# METHODS

## Bioinformatics analysis of sequence and fold variations

For the design of hybrid molecules, overlapping fragments of the parental allergens Bet v 1.0102, Cor a 1.0401, and Mal d 1.0108 were recombined. Structural models of Bet v 1.0102, Cor a 1.0401, Mal d 1.0108, MBC, and MBC4 were built by using MODELLER.[E1] The PDB entry 4a84 was selected to obtain a common structural baseline for all models. This PDB entry was identified by using a BLAST search with all 5 sequences as queries. Protein Data Bank (PDB) entry 4a84 was the hit with the best rank sum. Subsequently, for each target, 100 models were generated by using MODELLER in automodel mode. The models were then scored by using 2 statistical scoring functions, as provided by MAESTRO software.[E2] The corresponding configuration files are available from the MAESTRO Web site (https://biwww.che.sbg.ac.at/maestro/). The resulting scores reflect how well a certain sequence fits to a given fold. Structural similarities between the different models were determined with the MatchMaker tool, as provided by using UCSF Chimera.[E3]

## Cloning of MBC and MBC4

MBC was cloned by combining 3 overlapping fragments from Mal d 1.0108 (AF126402), Bet v 1.0102 (X77266), and Cor a 1.0401 (AF136945), respectively. Fragments were amplified by using PCR from parental allergens with the primers MBC forward (5′CATGCCATGGgtgtgtacaccttcgagaac3′) and MBC reverse (5′CCGGAATTCTTAacagtaggcatcagggtgTG3′), MalBet ward (5′CAAGCAAGCTGAAATCCTTGAAGGAAACGGTGGCCCCGGAACC ATCAAGAAGATCA3′), BetCor reverse (5′CATTGATTGAAGCGTTGC CCTTGGTGTGGTACTTGTTGC3′), BetCor forward (5′GCAACAAG TACCACACCAAGGGCAACGCTTCAATCAATG3′), and MBC reverse (5′CCGGAATTCTTAACAGTAGGCATCAGGGTGTG3′); restriction sites are underlined. Thereafter, PCR products were separated on a 1.4% agarose gel, and bands were excised and centrifuged at 14,000*g* for 20 minutes at 4°C and subjected in a 1:1:1 ratio to 5 cycles of a primerless PCR. After addition of the primer pair MBC forward and MBC reverse, reassembled genes were amplified and cloned into a pET28b vector (Novagen, Merck Millipore).

MBC4 was generated by using PCR with MBC as a template. The primer pairs MBC forward and MBC4, BM4 forward (5′GCAACCC CTagTGGAaGcaCCATCaaGAgtATCAGCAAC3′), BM4 reverse (5′GTT GCTGATacTCttGATGGtgCtTCCActAGGGGTTGC 3′), and MBC4 reverse (5′CCGGAATTCTTACGA GTAGGCATCAGGGTG3′) were used to amplify 2 fragments containing the desired mutations.[E4] Both fragments were separated on a 1.4% agarose gel, and bands were excised and centrifuged at 14,000*g* for 20 minutes at 4°C and subjected in a 1:1 ratio to 5 cycles of a primerless PCR. After addition of the primer pair MBC forward and MBC4 reverse, reassembled genes were amplified and cloned into a pET28b vector (Novagen).

## Recombinant production of MBC and MBC4

MBC was produced as a recombinant protein in *E coli* BL21 Star (DE3; Thermo Fisher). Freshly transformed cells were grown in LB medium supplemented with 2 mmol/L $MgSO_4$, 1% (vol/vol) glycerol, 0.2% (wt/vol) ammonium sulfate, 10 mmol/L sodium phosphate (pH 7.4), and 25 mg/L kanamycin to an $OD_{600}$ of 0.8 at 37°C and thereafter induced with 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside for 5 hours at 37°C. After harvesting, cells were resuspended in 20 mmol/L NaP (pH 8) and 0.5 mol/L urea, followed by 3 consecutive freeze/thaw cycles. Thereafter, 50 mmol/L $NaH_2PO_4$ and 0.5 mol/L NaCl were added on ice under constant stirring. The protein was subjected to a first purification step with a Phenyl Sepharose Column (GE Healthcare). MBC-containing fractions were dialyzed against 20 mmol/L imidazole (pH 7.4) and further purified with a DEAE column (GE Healthcare). Final polishing was performed with a Superdex 75 10/300 column (GE Healthcare). Purified proteins were stored at −20°C in 10 mmol/L NaP (pH 7.4).

Recombinant MBC4 was purified from bacterial inclusion bodies. Therefore the MBC4 construct was transformed into competent *E coli* BL21 Star (DE3) cells (Thermo Fisher). Positive transformants were cultured in LB medium supplemented with 2 mmol/L $MgSO_4$, 1% (vol/vol) glycerol, 0.2% (wt/vol) ammonium sulfate, 10 mmol/L sodium phosphate (pH 7.4), and 25 mg/L kanamycin. Protein expression was induced at an $OD_{600}$ of 0.7 by addition of 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside and cultured for 5 hours at 37°C. Bacterial cells were harvested after 6 hours of expression at 37°C by means of centrifugation at 4000*g* for 20 minutes and thereafter resuspended in 50 mmol/L Tris base, 1 mmol/L EDTA, and 0.1% Triton X-100. Lysis was performed by using 3 consecutive freeze/thaw cycles in liquid nitrogen and at 37°C, respectively. The pellet was washed with 50 mmol/L Tris base, 1 mmol/L EDTA, and 1% Triton X-100, followed by a wash step in 5 mmol/L sodium phosphate buffer (pH 7.4) containing 25% ethanol. Finally, the pellet was resolved in 20 mmol/L sodium phosphate buffer (pH 7.4) and 6 mol/L urea. The protein solution was filtered through a Millex-HPF HV filter unit (Merck Millipore) and loaded onto a Q FF anion exchange column (GE Healthcare). The flowthrough was collected, and the urea concentration was reduced to 4 mol/L. After addition of 2.5 mol/L NaCl, the protein solution was loaded on a Capto-Phenyl Column (GE Healthcare). Elution was performed with a 100-mL gradient to 20 mmol/L sodium phosphate buffer (pH 7.4) and 6 mol/L urea. Thereafter, the protein was renatured gradually by means of dialysis against 10 mmol/L sodium phosphate buffer (pH 7.4) and 4 mol/L urea, 3 mol/L urea, 1.5 mol/L urea, and 0 mol/L urea, respectively, and thereafter stored at −20°C.

## Antibody-binding analyses

ELISA experiments were performed to measure human serum IgE binding to parental allergens, as well as MBC4. Therefore MaxiSorp plates (Nunc, Thermo Fisher) were coated with 100 ng per well of protein antigen in 50 μL of PBS overnight at 4°C. After washing and blocking, plates were incubated with 1:10 diluted human serum samples in Tris-buffered saline (pH 7.4), 0.05% (vol/vol) Tween, and 0.5% (wt/vol) BSA overnight at 4°C. Bound human IgE was detected with an alkaline phosphatase (AP)–conjugated mouse anti-human IgE antibody (BD Biosciences, Franklin Lakes, NJ). Measurements were performed on a Tecan Sunrise (Tecan, Mannedorf, Switzerland) plate reader at 405 nm by using the substrate 4-nitrophenyl phosphate (Applichem GmbH, Darmstadt, Germany). Both murine $IgG_1$ and $IgG_{2a}$ were detected with AP-conjugated rat anti mouse $IgG_1$ antibodies or AP-conjugated rat anti-mouse $IgG_{2a}$ antibodies, respectively (both from Southern Biotech, Birmingham, Ala). After incubation for 1 hour at 37°C and 1 hour at 4°C, colorimetric detection with 10 mmol/L 4-nitrophenyl phosphate (Applichem GmbH) was performed, as previously described.

Mediator release assays with human IgE antibodies were performed with RBL-2H3 cells transfected with the α chain of human FcεRI. Cells were passively sensitized with patients' sera diluted in 1:20 in tissue culture medium overnight at 37°C and 7% $CO_2$.[E4] After several washing steps, antigen was added in serial dilutions in Tyrode buffer (Sigma, St Louis, Mo) supplemented with 1 g/L sodium bicarbonate, 0.1% (wt/vol) BSA, and 50% (vol/vol) deuterium oxide (Sigma). Antigen-dependent β-hexosaminidase release into the supernatant was measured after 1 hour of incubation at 37°C by means of enzymatic cleavage of the fluorogenic substrate 4-methylumbelliferyl-N-acetyl-β-glucosaminide and expressed as a percentage of total enzyme content of Triton X-100–treated cells. Alternatively, release assays were performed with RBL-2H3 cells, and passively sensitized for 1 hour at 37°C and 7% $CO_2$ with a pool of murine sera 1:25 diluted in tissue culture medium, according to the protocol previously described.[E5]

### REFERENCES

E1. Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 1993;234:779-815.

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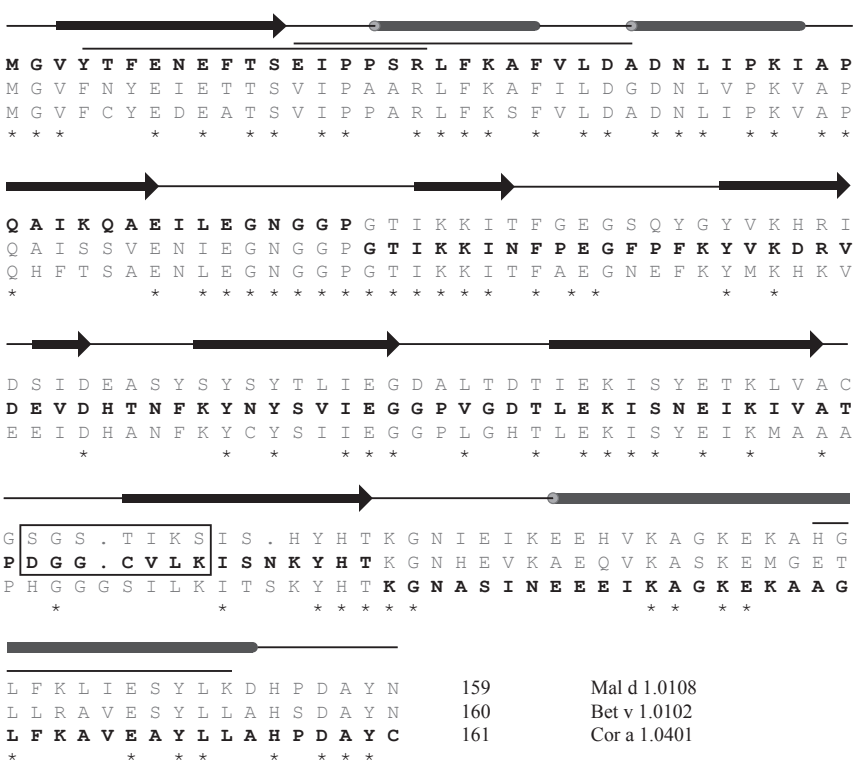
**A**




**B** Amino acid sequence of MBC

MGVYTFENEFTSEIPPSRLFKAFVLDADNLIPKIAP
QAIKQAEILEGNGGPGTIKKINFPEGFPFKYVKDRV
DEVDHTNFKYNYSVIEGGPVGDTLEKISNEIKIVAT
PDGGCVLKISNKYHTKGNASINEEEIKAGKEKAAGL
FKAVEAYLLAHPDAYS

Mal d 1.0108 (residue M1-P51)
Bet v 1.0201 (residue G52-T123)
Cor a 1.0401 (residue K124-S160)

**C** Amino acid sequence of MBC4

MGVYTFENEFTSEIPPSRLFKAFVLDADNLIPKIAP
QAIKQAEILEGNGGPGTIKKINFPEGFPFKYVKDRV
DEVDHTNFKYNYSVIEGGPVGDTLEKISNEIKIVAT
PSGSTIKSISNKYHTKGNASINEEEIKAGKEKAAGL
FKAVEAYLLAHPDAYS

Mal d 1.0108 (residue M1-P51)
Bet v 1.0201 (residue G52-T123)
Cor a 1.0401 (residue K124-S160)
Mutation site (residue S110-S116)

**FIG E1. A,** Alignment of amino acid sequences of Mal d 1.0108 (UniProt ID Q9SYW3), Bet v 1.0102 (UniProt ID P43177), and Cor a 1.0401 (UniProt ID Q9SWR4). **B,** *Boldface letters* indicate the combined sequences to form the hybrid MBC. *Arrows* and *tubes* describe β-sheets and α-helices, respectively. **C,** Seven amino acids from Mal d 1.0108 were introduced into the Bet v 1.0102 sequences, as indicated by the *box*, to generate MBC4.

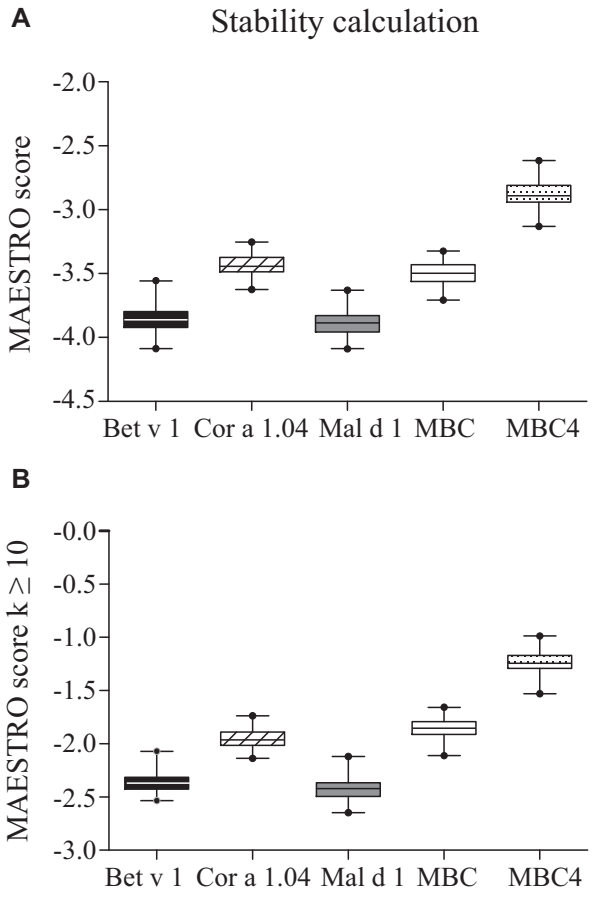



**FIG E2**. MAESTRO statistical scoring functions applied to the different sequences combined with a canonical Bet v 1 fold. Higher scores indicate lower sequence-structure compatibility. **A,** All contact and pair interactions applied. **B,** Local pair interactions (sequence distance, <10) omitted.

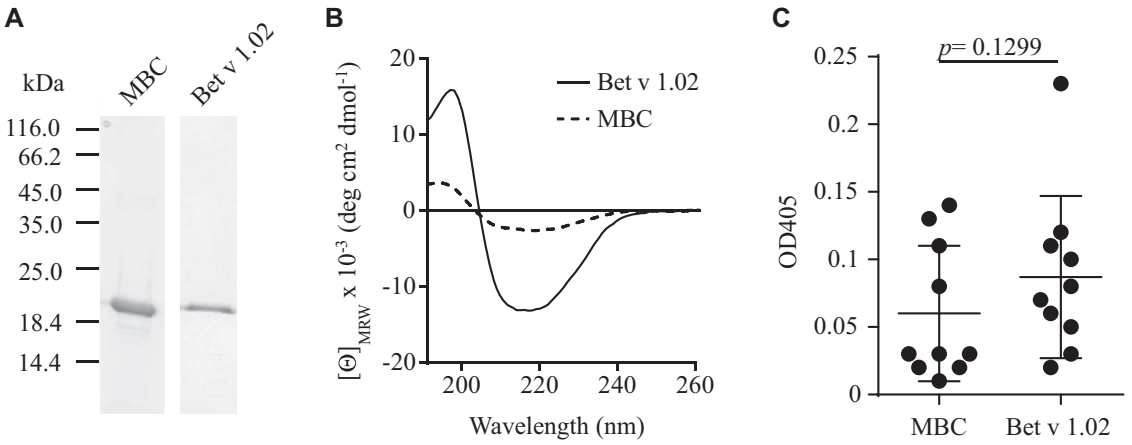



**FIG E3.** **A,** Coomassie-stained 15% SDS-PAGE of purified proteins. **B,** CD spectra of MBC and Bet v 1.0102 were recorded at 20°C and are presented as mean residue molar ellipticity after baseline correction. **C,** IgE ELISA with sera from patients with birch pollen allergy (n = 10). *P* values were calculated with paired-sample *t* tests.

**A**

| protein | theoretical mass [Da] | experimental mass [Da] | Δm [Da] |
|---|---|---|---|
| Bet v 1.0101 | 17439.6 | 17439.7 | 0.1 |
| Cor a 1.0401 | 17621.0 | 17620.6 | 0.4 |
| Mal d 1.0108 | 17548.7 | 17548.2 | 0.5 |
| MBC4 | 17409.6 | 17409.7 | 0.1 |

**B**

| protein | EU/mg |
|---|---|
| Bet v 1.0101 | 1.12 |
| Cor a 1.0401 | 2.50 |
| Mal d 1.0108 | 1.21 |
| MBC4 | 0.99 |

**C**

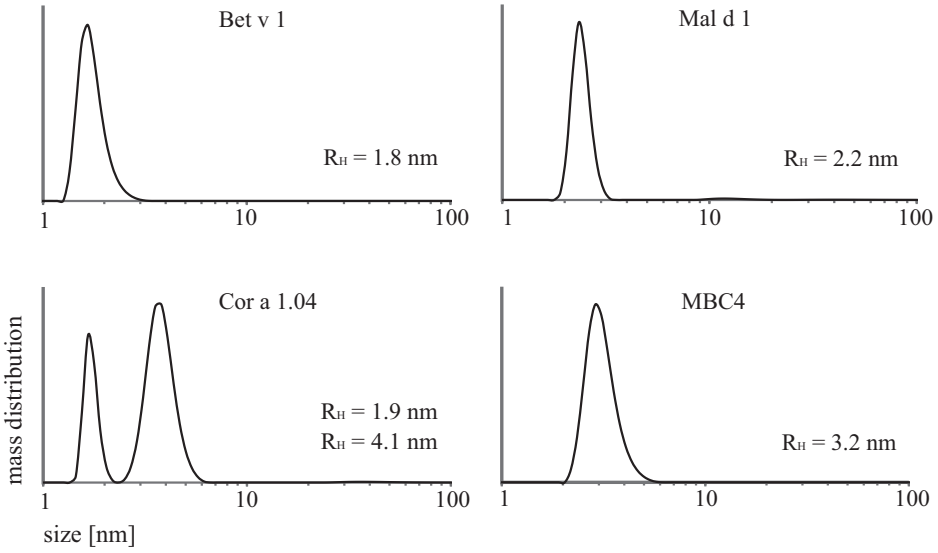



**FIG E4. A** and **B,** Average protein masses were determined by using mass spectrometry (Fig E4, *A*), and endotoxin levels of recombinant proteins were analysed by using the reporter cell line HEK-Blue mTLR4 (Fig E4, *B*). **C,** DLS measurements were performed to determine the hydrodynamic radius of proteins and aggregates thereof in solution.

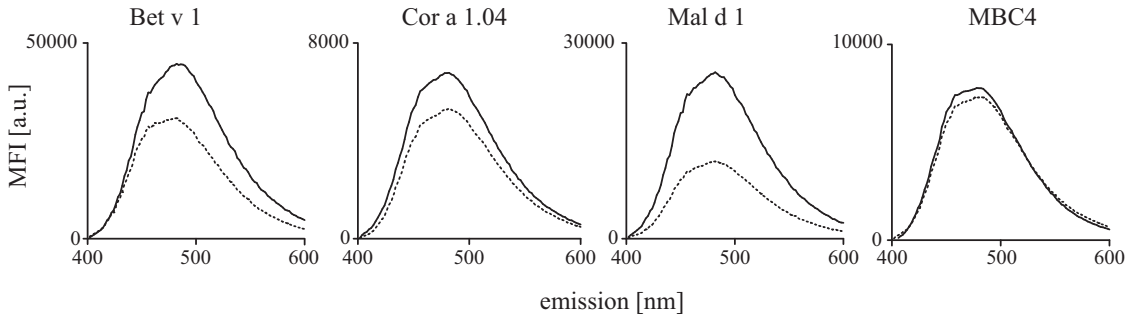



**FIG E5.** ANS displacement assays were performed before *(solid line)* or after (*dotted line*) preincubation of proteins with Na-deoxycholate in a 1:10 molar ratio (protein/ligand). Measurements were recorded at an excitation wavelength of 370 nm, whereas emission spectra were recorded from 400 to 600 nm. *MFI*, Mean fluorescence intentisy.

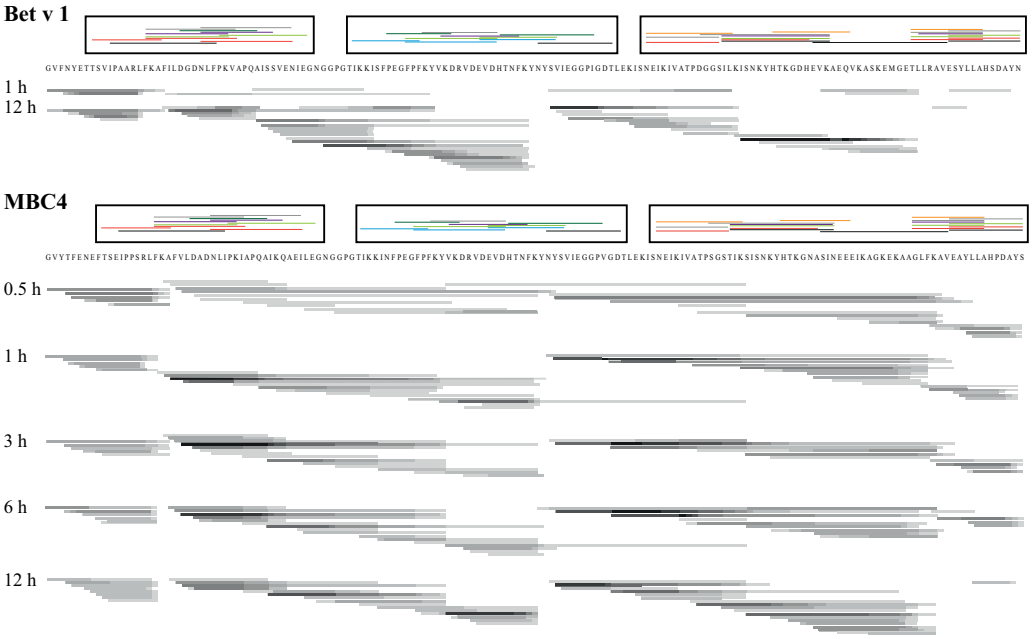



**FIG E6.** *Bars underneath protein sequences* show peptide clusters derived from *in vitro* endosomal/lysosomal proteolysis: black, frequently occurring peptides; gray, peptides of medium frequency; light gray, rare peptides. *Peptide clusters above each sequence (boxed)* represent TCL-reactive peptides from donors with birch pollen allergy (each color represents 1 donor).

**TABLE E1.** List of patients included in the study

| | | Total IgE | Birch pollen–specific IgE | | Food-specific IgE | | SPT | | | Diagnosis | | PFS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient no. | Code | kU/L | Class | PRU | kU/L | Class | B | Ap | Hn | AS | Po | Ap | Hn | Pn | Ca | Ki | Ro |
| 1 | 1 | 537 | 4 | 44.2 | Apple (2.49)<br>Hazelnut (1.0) | 2<br>2 | ++ | +++ | +++ | | • | • | • | • | | | • |
| 2 | 5 | 721 | 4 | 39.5 | Apple (7.41)<br>Hazelnut (10.6) | 3<br>3 | + | + | + | • | • | • | • | | • | • | |
| 3 | 6 | 8.38 | 3 | 42.6 | Apple (0.82) | 2 | + | +++ | + | • | • | • | • | | • | • | |
| 4 | 8 | 1028 | NA | NA | Apple (4.64)<br>Hazelnut (0.56) | 3<br>1 | +++ | | | • | • | • | • | | • | | • |
| 5 | 10 | 115 | 4 | 21.4 | NA | | + | | | • | • | • | • | | | | • |
| 6 | 20 | 16.4 | 3 | 8.24 | Apple (1.03) | 2 | +++ | +++ | +++ | | • | • | • | | | | • |
| 7 | 28 | 49.9 | 4 | 10.9 | Apple (1.26) | | +++ | | | | • | • | • | | | | • |
| 8 | 33 | 1210 | 5 | 73.7 | Apple (11.5)<br>Hazelnut (0.89) | 3<br>2 | +++ | | | • | • | • | • | | | | • |
| 9 | 34 | 217 | NA | NA | NA | | + | | +++ | | • | • | • | | | | • |
| 10 | 36 | 92.7 | 4 | 20 | NA | | +++ | + | +++ | | • | • | • | | | | |
| 11 | 65 | 116 | NA | NA | Apple (6.41)<br>Hazelnut (2.84) | 3<br>2 | +++ | +++ | +++ | • | • | • | • | | | • | • |
| 12 | 66 | NA | 6 | >100 | Apple (27.4)<br>Hazelnut (3.59) | 4<br>3 | + | | | | • | • | • | | | • | • |
| 13 | 67 | 594 | 6 | >100 | Apple (30.1)<br>Hazelnut (7.59) | 4<br>3 | + | +++ | +++ | | • | • | • | | • | | • |
| 14 | 95 | 491 | 6 | >100 | Apple (8.66)<br>Hazelnut (4.4) | 3<br>3 | +++ | +++ | +++ | | • | • | • | | | | • |
| 15 | 103 | 107 | 4 | 24.09 | Apple (6.16)<br>Hazelnut (1.91) | 4<br>3 | +++ | ++ | + | | • | • | • | | | | |
| 16 | 108 | 426 | NA | NA | Apple (15.3)<br>Hazelnut (4.15) | 3<br>3 | ++ | +++ | | • | • | | | | | | |
| 17 | 117 | 329 | 5 | 80.5 | Apple (24.2)<br>Hazelnut (5.33) | 4<br>3 | + | ++ | + | | • | • | • | | | | • |
| 18 | 131 | 29 | 4 | 135 | Apple (3.59)<br>Hazelnut (0.78) | 3<br>2 | NA | NA | NA | • | • | • | • | | • | • | |
| 19 | 645843 | 51.1 | 5 | | | | | | | • | • | | | | | | |
| 20 | 646859 | 51.6 | 5 | | | | | | | | • | | | | | | |
| 21 | 646494 | 73.6 | 5 | | | | | | | | • | | | | | | |
| 22 | 646951 | >100 | 6 | | | | | | | • | • | • | | | | • | |
| 23 | 646966 | 93.6 | 5 | | | | | | | | • | • | | | | | • |
| 24 | 647859 | 59.3 | 5 | | | | | | | | • | • | | | | | |

+, Wheal-and-flare reaction ≥50% of the histamine control; ++, wheal-and-flare reaction equal to the histamine control; +++, wheal-and-flare reaction > than the histamine control; *Ap*, apple; *AS*, Asthma; *B*, birch; *Ca*, carrot; *Hn*, hazelnut; *Ki*, kiwi; *NA*, not available; *Pn*, peanut; *Po*, pollinosis; *Ro*, other rosacea.

**TABLE E2.** Proliferative response of human TCLs derived from PBMCs from donors with birch pollen allergy

| | Patient No. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Protein** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **Median** |
| Bet v 1.0101 | | | | | | | | | | |
| 1 μg/w | 32.4 | 4.4 | 2.5 | 54.4 | 20.0 | 2.5 | 9.1 | 133.9 | 362.4 | 20.0 |
| 0.5 μg/w | 29.8 | 5.7 | 2.8 | 50.9 | 19.7 | 2.3 | 13.7 | 130.9 | 246.2 | 19.7 |
| 0.25 μg/w | 24.3 | 3.4 | 2.7 | 51.5 | 17.1 | 2.5 | 8.6 | 129.1 | 99.4 | 17.1 |
| 0.13 μg/w | | 2.7 | 2.6 | 55.4 | 16.4 | 2.8 | 9.5 | 135.1 | | 9.5 |
| Cor a 1.0401 | | | | | | | | | | |
| 1 μg/w | 1.1 | 0.0 | 0.4 | 6.3 | 1.6 | 1.8 | 2.7 | 1.2 | 2.6 | 1.6 |
| 0.5 μg/w | 1.3 | 0.2 | 0.6 | 6.5 | 1.8 | 2.5 | 1.7 | 1.0 | 2.0 | 1.7 |
| 0.25 μg/w | 0.7 | 0.5 | 0.8 | 7.1 | 2.1 | 2.3 | 2.8 | 1.9 | 1.6 | 1.9 |
| 0.13 μg/w | | 0.3 | 1.4 | 4.5 | 1.7 | 2.2 | 2.5 | 2.0 | | 2.0 |
| Mal d 1.0108 | | | | | | | | | | |
| 1 μg/w | 5.6 | 0.2 | 0.5 | 5.3 | 0.3 | 0.8 | 1.3 | 3.2 | 1.2 | 1.2 |
| 0.5 μg/w | 4.4 | 0.3 | 0.7 | 3.6 | 0.6 | 1.0 | 1.6 | 2.0 | 1.7 | 1.6 |
| 0.25 μg/w | 4.4 | 0.2 | 1.2 | 1.7 | 1.0 | 1.0 | 1.4 | 2.3 | 3.4 | 1.4 |
| 0.13 μg/w | | 0.1 | 0.7 | 1.5 | 1.1 | 0.9 | 1.5 | 2.1 | | 1.1 |
| MBC4 | | | | | | | | | | |
| 1 μg/w | 10.7 | 1.7 | 1.3 | 35.3 | 4.5 | 2.2 | 10.7 | 2.8 | 935.2 | 4.5 |
| 0.5 μg/w | 12.2 | 1.5 | 2.0 | 39.7 | 5.3 | 2.8 | 10.1 | 1.2 | 1123.5 | 5.3 |
| 0.25 μg/w | 12.8 | 1.4 | 2.0 | 37.3 | 4.6 | 2.3 | 8.8 | 0.6 | 1180.3 | 4.6 |
| 0.13 μg/w | | 2.4 | 1.9 | 37.5 | 6.6 | 2.9 | 10.8 | 0.4 | | 2.9 |

Stimulation indices are shown.
/w, Well.