

1 ONLINE REPOSITORY

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

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## 23 **Supplementary Materials and Methods**

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

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

### 33 Aqueous birch pollen extract

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

### 38 Purification of natural Bet v 1

39 For the purification of natural Bet v 1 (nBet v 1), an extract of 5 mg of *Betula pendula* (Allergon AB,  
40 Ängelholm, Sweden) pollen, prepared in a buffer consisting of 50 ml of endotoxin-free H<sub>2</sub>O and 0.5 M  
41 NaCl, was shaken for 5 min at room temperature at 1,400 rpm and then centrifuged for 5 min at  
42 12,000 x g at 4°C; the resulting supernatant was filtered (0.45-µm filter, GE Healthcare Biosciences,  
43 Little Chalfont, UK). Natural Bet v 1 was purified by a combination of hydrophobic chromatography  
44 on a 10-ml phenyl Sepharose column and size-exclusion chromatography using a Superdex 75 10/300  
45 GL column (both from GE Healthcare Biosciences). The purified protein was physicochemically  
46 characterized and stored at -20°C. The nBet v 1 preparation represented a heterogeneous mixture of  
47 the following isoforms, as determined by mass spectrometry: Bet v 1a (MS-score 492.42, coverage  
48 93.13), Bet v 1f (MS-score 507.42, coverage 73.75), Bet v 1g (MS-score 401.48, coverage 70.63), Bet v  
49 1m (MS-score 454.27, coverage 67.50) and several other Bet v 1-derived fragments.

### 50 Determination of LPS level in BPE

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

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

#### 65 In vitro antigen uptake and maturation of mBMDCs

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

#### 90 Stimulation of human moDCs and analysis of cytokine profile

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

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

#### 107 *In vitro* stimulation of human naïve CD4<sup>+</sup> T-cells and analysis of mRNA expression

108 Naïve CD4<sup>+</sup> T-cells were isolated from PBMCs of healthy and anonymous blood donors by magnetic  
109 cell separation according to manufacturer's instructions (Naïve CD4<sup>+</sup> T-cell Isolation Kit II, Miltenyi  
110 Biotec, Bergisch Gladbach, Germany). The blood samples were provided by the university hospital for  
111 blood group serology and transfusion medicine in Salzburg. T-cells were seeded in 48-well plates at a  
112 density of  $2 \times 10^6$  cells per mL in IMDM (PAA Laboratories GmbH, Austria) supplemented with 5% FCS  
113 i.a. (PAA), 2 mM L-Glutamine, 100 U/mL penicillin (Sigma-Aldrich, Inc., St. Louis, MO, USA), 100 µg/mL  
114 streptomycin (Sigma) and stimulated with 0.1 µg/mL αCD3 (Anti-Human CD3 Functional Grade®  
115 Purified, Clone: OKT3, eBioscience), 2.5 µg/mL αCD28 (Purified NA/LE Mouse Anti-Human CD28, BD  
116 Pharmingen) and 0.01% and 0.1% of BPE. After 7 days cells were re-stimulated for another 6 hours  
117 and T-cell cytokine mRNA expression was measured by qPCR. Total RNA was isolated by using TRI  
118 Reagent (Sigma) and reverse-transcribed into cDNA with RevertAid H Minus Reverse Transcriptase  
119 (Thermo Fisher Scientific). mRNA expression was analyzed relatively to the large ribosomal protein  
120 P0 (RPLP0) by quantitative real-time PCR (Rotor-Gene Q, Rotor-Gene Q Series Software, Quiagen,  
121 Hilden, Germany) with iQ™ SYBR® Green Supermix (Bio-Rad, CA, USA) and the following primers:  
122 RPLP0: forward 5' - GGCACCATTGAAATCCTGAGTGATGTG - 3', reverse 5' -  
123 TTGCGGACACCCTCCAGGAAG - 3', IL-5: forward 5' - CCTTGGCACTGCTTTCTACTCATCG - 3', reverse 5' -  
124 GGGTTTACTCTCCGTCTTCTCTCCACA - 3', IL-13: forward 5' - TGTGCCTCCCTCTACAGCCCTCAG - 3',  
125 reverse 5' - TCAGCATCTCTGGGTCTTCTCG - 3', IL-2: forward 5' -TCCCAAACCTACCAGGATGCTCAC - 3',  
126 reverse 5' -AATGTTGTTTCAGATCCCTTTAGTTCCAGA - 3', TNF-α: forward 5'-  
127 CAAGCCTGTAGCCCATGTTG -3', reverse 5'- GAGGTTGACCTTGGTCTGGTA -3'. Statistical significance  
128 was determined by one-way ANOVA, Tukey post-test (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).  
129 According to the Austrian national regulations, informed consent in the case of anonymous blood  
130 cells discarded after plasmapheresis (buffy coats) is not required. Thus, there is no requirement for  
131 additional approval by the local ethics committee.

132

#### 133 Bet v 1 depletion by immunoprecipitation

134 Depletion was performed by immunoprecipitation using mouse monoclonal anti-Bet v 1.0101  
135 antibodies, which were generated by hybridoma technology and purified using Bet v 1.0101-coupled  
136 NHS Sepharose™ 4 Fast Flow (GE Healthcare Biosciences, Little Chalfont, UK) material. A total of 1 ml  
137 of Protein G Sepharose™ 4 Fast Flow (also GE Healthcare Biosciences) was pre-incubated with PBS  
138 containing 0.1% BSA to saturate the nonspecific binding sites of the Protein G Sepharose™ material.  
139 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  
140 shaken overnight at 4°C. The suspension was incubated with 1 ml of the Protein G Sepharose™ slurry  
141 over 30-60 min at room temperature. The suspension was centrifuged for 15 min at 14,000 x g, and  
142 the supernatant containing the Bet v 1-depleted extract was collected. The concentration of the  
143 depleted extract was determined by Bradford assay. The depleted extract was analyzed by sodium  
144 dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. For the  
145 immunoblot assay, the same monoclonal anti-Bet v 1.0101 antibody was used as a primary antibody.  
146 As a secondary antibody, an alkaline phosphatase-conjugated rabbit anti-mouse IgG + IgM antibody  
147 (Jackson ImmunoResearch Europe Ltd., Oaks Drive Newmarket, Suffolk, UK) was used at a

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

#### 157 Immunization of 4get mice

158 4get mice (Jackson laboratory, Bar Harbor, ME, USA) were immunized with either BPE (n=5) or rBet v  
159 1 (n=3) in PBS, without added adjuvants. For the immunizations, 65 µg of BPE were injected, and the  
160 amount of injectable rBet v 1 was calculated according to the total Bet v 1 quantified within the BPE.  
161 Skin-draining inguinal lymph nodes of immunized mice, as well as of naïve mice were collected.  
162 Lymphocytes were stained with an APC-conjugated anti-mouse CD4 antibody and analyzed for IL-  
163 4/eGFP expression using flow cytometry (BD Biosciences, San Jose, CA, USA). In a second experiment,  
164 a different batch of BPE (n=5) was used and compared with a Bet v 1-depleted version of this extract  
165 (nBet v 1-depleted extract, n=5) as well as a reconstituted version of the depleted extract (rBet v 1-  
166 replaced extract, n=5). The results were compared with those of naïve 4get mice. Statistical analysis  
167 was performed using one-way ANOVA with a Bonferroni post-test to compare all groups (\* $P \leq 0.05$ ;  
168 \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ). All *in vivo* experiments were performed according to national guidelines  
169 approved by the Austrian Federal Ministry (BMWF-66.012/0010-II/3b/2013).

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#### 171 **References**

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183

184 **Figure Legends**

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

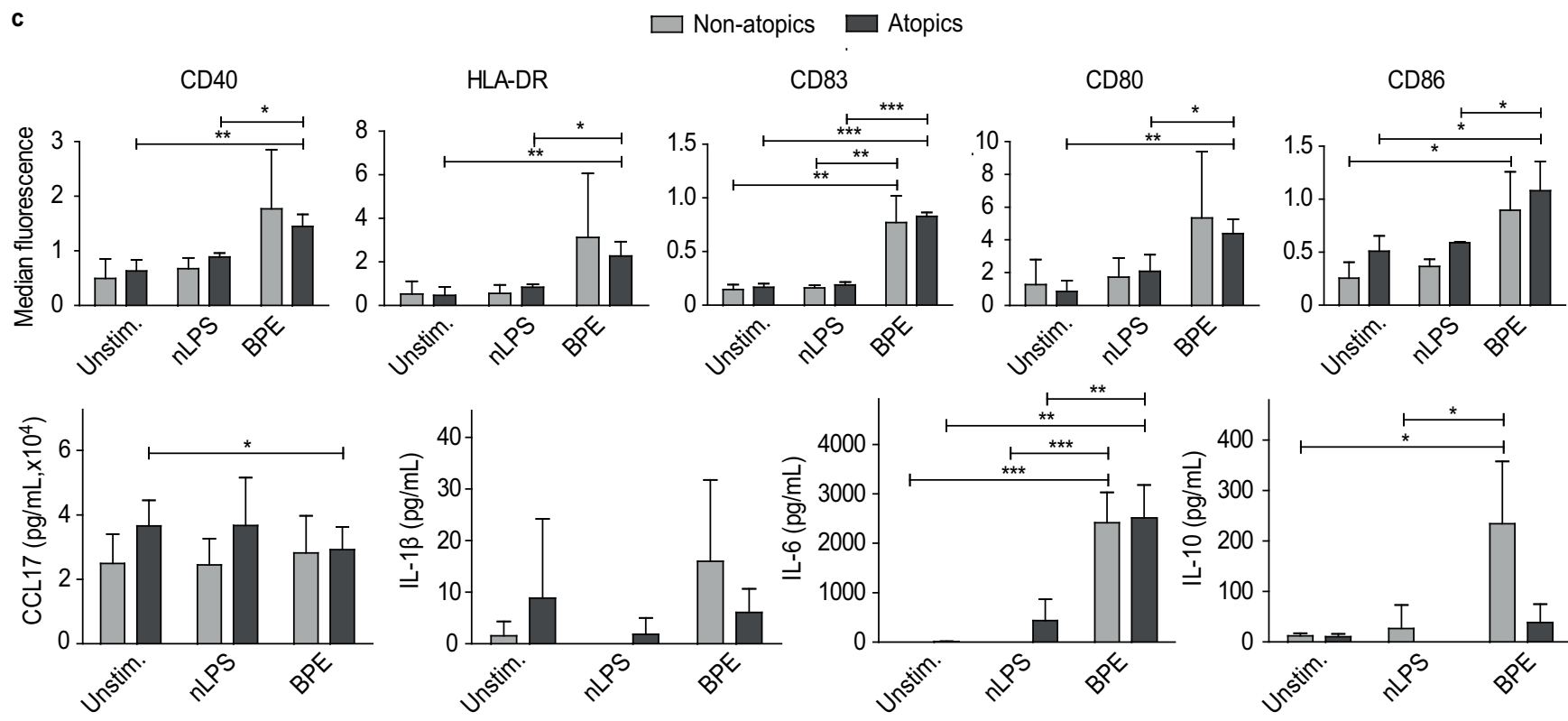
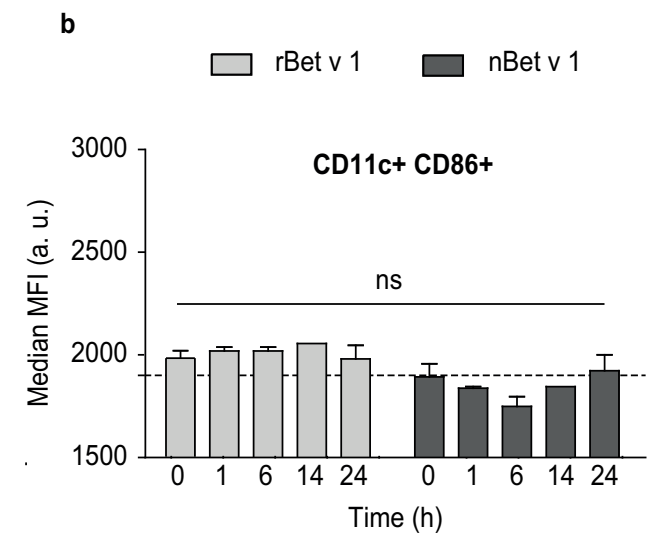
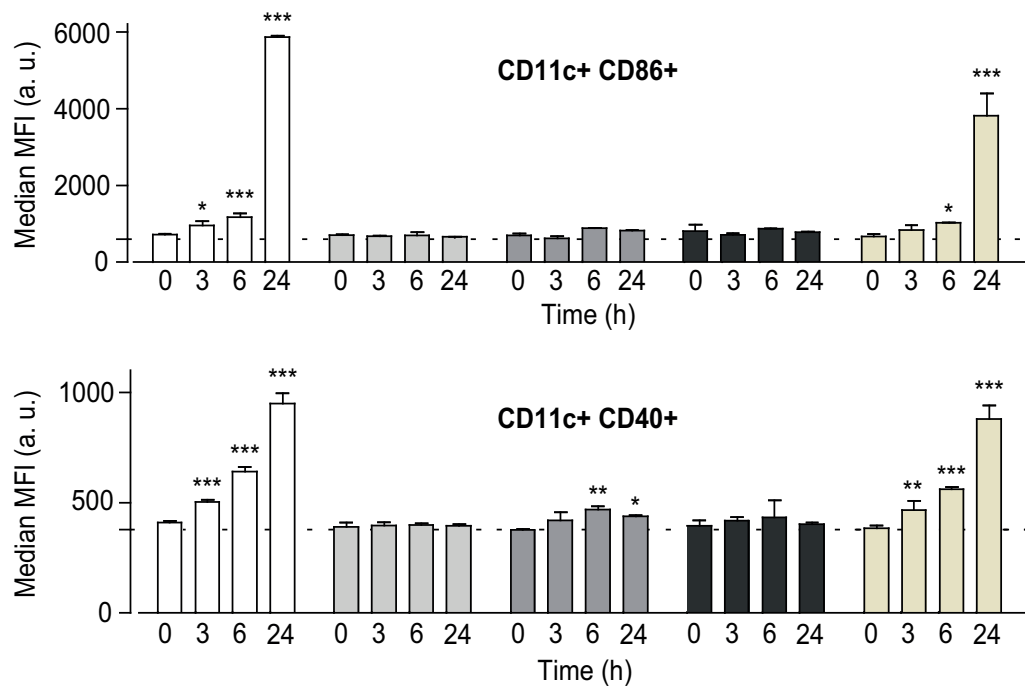
191 **FIG E2.** Activation (a) and cytokine expression (b) of human moDCs induced by rBet v 1. Error bars  
192 indicate mean and SEM (\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ).

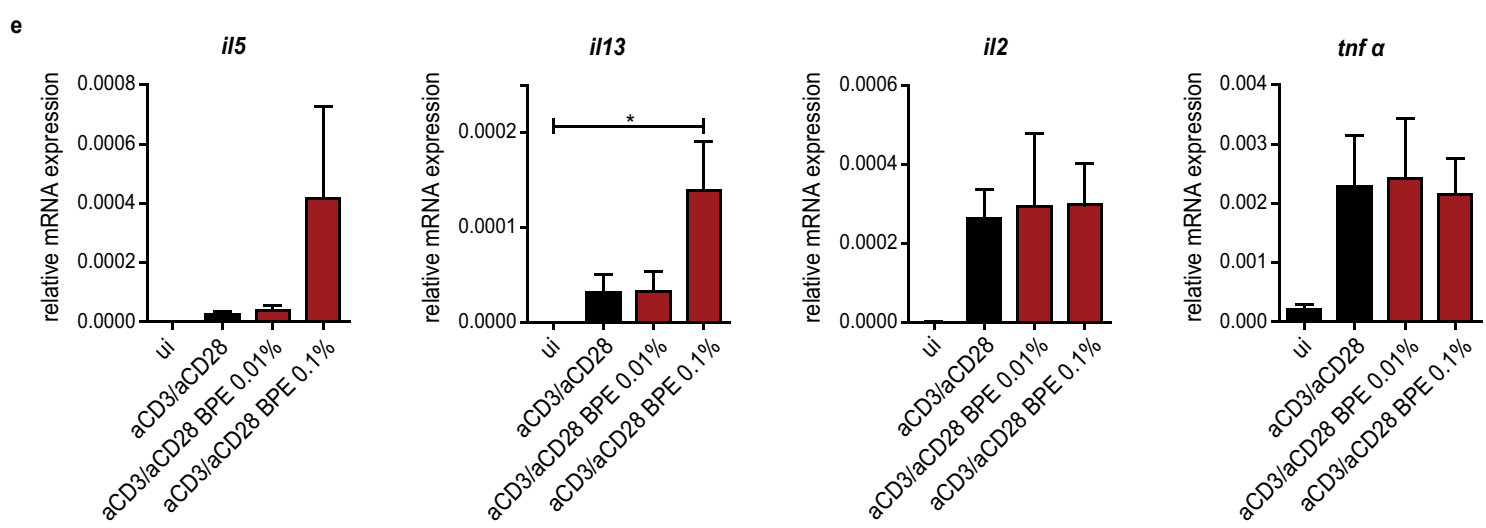
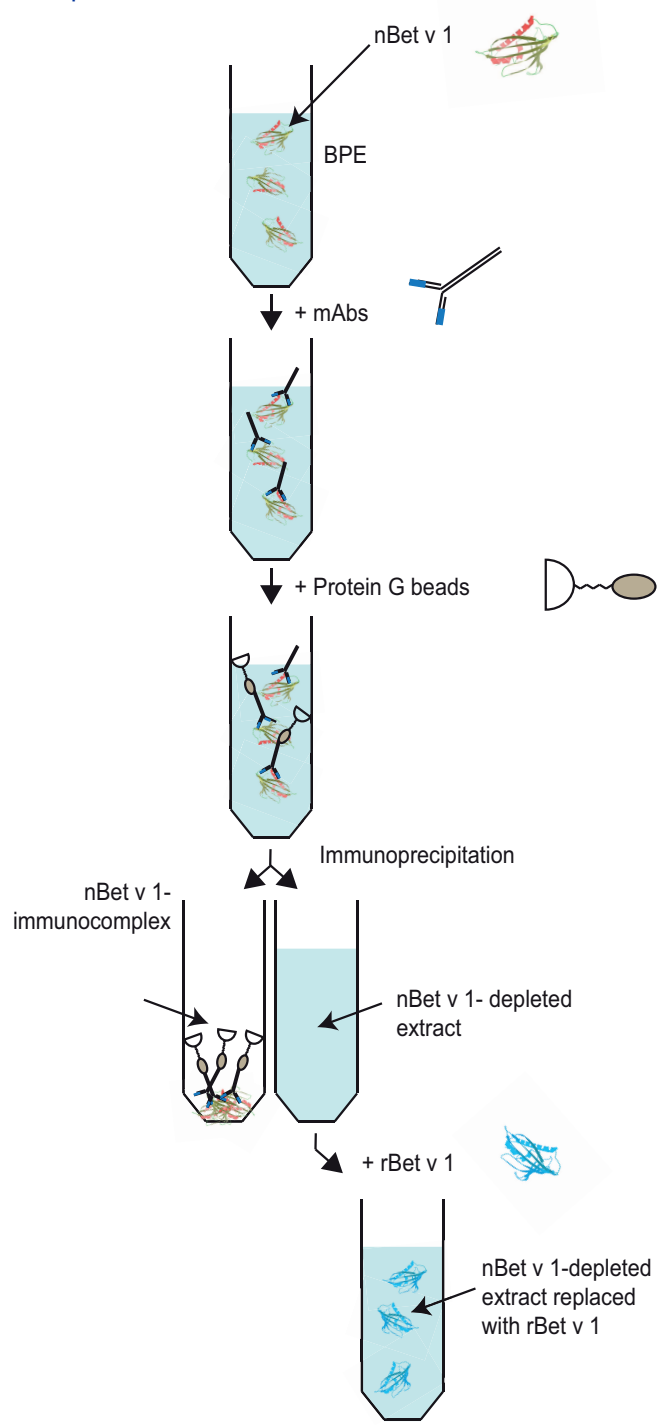
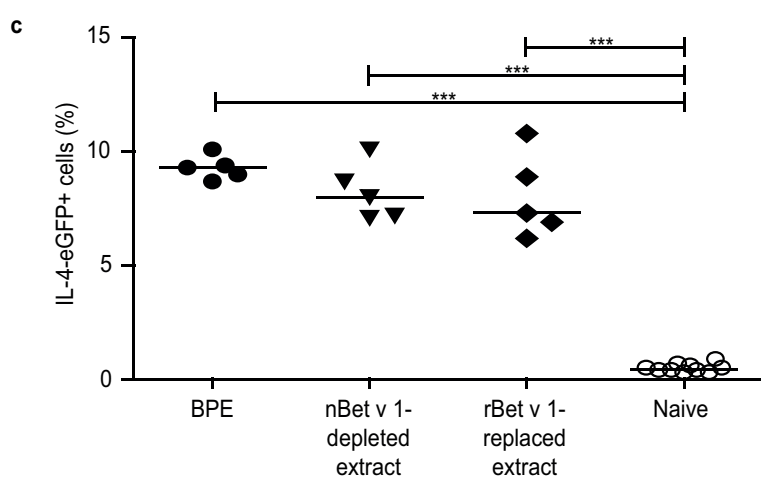
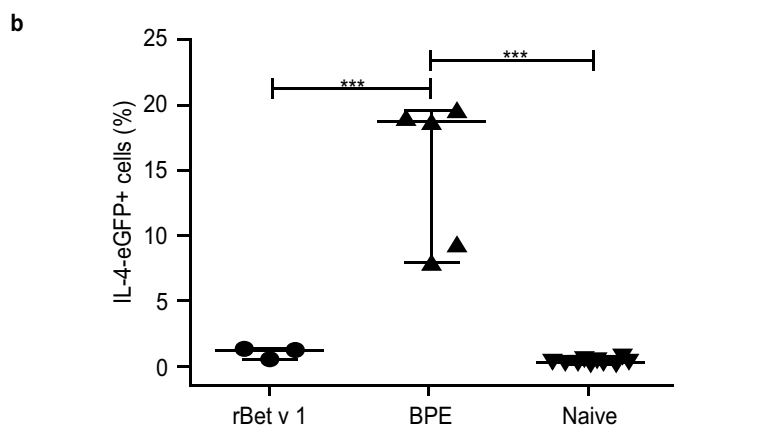
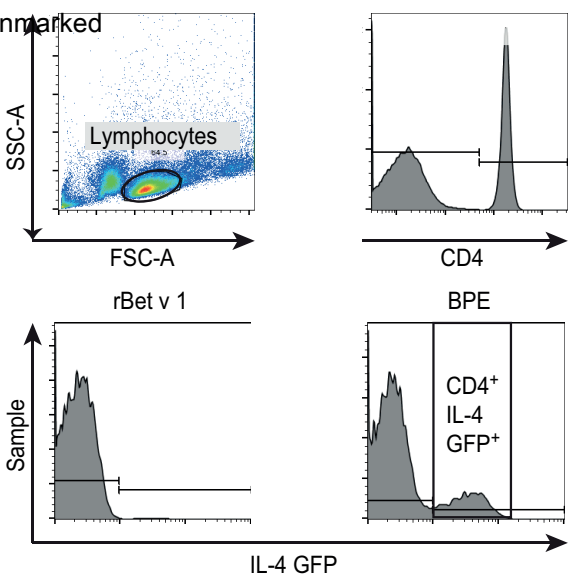
193 **FIG E3.** Depletion of nBet v 1 from birch pollen extract and replacement with rBet v 1. The birch  
194 pollen extract was depleted of Bet v 1 by immunoprecipitation using allergen-specific monoclonal  
195 antibodies. With this procedure, it was possible to reduce the amount of nBet v 1 measured within  
196 the extract from 12.5% to 2%. SDS-PAGE analysis of the samples used in the 4get experiment (Fig 2b):  
197 rBet v 1, birch pollen extract, nBet v 1-depleted extract and rBet v 1-replaced extract (a). Immunoblot  
198 of SDS-PAGE samples using a monoclonal mouse anti-Bet v 1 antibody as the primary antibody (b).  
199 The reduction of nBet v 1 within the nBet v 1-depleted extract was quantified by sandwich ELISA (c).  
200 The rBet v 1-replaced extract was reconstituted equivalently to the 12.5% of nBet v 1 found in the  
201 original extract. The question whether or not all nBet v 1 isoforms were reduced by  
202 immunoprecipitation was addressed by mass spectrometry (d). For this purpose, the control proteins  
203 Bet v 2 and Bet v 7 were used to normalize the mass spectrometry data. All the isoforms found in the  
204 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  
205 after the immunoprecipitation.

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

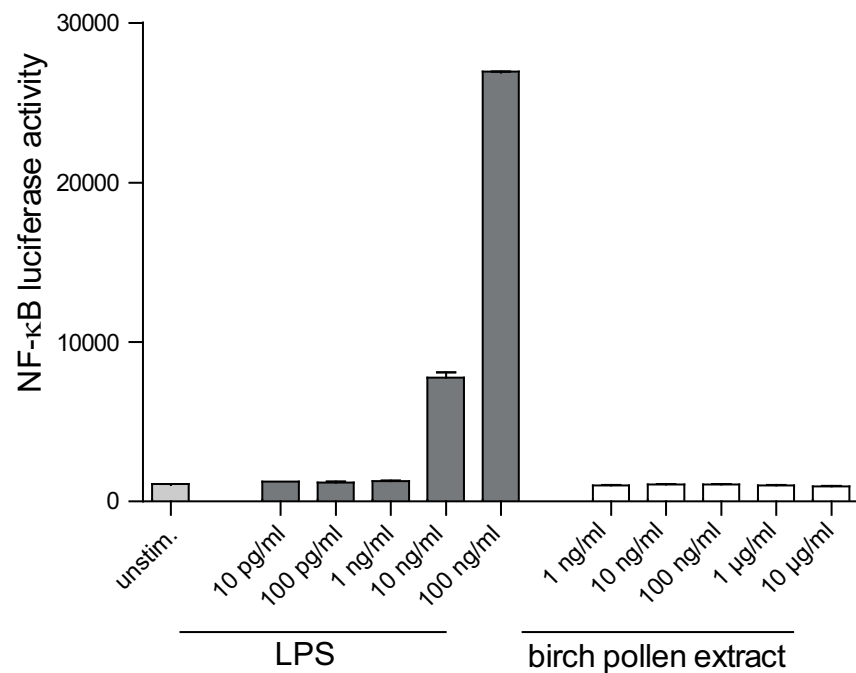
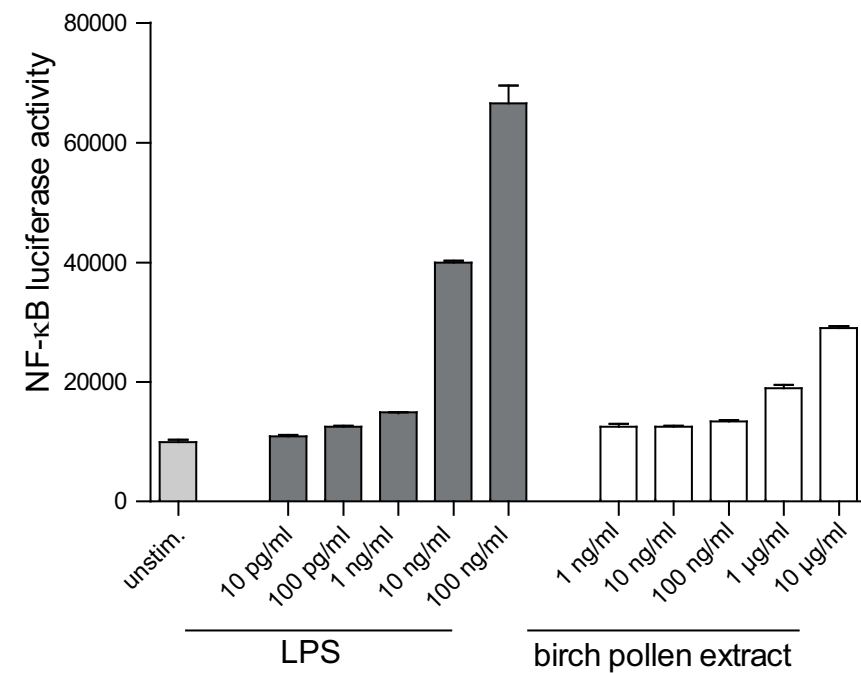
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□ BPE    ■ rBet v 1    ■ rBet v 1+nLPS    ■ nLPS    ■ LPS 100ng/ml

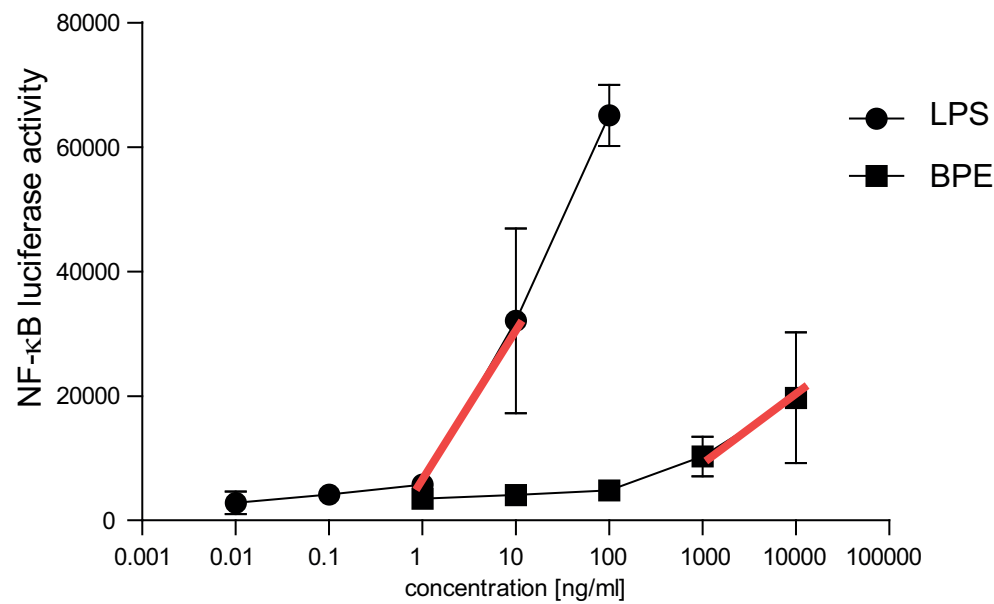


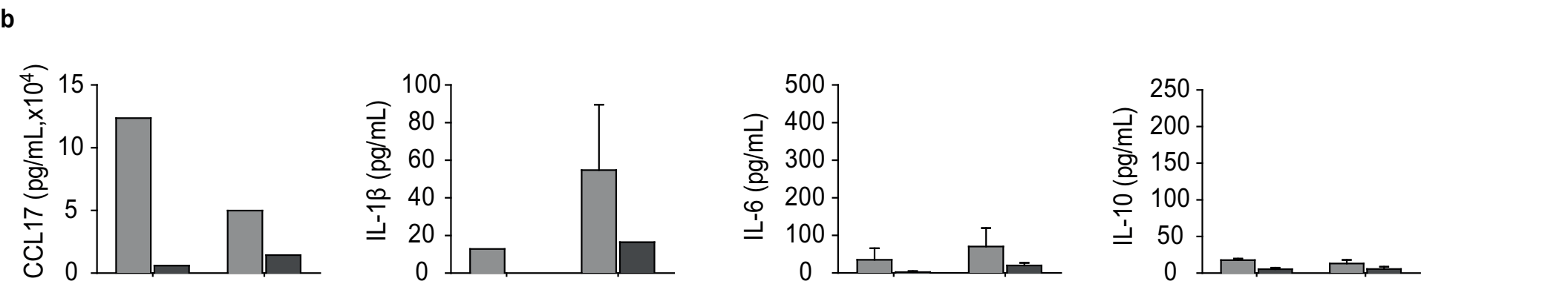
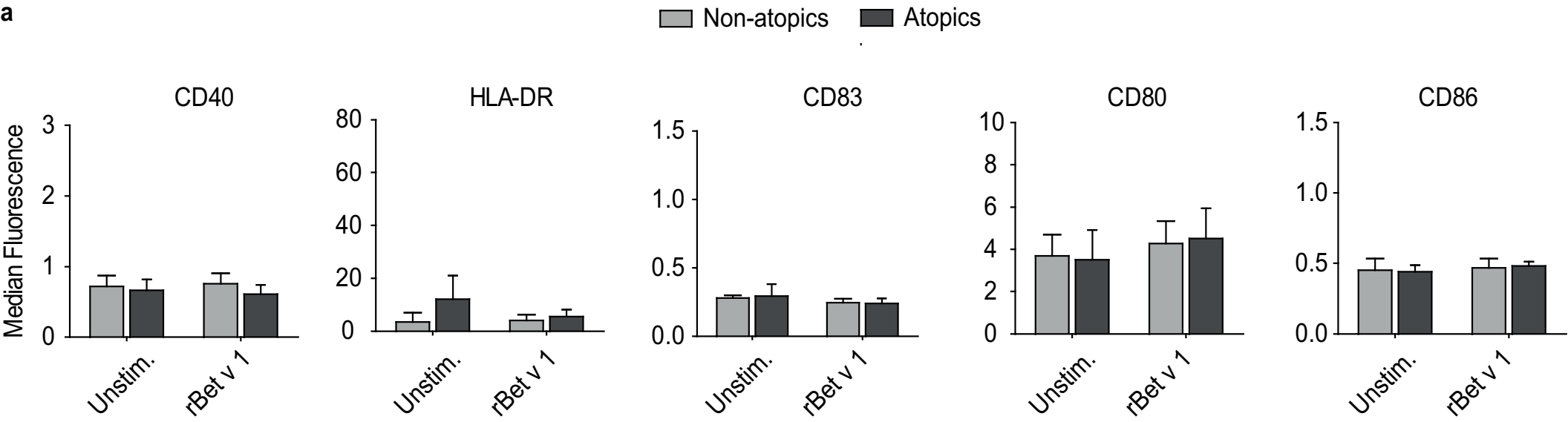


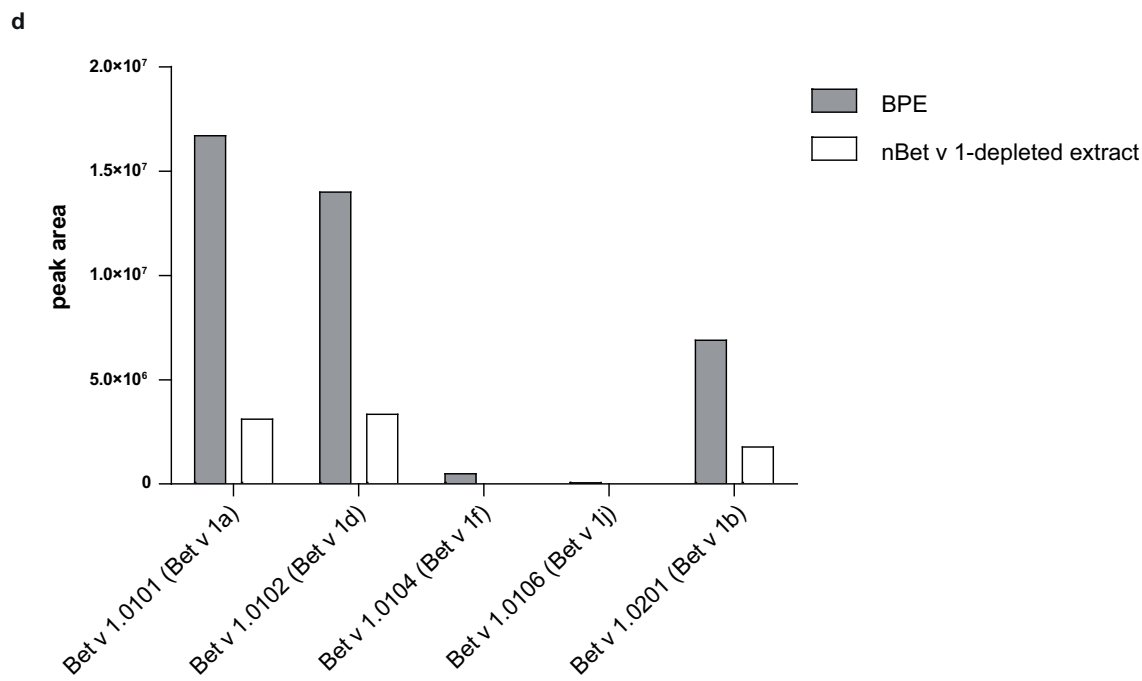
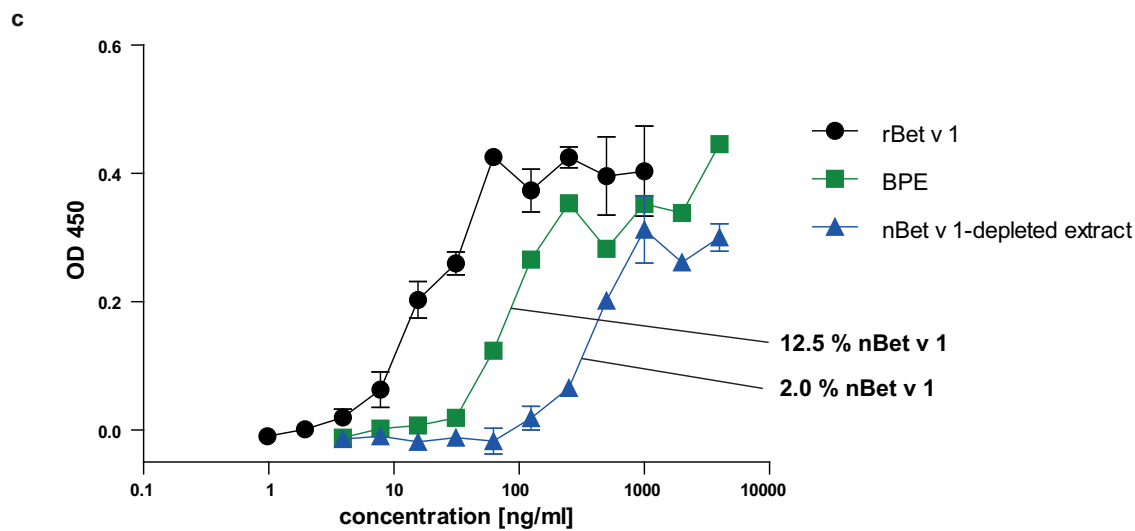
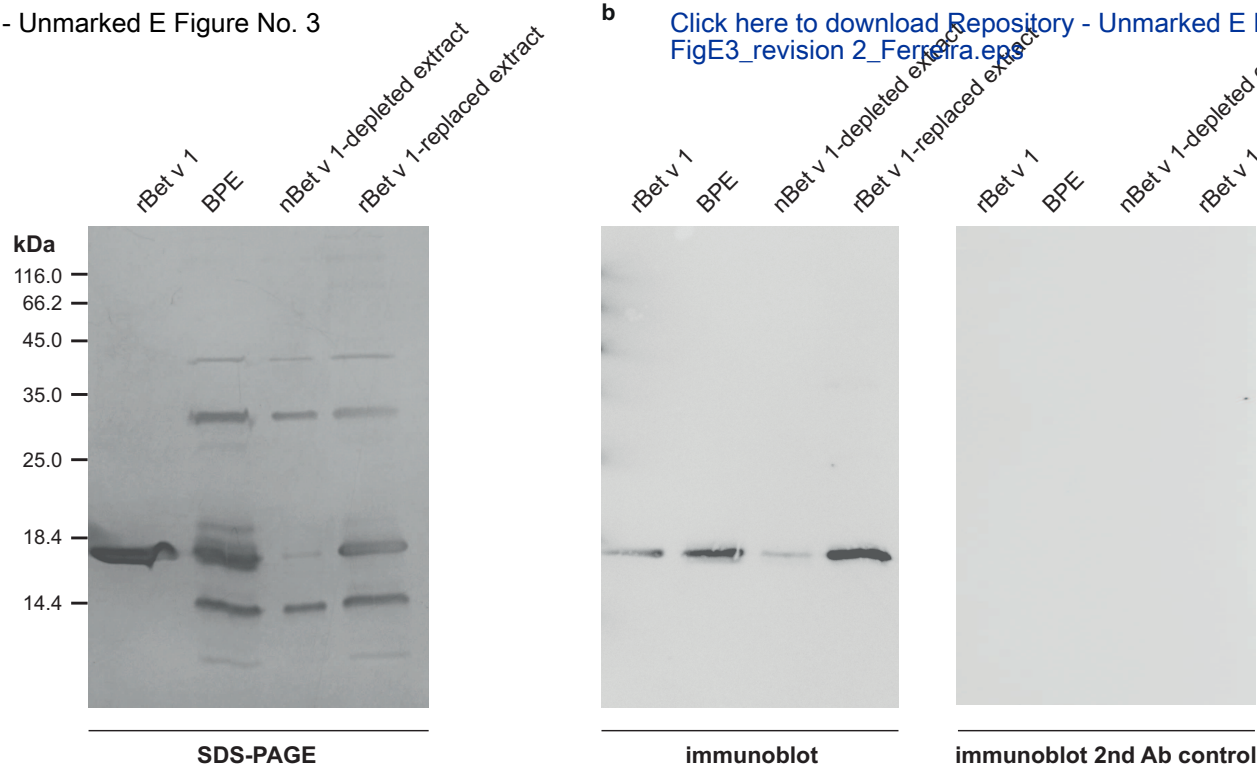


**TLR2/CD14****TLR4/CD14/MD2**

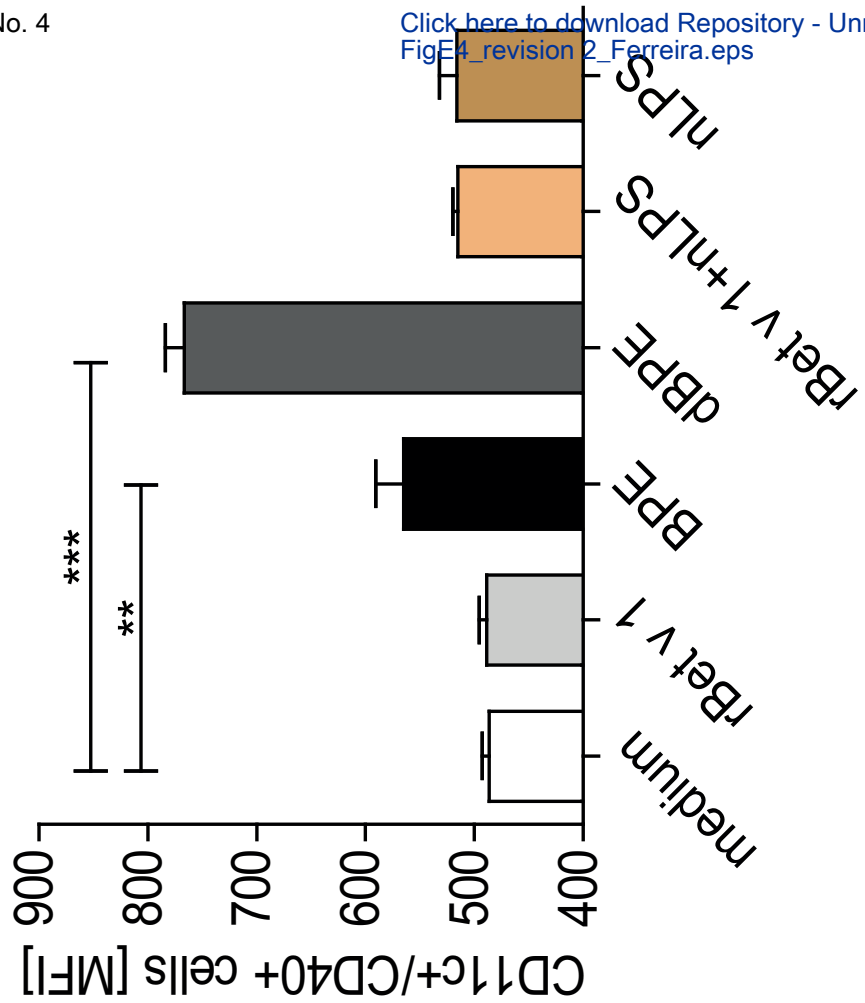
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**TLR4/CD14/MD2**





**CD40**



**CD80**

