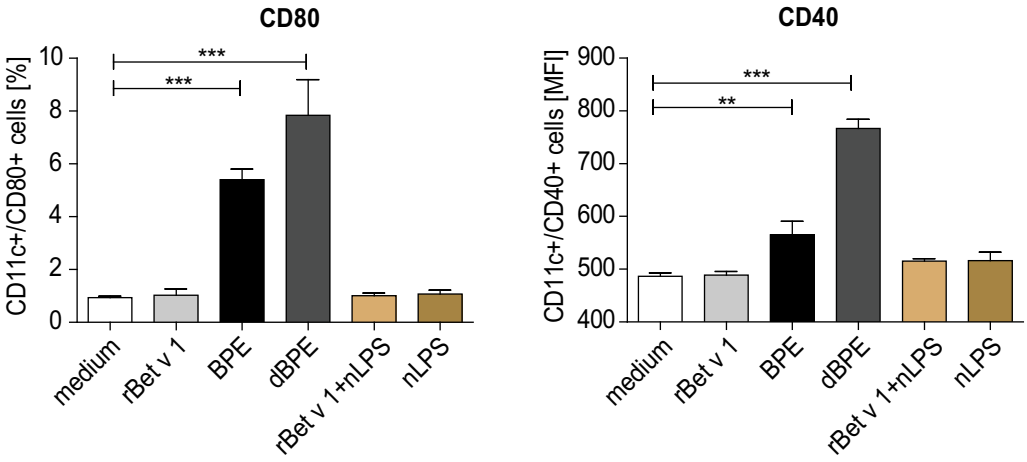

FIG E4. Activation of $CD11c^+$ mBMDCs isolated from 4get mice on stimulation with BPE, nBet v 1–depleted BPE (dBPE), or rBet v 1 ± nLPS for 24 hours. The expression of the surface activation markers CD80 and CD40 was analyzed.